AVERTISSEMENT

Ce document est le fruit d'un long travail approuvé par le jury de soutenance et mis à disposition de l'ensemble de la communauté universitaire élargie.

Il est soumis à la propriété intellectuelle de l'auteur. Ceci implique une obligation de citation et de référencement lors de l'utilisation de ce document.

D'autre part, toute contrefaçon, plagiat, reproduction illicite encourt une poursuite pénale.

Contact : ddoc-theses-contact@univ-lorraine.fr

LIENS

Code de la Propriété Intellectuelle. articles L 122. 4
Code de la Propriété Intellectuelle. articles L 335.2- L 335.10
http://www.cfcopies.com/V2/leg/leg_droi.php
http://www.culture.gouv.fr/culture/infos-pratiques/droits/protection.htm

PRES DE L'UNIVERSITE DE LORRAINE

INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE

Ecole Nationale Supérieure d'Agronomie et des Industries Alimentaires

Laboratoire d'Ingénierie des Biomolécules

THESE

Présentée devant l'Institut National Polytechnique de Lorraine

Muhammad IMRAN

Pour obtenir le grade de Docteur de l'INPL

Spécialité : Procédés Biotechnologiques et Alimentaires

Enrobages actifs contenants des peptides antimicrobiens nano-vectorisés

Active packaging containing nano-vectorized antimicrobial peptides

---

Soutenue publiquement le 26 Avril 2011 devant la commission d'examen

Rapporteurs :

Mme. Amparo CHIRALT BOIX	Professeur, UPV, Spain
M. Séamus FANNING	Professeur, UCD, Ireland

Examinateurs :

M. Faqir Muhammad ANJUM	Professeur, NIFSAT-UAF, Pakistan
M. Pascal DEGRAEVE	Professeur, UL1, France
M. Stéphane DESOBRY	Professeur (Directeur de thèse), INPL, France
Mme. Anne-Marie REVOL-JUNELLES	MdC-HDR (Co-directeur de thèse), INPL, France

PRES DE L'UNIVERSITE DE LORRAINE

INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE

Ecole Nationale Supérieure d'Agronomie et des Industries Alimentaires

Laboratoire d'Ingénierie des Biomolécules

THESE

Présentée devant l'Institut National Polytechnique de Lorraine

Muhammad IMRAN

Pour obtenir le grade de Docteur de l'INPL

Spécialité : Procédés Biotechnologiques et Alimentaires

Enrobages actifs contenants des peptides antimicrobiens nano-vectorisés

Active packaging containing nano-vectorized antimicrobial peptides

---

Soutenue publiquement le 26 Avril 2011 devant la commission d'examen

Rapporteurs :

Mme. Amparo CHIRALT BOIX	Professeur, UPV, Spain
M. Séamus FANNING	Professeur, UCD, Ireland

Examinateurs :

M. Faqir Muhammad ANJUM	Professeur, NIFSAT-UAF, Pakistan
M. Pascal DEGRAEVE	Professeur, UL1, France
M. Stéphane DESOBRY	Professeur (Directeur de thèse), INPL, France
Mme. Anne-Marie REVOL-JUNELLES	MdC-HDR (Co-directeur de thèse), INPL, France

Drinking **Nature** is an unquenchable thirst.

Berri Clove

Curiosity has its own reason for existing.

I have no special talent,

I am only passionately curious.

Albert Einstein

Avant Propos

Je souhaite tout d'abord remercier le 'Higher Education Commission (HEC)' du Pakistan d'avoir accepté de financer ce travail de thèse qui a été réalisé au sein du Laboratoire d'Ingénierie des Biomolécules de l'Ecole Nationale Supérieur d'Agronomie et des Industries Alimentaires de Nancy, sous la direction de Monsieur Stéphane Desobry, professeur et la co-direction de Madame Anne-Marie Revol-Junelles, Maître de conférences HDR à l'ENSAIA.

Ma reconnaissance le plus sincère au Professeur Stéphane Desobry pour son encadrement, sa compréhension, son soutien et la grande confiance qu'il m'a témoigné tout au long de la thèse. Sa concepte à être optimiste et plein d'espoir, son aptitude à la neutralisation de l'énergie négative en bonne volonté, son approche utopique d'apporter le bonheur à tous, ses gestes de routine et d'humeur portant les ondes magnétiques de positivité, et ses manières simples d' « être humain » m'aideront tout au long de ma vie, professionnellement et personnellement.

Je remercie très chaleureusement Madame Anne-Marie Revol-Junelles pour son encadrement, sa disponibilité, sa sympathie, ses conseils précieux ainsi que pour avoir patiemment corrigé ce document et les articles. Je l'apprécie beaucoup pour m'avoir faite apprendre le sens du mot «organisation» à la fois pour le travail expérimental et la transformation des idées, et son approche réaliste, tout en traitant de la science.

Je remercie également les membres de jury qui ont accepté de juger ce travail : Madame Amparo Chiralt, Professeur à l'Universidad Politécnica de Valencia et le Monsieur Séamus Fanning, Professeur à l'Université Collège de Dublin.

Je tiens à remercier le Monsieur Pascal Degraeve, Directeur du département Génie Biologique de l'IUT de Bourg en Bresse et le Monsieur Faqir Muhammad Anjum, Directeur de National Institute of Food Science and Technology de Faisalabad, d'avoir accepté de prendre part à ce jury.

Mes sincères remerciements vont également à Michel Linder, Elmira Arab Tehrany, Muriel Jacquot, Jordane Jasniewski, Catherine Cailliez-Grimal et Frédéric Borges pour leur aide, leurs conseils et leur patience.

Je remercie le Dr. Eefjan Breukink, Professeur à l'Université de l'Utrecht, pour m'avoir accueilli en stage dans son laboratoire et pour l'aide apportée dans la réalisation des analyses de marquage fluorescent. Mes remerciements s'adressent également aux responsables des différent laboratoires pour m'avoir rendu accessible les différents techniques, surtout Emmanuel Guédon (CCDS au LSGC), Cedric Paris (LCMS), Dominique Dumas (CLSM à la Faculté de medecine) et Christoph Stenger (Lipidomix), Gregory Francius (AFM au LCPME), Jaafar Ghanbaja et Jacquline Chanél (TEM, SEM au SCMEM et FdM), Aurélie François (Spectrofluorimeter au CRAN), Jean-Michel Girardet et Christelle Harscoat (semi-prep HPLC à l'URAFPA-UHP et SVS), *Céline* CAILLET (zeta-sizer au LEM)… Un grand merci pour m'avoir communiqué votre passion, vos discussions enrichissant et vos précieux conseils.

Je tiens à remercier l'ensemble du personnel de l'ENSAIA qui me connaît et particulièrement celui du LIBio, et surtout Marie-Noële Maucourt (pour ses Tic-Tac), Anne-Laplace, Carole Jeandel, Mlle Turban, Delphine, Myriam, Paul, et Marlies de Bruin (Pays-Bas).

Je remercie l'ensemble des thésards : Agnieszka, Majid, Guillaume, Smail, Lynn, Charbel, Javeed (l'équipe emballage) ; Behnoush, Nabila, Pascale, Kassem (l'équipe lipide) ; Abdur-Rehman, Inam, Amira, Thibaut, Mireille, (l'équipe microbiologie) ; Laetitia, Raza, Leila et des stagiaires du LIBio (surtout Noémie, Soumaya, Catherine, Amira, Mamadou) qui ont contribué, de près ou de loin, à la réalisation de cette étude, dans une ambiance toujours amicale et stimulante. Je remercie aussi les jeunes chercheurs du laboratoire, venus de France ou d'ailleurs, avec qui j'ai eu l'occasion de partager de bons moments inoubliables.

Je voudrais aussi remercier les amis (surtout Shani) qui m'ont accompagné tout au long de cette aventure et ont constitué ma deuxième famille en France.

Je terminerai en exprimant de sincères remerciements à ma famille pour leur écoute, leur soutien et leurs encouragements tout au long de mes pérégrinations doctorales, en particulier mon cher Abbu G, ma très belle Mamma jani, mes sœurs, mes frères Mudassar et Tayyab, mes beaux-frères, mes nièces Urooj et Samar, mes petit neveux Ans, Avais et Hussy, mon oncle et Imran (self-talking Buddy).

En fin, je remercie tous ceux qui font progresser la SCIENCE, sincèrement....

Table des matières

1. Introduction

Introduction

La sécurité des aliments occupe une place de plus en plus importante à travers le monde. L'emballage - ou l'enrobage - actif est une source d'inspiration, car il propose de nombreuses potentialités pour améliorer la sécurité et prolonger la durée de vie des produits alimentaires. Il permet à l'industrie alimentaire d'associer des fonctions antimicrobiennes, liés la présence des agents actifs, à celles préexistantes de l'emballage. Un des facteurs critiques d'altération des aliments est la possibilité d'une contamination de surface. Cependant, l'inhibition des micro-organismes peut être améliorée en maintenant une forte concentration d'agents actifs au niveau des surfaces des aliments (enrobage et emballage) et en limitant leur diffusion au cœur du produit. Les bactériocines peuvent être intéressantes pour la bio-préservation des aliments à un niveau industriel en raison de leur thermostabilité, leur large spectre d'action vis-à-vis de nombreux pathogènes alimentaires, et leur activité antimicrobienne à faible concentration sur une large gamme de pH. Parmi toutes les bactériocines découvertes ces dernières années seule la nisine, un peptide de 34 acides aminés produit par certaines souches de *Lactococcus lactis* subsp. *lactis* est autorisée comme additif (E234) par l'Organisation Mondiale de la Santé. De ce fait, les applications de la nisine dans l'industrie de l'emballage alimentaire sont nombreuses en raison de son efficacité à contrôler le développement de pathogènes alimentaires Gram-positif, son statut GRAS (Generally Recognized as Safe) et son étiquette « additif bio».

Les nanotechnologies possèdent la potentialité d'améliorer la sécurité, les procédés et l'emballage alimentaires. La nano-encapsulation d'agents actifs, comme la nisine, est un concept innovant permettant de protéger les agents actifs d'une dégradation éventuelle pendant le procédé de fabrication de l'aliment et son stockage ou liée à des interactions avec la matrice alimentaire complexe. Cette technologie permet d'améliorer la répartition de l'agent actif et un relargage progressif entrainant ainsi une action à long terme dans des produits alimentaires. Les liposomes sont particulièrement adaptés à une utilisation en industrie alimentaire en tant que système de libération de composés actifs car ils sont bien caractérisés, facilement réalisables, très modulables dans leur propriétés de transporteur, fortement bio-compatibles et sont considérés comme des matériaux GRAS. Cependant, peu d'informations sont disponibles sur la méthodologie à suivre pour l'encapsulation d'agents antimicrobiens. Pour améliorer la sécurité alimentaire en incorporant de la nisine dans des liposomes à un niveau nano, un nouveau concept

de microfluidisation, utilisant un système de destruction des cellules en continu (Continuous Cell Disrupteur System - CCDS) est un des objectifs de ce travail ; ce système est efficace, rapide, sans danger pour l'environnement et applicable à grande échelle pour la production industrielle de système transporteur de dimension nano.

Une fois l'immobilisation de la nisine dans des nano liposomes réalisées, la problématique est d'obtenir une charge maximale d'agents actifs à la surface de l'aliment et une stabilité au cours du temps des liposomes incorporés dans l'aliment. Cette problématique a été résolue en fusionnant les deux concepts de relargage lent par nano-encapsulation de la nisine et immobilisation de ces complexes dans un bio-polymère. Cette approche permet de formuler la nouvelle génération de films biodégradables contenant soit des agents actifs, soit des agents actifs nano-encapsulés, soit les deux. Ainsi, la nisine peut migrer à travers les liposomes *via* la formation de pores et à travers le bio-polymère, qui garantit ainsi la disponibilité d'une nisine active pendant toute la durée de conservation du produit alimentaire.

Le suivi du relargage de la nisine à travers les bio-membranes ou les systèmes de transport, l'étude *in vivo* des mécanismes d'action de la nisine vis-à-vis de bactéries pathogènes, nécessitent de pouvoir localiser de façon précise et quantifier la nisine. Ces dernières années, le marquage de biomolécules par des marqueurs fluorescents est apparu comme une méthodologie innovante à des fins analytiques en microbiologie alimentaire, médecine ou pharmacie en raison de sa précision, sa rapidité, ses limites de détection très faibles, son faible coût d'extraction, sa possibilité de détection *in vivo*… Ainsi le marquage de la nisine Z, non encore documentée à ce jour, avec un marqueur fluorescent a été optimisé et caractérisé par des méthodes de chromatographie liquide couplés à un spectre de masse (LC/MS). L'obtention de nisine ainsi marquée a permis d'atteindre les objectifs précités.

Prédire la diffusion de la nisine à travers l'emballage vers des systèmes alimentaires modèles est nécessaire pour déterminer ses potentialités d'activité antimicrobienne dans l'aliment, car la nisine a besoin d'atteindre le micro-organisme pour exercer son action. Ainsi, les valeurs du coefficient de diffusion (D) et du coefficient de partition (K) de l'agent antimicrobien dans le film d'emballage donnent des informations sur la durée d'efficacité du composé vis-à-vis des bactéries cibles présentes à la surface de l'aliment. Actuellement, la demande croissante pour la protection et la conservation de l'environnement encourage le développement de films

comestibles ou fabriqués à partir de matériaux biodégradables pour apporter des caractéristiques d'éco-compatibilité. Cependant, il n'existe pas d'études publiées comparant le relargage de la nisine de l'emballage réalisé avec différent bio-polymères vers l'aliment. Cet aspect a été étudié pour des bio-polymères de différents origines et composition incluant des dérivés de la cellulose tel que l'Hydroxy Propyle Méthyle Cellulose (HPMC), des dérivés de protéines animales tels que des caséinate de sodium (CS), des dérivés de cytosquelette de crustacés tels que le chitosane (CTS) et des dérivés de monomères de l'acide lactique tel que l'acide poly-lactique (PLA). Leur capacité relative et leur efficacité à être des supports d'agents actifs ont été évalués et comparées. L'efficacité à prédire les valeurs de D et K dépendant fortement de l'efficacité/sensibilité des méthodes de quantification de l'agent antibactérien, la nisine marquée à la fluorescéine a été utilisée pour évaluer les transferts.

Toutefois, les systèmes de libération de dimension nanométrique peuvent varier dans leur capacité à encapsuler une molécule telle que la nisine et dans leur capacité à délivrer cette molécule. Les études des interactions entre l'agent actif encapsulé et le biopolymère support de cette encapsulation sont importantes pour évaluer la capacité d'un système à libérer la nisine. Dans ce contexte, la composition de la matrice de biopolymère, les conditions de process, la charge électrophorétique, la nature du milieu simulant l'aliment, la température peuvent être modifiées de façon à acquérir la biodisponibilité désirée de l'agent actif pour une grande diversité de produits alimentaires.

L'objectif global de ce travail a été de mettre au point des modèles de nisine fluorescente et d'encapsulation de nisine pour l'étude des transferts moléculaires dans différents emballage actifs à base de biopolymères et dans l'aliment. Les verrous scientifiques était liés au greffage de fluorochrome de faible masse moléculaire sur la nisine, la structuration de liposomes multilamellaires permettant l'obtention d'un relargage de cinétique contrôlés de la nisine dans un matrice biologique (biomatériau et aliments simulés). Ce sujet a donc couvert les secteurs de la chimie, de la physique et de la microbiologie.

Introduction

Ever since '*Eve*' started it all by offering '*Adam*' the *Apple*, food and its safety has turn out to be an ever more significant concern world-wide. Active food packaging/coating is an inspirational advancement, as it carries significant potential of improving food safety and prolonging the shelf life of food products. It permits the food industry to unite the preservative functions of active agents with the protective functions of pre-existing packaging notion. One of the critical factors affecting food spoilage is the microbial growth at food surface. However, microbial inhibition can be improved by maintaining a high concentration of active agents at food surfaces (coating, wrapping, and packaging) and by limiting their diffusion into the food core. Bacteriocins can be interesting food bio-preservatives at industrial scale due to their thermostability, their broad-spectrum against food borne pathogens, and their antimicrobial activity at low concentrations over a wide range of pH. Among all the bacteriocins only nisin, a 34-residue-long peptide produced by *Lactococcus lactis* is allowed to be used as a food preservative (E234) by World Health Organization. Consequently, applications of nisin in food packaging industry are increasingly high due to effective control over food borne pathogens (Gram-positive bacteria), generally recognized as safe (GRAS) status, and its 'bio-additive' tag.

Food nanotechnology has the potential to improve food safety and bio-security, food processing, food packaging and functional ingredient concepts. Nano-encapsulation of active agents including nisin is an innovative concept to protect the active ingredient against possible denaturation during processing, storage and by interaction with complex food system; to improve the distribution of the active ingredient, to provide gradual release, and thus act as a long-term preservative in pharmaceutical and food systems. Liposomes are particularly well suited for use in the food industry as delivery systems because they are well characterized, easily made, highly versatile in their carrier properties, highly biocompatible, and GRAS materials. However, little information is available on the methodology of encapsulation for antimicrobial agents. To improve the food safety by liposomal encapsulation of bacteriocin at nano-scale, a new concept of microfluidic approach using continuous cell disruption system (CCDS) is optimized during the present study, which is effective, rapid, environment friendly and suitable for large-scale industrial production of nano-carrier systems.

Once immobilizing the nisin in/as bioactive packaging films and its liposomal encapsulation as nano-delivery system was successfully accomplished, the next question was how to deal with maximum bacterial load at food surface and stability kinetics of liposomes incorporated in food. Thus further improvement was envisaged by the fusion the above-mentioned two concepts of slow release i.e. nisin nanoencapsulation and subsequent biopolymer immobilization to formulate the next generation biodegradable films embedded with either active agent, nano-encapsulated active agent or both of them. Thus, nisin migrate through the liposome by pore formation and further from the network of polymer film which may guarantee the enhanced availability of fresh/active nisin during conservation.

The advanced study of nisin detection, quantification, and release from biomembranes or nano-carrier systems, target delivery and *in vivo* mechanism of action against potential pathogens requires precise localization and quantification approach. In recent years, the labeling of biomolecules by fluorescent markers has emerged as innovative methodology for bio-analytical purposes in food microbiology, medicine and pharmaceutics due to the advantages of precision, rapidity, wide detection limits, no extraction cost, in vivo recognition… So labeling of nisin Z, not reported till date, with fluorescent marker was optimized and characterized through recently developed method of liquid chromatography/mass spectrometry to realize the above-mentioned objectives.

Predicting nisin diffusion from packaging to the model food system is necessary to control its antimicrobial activity, since nisin needs to reach microorganisms to exercise its potential. Likewise, diffusion coefficient (D) and partition coefficient (K) values for antimicrobials in the packaging films provide information about how long the antimicrobials are efficient against target microorganisms at the surface of the films. Currently, the increased awareness for environmental conservation and protection has endorsed the development of edible coatings and films from biodegradable materials to meet user-friendly and eco-friendly attributes and serve as active agent carriers. However, there is no published work on the release of nisin from packaging to food while comparing different biopolymers. This interesting aspect was studied for biodegradable polymers with distinct origin and composition including; cellulose derivative hydroxypropyl methylcellulose (HPMC), dairy animal's protein based sodium caseinate (SC), marine animal's exoskeleton based chitosan (CTS), and chemically synthesized from bio-derived

monomers based poly-lactic acid (PLA) to judge their relative capacity and efficiency as active agent carrier. As the efficacy of predicting the values of D and K depends mainly on the efficacy/sensitivity of antimicrobial agent quantification method, fluorescein labeled nisin previously fabricated was utilized to study mass transfer by rapid fluorescence spectrometry.

Likewise, the nano-delivery system may vary in their ability to encapsulate, sustain the pore-forming ability of nisin, and deliver at target time or environment. The interaction study between encapsulated active agent and encapsulating biopolymer is of significant importance concerning the superior delivery of nisin to improve food safety. In this context, the composition of encapsulating biopolymer, processing conditions, electrophoretic charge, nature of food stimulant medium, temperature (…) can be altered to acquire suitable bioavailability of active agent for a wide variety or specific food products.

The overall objective of the present work was to optimize and develop fluorescent labeling and encapsulation of nisin for molecular transfer study in different packaging based on biopolymers and in the food. The scientific obstacles were related to the grafting of low molecular weight fluorochrome on nisin, the structuring of multilamellar liposomes to obtain the controlled release kinetics of nisin in a biological matrix (simulated food and biomaterial). Hence, the topic has covered the areas of chemistry, physics and micobiology.

Publications:

1)- Imran M., Revol-Junelles A-M., Martyn A., Tehrany E.A., Jacquot M., Linder M., Desobry S. 2010. Active food packaging evolution: Transformation from micro- to nanotechnology *Critical reviews in food science and nutrition (50), 799–821*

2)- Jamshidian M., Tehrany E.A., Imran M., Jacquot M., Desobry S. 2010. Poly lactic acid: Production, applications, nanocomposites and release studies. *Comprehensive reviews in food science and nutrition (9), 552–571*

3)- Imran M., Revol-Junelles A-M., Desobry S. 2011. Predicting the future: Adaptations of food packaging trends via nanotechnology (*In: Modified Atmosphere and Active Packaging. Taylor & Francis Publishers)*

4)- Imran M., El-Fahmy S., Revol-Junelles A-M., Desobry S. 2010. Cellulose derivative based active coatings: Effects of nisin and plasticizer on physico-chemical and antimicrobial properties of hydroxypropyl methylcellulose films. *Carbohydrate polymer (81) 219-225*

5)- Imran M., Revol-Junelles A.M., Paris C., Guedon E., Linder M., Desobry S. Liposomal nanodelivery systems to encapsulate food biopreservative nisin by novel strategy of microfluidic format *(Colloids and Surfaces, Soumis 2011)*

6)- Imran M., Revol-Junelles A.M., René N., Jamshidian M., Akhtar M.J., Tehrany E.A., Jacquot M., Desobry S. Microstructure and physico-chemical evaluation of nanoemulsion-based antimicrobial peptides embedded in packaging films *(Food hydrocolloids, Accepté 2011)*

7)- Imran M., Revol-Junelles A.M., de Bruin M., Paris C., Breukink Eefjan, Desobry S. Fluorescent labeling of the lantibiotic peptide nisin Z: Purification, characterization and assessment of anti-listerial mechanism of action (*Food microbiology, en cours de soumission*)

8)- Imran M., Klouj A., Jamshidian M., Akhtar M.J., Revol-Junelles A.M., Desobry S.Controlled release of an antimicrobial bio-preservative nisin from bio-membranes: Comparison of HPMC, sodium caseinat, poly-lactic acid and chitosan efficacy for packaging applications (*Journal of controlled release, soumise*)

9)- Diffusion kinetics of pore-forming nano-vectorized bacteriocin in model membranes Muhammad Imran, Anne-Marie Revol-Junelles, Grégory Francius, Stéphane Desobry (ACS Nano, *En cours de soumission*)

Congrès Internationaux:

1)- Imran M., Revol-Junelles A-M., Linder M., Desobry S. Potential use of microfluidization for vectorization of nisin in nanoliposome: A novel template for active agent / drug nanoencapsulation. (NanoSMat-2009, 19-22 Oct 2009, Rome, ITALY): *Oral presentation*

2)- Imran M., Revol-Junelles A-M., Desobry S. Nano-encapsulation of biopreservatives for controlled release: Is it the future packaging trend? (CIFST conference, May 30-June 1, 2010, Winnipeg, Canada): *Oral presentation*

3)- Imran M., Revol-Junelles A-M., Desobry S. Enhancement of controlled release of the active agent by biopolymer-coated nanoliposome-encapsulated-nisin: A novel approach for food packaging applications. (IFT 2010, 17-21 July 2010, Chicago, USA): *Poster presentation*

4)- Imran M., El-Fahmy S., Revol-Junelles A-M., Jacquot M., Tehrany E.A., Desobry S. Physicochemical properties and bioactivity of composite films (Material Bioproduct interactions (MATBIM), 3-5 March 2010, Paris, France): *Oral presentation*

II. Synthèse Bibliographique

La synthèse bibliographique est divisée en trois chapitres : le premier chapitre est constitué d'un article de revue, publié en 2010 dans la revue *Critical reviews in food science and technology* et intitulé «Active food packaging evolution : Transformation from micro- to nanotechnology » ; le second chapitre est un extrait d'un deuxième article de revue paru en 2010 dans le journal *Comprehensive reviews in food science and nutrition* et intitulé « Poly lactic acid : production, applications, nanocomposites and release studies » ; le troisième chapitre est extrait d'un chapitre du livre *Modified Atmosphere and Active Packaging* rédigé en 2011 et intitulé « Predicting the future : Adaptations of food packaging trends via nanotechnology ».

Le premier chapitre présente les potentialités des nanotechnologies dans l'emballage actif. L'objectif est de présenter les nouvelles tendances des technologies décrites dans la littérature dans le domaine de l'emballage actif et d'examiner les nouvelles technologies utilisées pour fonctionnaliser le matériel actif nanovectorisé, en se référant de manière plus spécifique aux applications potentielles dans le domaine alimentaire. Ce chapitre met en avant le dernier concept technologique « 3-BIOS» qui se réfère aux notions Bioactif - Biodégradable - Bionanocomposite. Par ailleurs, ce chapitre présente les tendances et statuts récents des systèmes règlementaires et analyse le nouveau concept traitant de nanotechnologie alimentaire, qui peut directement ou indirectement améliorer la qualité et la sécurité des aliments.

Le second chapitre est une partie d'un article de revue portant sur l'acide poly lactique (PLA). Dans cet article, la production, les applications, les procédés technologiques utilisés, les modifications pouvant être apportées et la biodégradabilité de ce matériau sont décrits. Dans ce chapitre, seules les parties de cette publication en relation avec le travail de thèse sont présentées. Il s'agit des études de migration et de relargage des composés actifs à partir du PLA pour améliorer les applications du PLA.

Le troisième et dernier chapitre résume les différents mécanismes de libération de divers composés actifs (composés antioxydants ou antimicrobiens, enzymes, acides aminés, vitamines, acides gras essentiels) à partir de liposomes à un niveau micro ou nano. Ce chapitre décrit les phénomènes de relargage se déroulant au niveau moléculaire ou membranaire et analyse les différentes approches utilisées jusqu'à présent pour la quantification de l'efficacité de relargage des composés actifs encapsulés utilisés dans le secteur alimentaire.

The bibliography section comprises three parts: first chapter of review article entitling 'Active food packaging evolution: Transformation from micro- to nanotechnology; second chapter is the extract of another review article entitled as 'Poly-lactic acid: Production, applications, nanocomposites, and release studies'; and third chapter is the extract of chapter written for active packaging book entitled as 'Predicting the future: Adaptations of food packaging trends via nanotechnology'.

First chapter presents the potentials of nanotechnology in active food packaging. The objective is to provide a comprehensive introduction of novel trends in active food packaging, furthermore, to examine some of the newest technologies described in the literature to functionalise nano-structured active material with special reference to potential applications in food systems. It highlights the next technological concept of 3-BIOS blends with nanotechnology; which refers to Bioactive, Biodegradable and Bio-nanocomposite. Further, this section explains the trends and recent status of regulatory issues and reveals the emerging concepts dealing food nanotechnology, which can directly or indirectly improve the quality and safety of foods.

On the other hand, second review article focuses more specifically on Poly-lactic acid (PLA) regarding its production, applications, processing technologies, modifications, and biodegradability. However, in the interest of this thesis only an extract relating to the migration and release studies of active compounds from PLA active food packaging is mentioned; in addition, recent nanocomposites applied for improving PLA applications are assessed.

The third and final chapter of bibliography summarizes the different release mechanisms of diverse active agents (antioxidant, antimicrobial, enzyme, amino acids, vitamin, enzyme, fortifier, Essential fatty acids) from liposome at micro- or nanoscale. It also emphasizes the release phenomenon occurring at molecular or membrane level. Moreover it analyses the different approaches utilized till date for the quantification of release efficiency of encapsulated active agents being employed in food sector.

Chapitre 1

L'évolution des emballages alimentaires: Transformation de la micro à la nanotechnologie

Muhammad Imran, Anne-Marie Revol-Junelles, Agnieszka Martyn, Elmira Arab Tehrany, Muriel Jacquot, Michel Linder, and Stéphane Desobry

Laboratoire d'Ingénierie des Biomolécules, ENSAIA–INPL, Nancy Université, 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France

(Critical Reviews in Food Science and Nutrition- (50), 799–821. 2010)

Critical Reviews in Food Science and Nutrition, 50:799–821 (2010)
Copyright © Taylor and Francis Group, LLC
ISSN: 1040-8398 print / 1549-7852 online
DOI: 10.1080/10408398.2010.503694

Taylor & Francis
Taylor & Francis Group

Active Food Packaging Evolution: Transformation from Micro- to Nanotechnology

MUHAMMAD IMRAN, ANNE-MARIE REVOL-JUNELLES, AGNIESZKA MARTYN, ELMIRA ARAB TEHRANY, MURIEL JACQUOT, MICHEL LINDER, and STÉPHANE DESOBRY

Laboratoire d'Ingénierie des Biomolécules, ENSAIA–INPL, Nancy Université, 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France

Predicting which attributes consumers are willing to pay extra for has become straightforward in recent years. The demands for the prime necessity of food of natural quality, elevated safety, minimally processed, ready-to-eat, and longer shelf-life have turned out to be matters of paramount importance. The increased awareness of environmental conservation and the escalating rate of foodborne illnesses have driven the food industry to implement a more innovative solution, i.e. bioactive packaging. Owing to nanotechnology application in eco-favorable coatings and encapsulation systems, the probabilities of enhancing food quality, safety, stability, and efficiency have been augmented. In this review article, the collective results highlight the food nanotechnology potentials with special focus on its application in active packaging, novel nano- and microencapsulation techniques, regulatory issues, and socio-ethical scepticism between nano-technophiles and nano-technophobes. No one has yet indicated the comparison of data concerning food nano- versus micro-technology; therefore noteworthy results of recent investigations are interpreted in the context of bioactive packaging. The next technological revolution in the domain of food science and nutrition would be the 3-BIOS concept enabling a controlled release of active agents through bioactive, biodegradable, and bionanocomposite combined strategy.

Keywords nanoencapsulation, biodegradable, liposome, antimicrobial, regulatory issues, controlled release

INTRODUCTION

The word "necessity" has been transfigured into a more diabolical notion "Fear of death," and thus Fear of death is the mother of invention in the twenty-first century. This fear factor is assessed by the Centers for Disease Control and Prevention (CDC); Foodborne diseases cause approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States each year (Mead et al., 1999). More than 200 known diseases are transmitted through food. Acute gastroenteritis affects 250 to 350 million people in the United States annually and an estimated 25–30% of these cases are thought to be foodborne disease. Approximately one person out of four may experience some form of foodborne illnesses each year (McCabe-Sellers et al., 2004). Such incidences of foodborne illness are mounting in developing countries as well as in the developed world (Greig et al., 2007). The Foodborne Diseases Active Surveillance Network (Food Net) states that comparing 2007 with 2004–2006, the estimated incidence of infections caused by *Campylobacter, Listeria*, *Salmonella*, *Shigella*, *Vibrio*, and *Yersinia* did not decline significantly, and the incidence of *Cryptosporidium* infections increased by 44% (Vugia et al., 2008). At the same time these illness-outbreaks create an enormous social and economic burden due to food recalls. As a result of several food-related incidents and reported outbreaks worldwide, consumer confidence has begun to oscillate (Jevsnik et al., 2008; Sofos, 2008).

The post-process contamination caused by product mishandling and faulty packaging is responsible for about two-thirds of all microbiologically related class I recalls in the United States, with most of these recalls originating from contamination of ready-to-eat (RTE) food products (Cagri et al., 2004; Gounadaki et al., 2007). Post-processing protection using "active packaging and coatings" has been proposed as an

Address correspondence to: Prof. Stéphane Desobry. Laboratoire d'Ingénierie des Biomolécules, ENSAIA–INPL, Nancy Université, 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France. Tel:+33 (0) 3 83 59 58 80; Fax: +33 (0) 3 83 59 57 72. E-mail: Stephane.Desobry@ensaia.inpl-nancy.fr

innovative approach that can be applied to RTE products to minimize or prevent the growth of pathogenic microorganisms (Gandhi and Chikindas, 2007; Kristo et al., 2008; Min et al., 2008).

The future is indicated by the past, the present, and the consumer. During the last decade, consumer demand for foods which are natural, of high quality, have elevated safety, are minimally processed, have a longer shelf-life, and are easy-to-eat with a fresh taste and appearance has been regarded as a matter of cardinal importance (Sobrino-Lopez and Martin-Belloso, 2008). Meanwhile, increased awareness for environmental conservation and protection has promoted the development of edible coatings and films from biodegradable materials to maintain the quality of both fresh and processed food (Villalobos et al., 2006). Since the market for natural, minimally processed, and RTE foods is broadening, bio-active packaging is being implemented in strategies that actively contribute to food preservation and transformation concepts (Fernandez et al., 2008).

The invention of nanotechnology and its implementation in food products and active/smart packaging has been approved due to its enabling nature. This has the potential to revolutionize agriculture and food systems. Nanotechnology is a multidisciplinary approach that provides endless promising possibilities in supporting the lives of people (Baer et al., 2003; Khosravi-Darani et al., 2007; Un and Price, 2007). Nanoscience is currently enabling evolutionary changes in several technology areas but new paradigms will eventually have a much wider and revolutionary impact. Nanoscience is "the study of phenomena and manipulation of materials at atomic, molecular, and macromolecular scales (0.2–100 nm), where properties differ significantly from those at a larger scale," whereas nanotechnology is "the design, characterisation, production, and application of structures, devices, and systems by controlling the shape and size at the nanometre scale" (Royal Society and Royal Academy of Engineering, Nanoscience, and Nanotechnologies, 2004). The US definition is that "nanotechnology is the understanding and control of matter at dimensions of roughly 1 to 100 nanometers, where unique phenomena enable novel applications." Encompassing nanoscale science, engineering, and technology, nanotechnology involves imaging, measuring, modelling, and manipulating matter at this length scale (NNI, 2001).

This review focuses on the potential of nanotechnology in active food packaging. It highlights, examines, and compares the known and predictive benefits of nano- versus microtechnology. The objective is to endow with a comprehensive introduction of novel trends in active food packaging. Furthermore, reports of some of the newest technologies have been described in the literature to functionalize nanostructured active material, with a special reference to potential applications in food systems. This review article aims to understand the recent status of regulatory issues and reveals the emerging concepts dealing with food nanotechnology that would improve the quality of human life.

ACTIVE PACKAGING

In view of the fact that food safety has become an ever more significant international concern, active packaging is considered to be a rapidly emerging concept (Weber et al., 2002). Active packaging is defined as an intelligent or smart system that involves interactions between package or package components and food or internal gas atmosphere and complies with consumer demands for high quality, fresh-like, and safe products (Labuza and Breene, 1989). In particular, active packaging changes the condition of packaged food to extend shelf life or improve food safety or sensory properties, while maintaining its quality (De Krujif et al., 2002). The improvement of sensory properties is argued to relate to active packaging. This emphasizes that as an indirect effect of improved food safety, the final sensory attributes of the product at its time of consumption, is better than the foods preserved in non-active packaging. Active food packaging is an inspiring advancement, which permits the food industry to unite the preservative functions of antimicrobials with the protective functions of a pre-existing packaging notion (Mauriello et al., 2004; Scanell et al., 2000).

Bioactive packaging is progressively more experimented upon because it is believed to have a significant potential in improving food safety and prolonging the shelf life of food products (Quintavalla and Vicini, 2002; Vermeiren et al., 1999). To date, various distinguished reviews have laid emphasis on the worth of active packaging for safer, healthier, and higher quality foods (Cagri et al., 2004; Coma, 2008; Joerger, 2007). Details about the O_2 scavenging system, the moisture absorption system, CO_2, and ethanol generation can be obtained from other reviews (Vermeiren et al., 1999; Ozdemir and Floros, 2004; Suppakul et al., 2003).

Active packaging realizes certain extraordinary but vital functions other than providing an inert barrier between the product and external conditions. The principal rewards that active food packaging brings are—the efficient control of surface contamination where the microbial growth predominantly originates; protecting food stuff with high water activity such as fish and sea food (Millete et al., 2007); comparatively prolonged retention of antimicrobial activity (Scanell et al., 2000); extension of shelf-life of foods by residual activity over time (Mauriello et al., 2004); reducing the risk of pathogen development, i.e., log reduction (Scanell et al., 2000); restrain partial inactivation of active substances by product constituents cross-reaction (Mauriello et al., 2005); controlled diffusion of bactericidal or bacteriostatic active agents; target specific microorganism population to provide higher safety (Quintavalla and Vicini, 2002); a comparatively low use of preservative agents; simplification of the production process by combining the addition of preservatives and the packaging step; improving sensorial properties (Vermeiren et al., 2002); the ability to inhibit the germination of spores; applicable to ready-to-eat food stuff (Janes et al., 2002); imperative control of post-process contamination (Kristo et al., 2008); and above all, prevent economic loss and possible deaths due to foodborne infections.

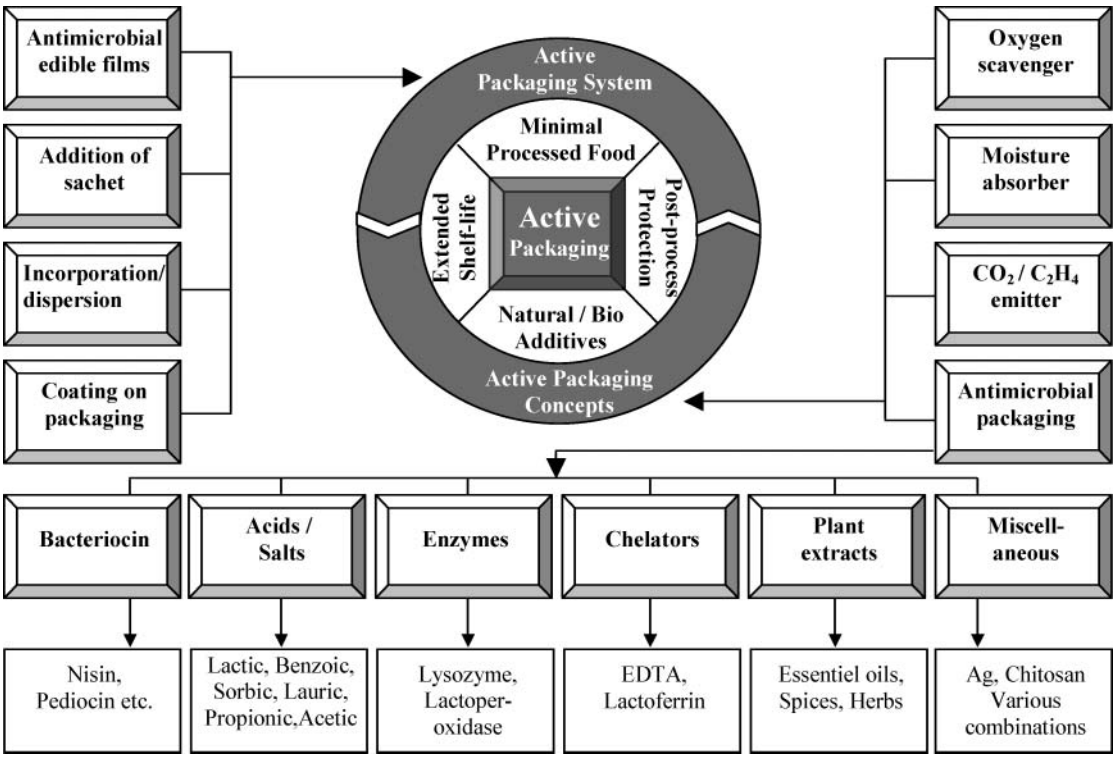


Figure 1 Active food packaging systems, concepts and application matrix.

As the main focus of this review is to analyze the worth of nanotechnology in active food packaging, therefore rather than discussing details of individual aspects of active packaging, just the specific key points of active food packaging are summarized (Fig. 1).

A whole range of active additives have been successfully incorporated in packaging material to confer antimicrobial activity including silver substituted zeolite (Del Nobile et al., 2004), organic acids and their salts, bacteriocin such as nisin and pediocin (Franklin et al., 2004), enzymes such as lysozyme (Min et al., 2008; Conte et al., 2006), chelators like EDTA and lactoferrin (Al-Nablusi and Holley, 2006; Hoffman et al., 2001); the organic compound triclosan (Vermeiren et al., 2002), plant extracts (Joerger, 2007), chitosan (Rhim et al., 2006), and a combination of a few of the above mentioned as hurdle technology (Kristo et al., 2008).

The regulatory systems for active packaging employed in the United States and Europe is apparently similar, but each has its own special set of exemptions. In brief, the US approach considers that "the dose makes the poison" so that toxicological justification is not needed, while the European approach starts from the principle that there must be toxicological data on all substances regardless of the level of anticipated exposure (Heckman, 2005). Most of the active agents are judged as food contact material instead of food additive (EU Regulation, 2004) A European study named EU FAIR R&D programme (1999), initiated amendments to European legislation for food-contact materials to establish and implement active and intelligent systems regulations for packaged food. The legal consequences of a new EU framework regulation on food contact materials, which include controls on active/intelligent packaging and its eventual benefits for the consumer and industry have been thoroughly reviewed (Weber et al., 2002; De Jong et al., 2005; Rasmussen and MacLellan, 2001).

Safe uses of active and intelligent packaging have been recently integrated by Regulation 450/2009/EC. The new Regulation establishes specific requirements also for the marketing of active and intelligent materials and articles intended to come into contact with food. It is mentioned that the substances responsible for the active and intelligent functions can either be contained in separate containers (e.g. oxygen absorbers is small sachets) or be directly incorporated in the packaging material (e.g. oxygen absorbing films). Moreover, the materials may be composed of one or more layers or parts of different types of materials, such as plastics, paper, and board as well as coatings and varnishes. Only the active and intelligent "components" should be subjected to authorization. The term "active component" means a system based on individual substance or a combination of substances which cause the active function of an active material or article. It may release substances or absorb substances into or from the packaged food or the environment surrounding the food. The community list of authorized substances that can be used to manufacture an active or intelligent component of materials shall therefore be established, once the European Food Safety Authority (EFSA) has performed a risk assessment and has issued an opinion on each substance (http://ec.europa.eu/food/food/chemicalsafety/foodcontact/). EFSA guidelines explain which factors the authorities will take into account when making safety assessments. This includes for example the toxicological properties of the product and the

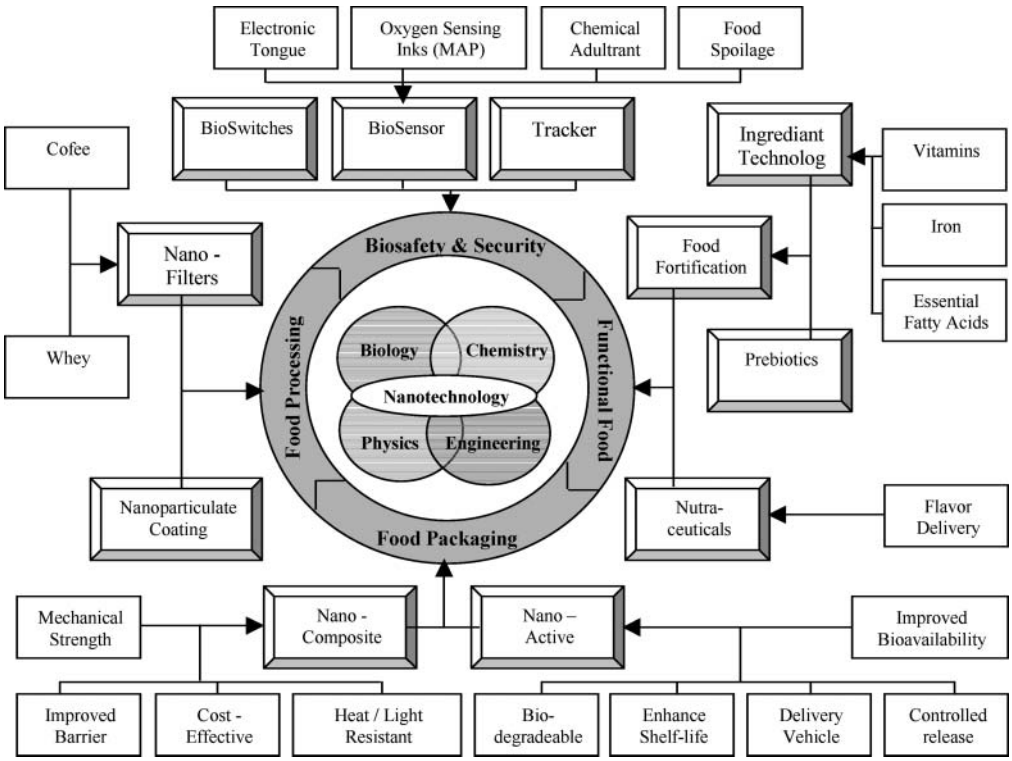


Figure 2 Vital nanotechnology application scopes in food.

extent to which they, or their breakdown products, could transfer into foods.

Further research on active food packaging materials is necessary not only to reveal the mechanisms of action of existing systems, but also to develop novel efficient active agents. Sufficient knowledge is required not only on the independent properties of the coating films and the antimicrobial compounds but also on their interactions. An upcoming center of attention will be the use of bio-derived active antimicrobial compounds with a wide spectrum of activity and low toxicity. Current trends suggest that active packaging will generally incorporate micro- or nanotechnology to accomplish more effective and efficient improvement. Parallel tests with diverse foods will be indispensable before we reach the ideal combination, working equally well for a majority of foods.

VITAL NANOTECHNOLOGY BENEFITS: APPLICATION SCOPES IN FOOD

The prefix "micro" was first used during the 1980s, and the prefix "nano" has been nominated to portray the current generation of dimension-reducing technologies. Nanotechnology generally refers to objects that are one-billionth of a meter in diameter. For the moment, an internationally standardized conception that defines nanotechnology does not exist yet. Nonetheless, we present the following depictions as more accepted ones.

Nanotechnology is important because it is cheap, relatively safe, clean, and the financial rewards are very high. Nanotechnology touches or will touch every aspect of our life (El Naschie, 2006). Nature has been performing "nanotechnological feats" for millions of years (Scott and Chen, 2008). The vital nanotechnological benefits in the food regime have been presented in Fig. 2.

In the food industry significant advancement by nanotechnology are: new functional materials; micro- and nanoscale processing; product development; and the design of methods and instrumentation for food safety and biosecurity (Moraru et al., 2003). At the Second International Food Nanoscience Conference (Chicago, USA, August 1, 2007), IFT's president Dennis Heldman asserted that the immense opportunities of nanoscience are possible in the areas of (i) food safety and biosecurity (ii) food processing (iii) food packaging (iv) ingredient technologies (Bugusu and Lubran, 2007). One can speculate that understanding the unique possessions of edible stuff of nanometer size will result in innovative, safer, healthier, and tastier foods. At the "*Nano4Food*" conference proceeding, Professor Stroeve emphasized that "Food-related nanotechnology research is already underway and could significantly affect our food supply within the next decade" (Nachay, 2007). Chau et al. (2007) have concluded that the global development of nanofoods is in fact in its initial stage, and yet a nanotechnology food market of US $20.4 billion in 2010, is expected.

Food nanotechnology has the potential to alter nutrient intake by broadening the number of enriched and fortified food products (Nickols-Richardson, 2007). Perspectives of nanoscale materials in the food industry have been reviewed well by Sanguansri and Augustin (2006) and Darnton-Hill and Nalubola (2002). The solubility of functional lipids (carotenoids, phytosterols, essential fatty acids, natural antioxidants) in food formulations

is a matter of foremost concern. Moreover, functional lipids with low water solubility may be prone to reduced bioavailability. Nanotechnology provides a good opportunity to improve the solubility of such active ingredients and to increase their bioavailability (Moraru et al., 2003). Omega-3 (highly unsaturated fatty acids) present in marine oils is responsible for their numerous beneficial effects on the retina, the cardiovascular system, the nervous system, etc. The problem with their direct incorporation into food products is their ease of oxidation and, therefore, the corresponding losses. For this reason they must be microencapsulated in most food applications (Kolanowski et al., 2007; 2006; Luff, 2007; Shahidi, 2000; Yep et al., 2002). With the advancement in technology, nano-encapsulation may enhance the stability and optimum release of these essential micro/macro-nutrients and thus play a vital role in increasing the survival rate from coronary heart diseases as a consequence of these nano-functional foods.

Malnutrition contributes to more than half of the deaths of children under five in developing nations. Several inexpensive agricultural and food applications of nanotechnology have the potential to decrease malnutrition, and thus infant mortality (Court et al., 2005). Flavored waters and milk fortified with vitamin, mineral, and other functional ingredients via nanoemulsion technology has gained a lot of importance. Carotenoids, in addition to their provitamin-A activity, have recently been implicated in the prevention of, or protection against serious human health disorders such as cancer, heart disease, macular degeneration, and cataracts. Physical stability of beta-carotene nanodispersions was superior during storage (Tan and Nakajima, 2005). Particle size is a determinant of iron (major malnutrition element) assimilation from feebly soluble Fe compounds. Decreasing the particle size of metallic Fe and ferric pyrophosphate added to foods increases Fe absorption (Rohner et al., 2007). Reducing poorly soluble Fe compounds to nanoscale may increase relative bioavailability and stability and thus their nutritional value. Thus, nano-ingredient food technology has the potential to get rid of major malnutrition dilemmas.

Several filtration technologies do not provide good quality, while with others the quality of the product is good but the cost is too high and needs to be cheap. With using nano-filters the investment (lower applied pressure, higher flux) and operation costs (lower pressure) of the equipment can be relatively low (Rektor and Vatai, 2004; Vincze and Vatai, 2004). More than one-third of the population of rural areas in Africa, Asia, and Latin America have no clean water. More than 2 million children die each year from water-related diseases. Inexpensive, easily transportable, and easily cleanable systems like nanomembranes and nano-clays purify, detoxify, and desalinate water more efficiently than conventional systems (Court et al., 2005).

Food NanoBioProcessing will achieve the goal of bioprocessing (utilize natural biological processes to generate a required compound from a specific waste/feedstock) with greater efficiency. The developments of devices that allow rapid identification of microbes present in feedstock are examples of research at the nanoscale that will increase the efficiency of bioprocessing (Scott and Chen, 2008). Nanosensors for the detection of pathogens and contaminants possibly will make manufacturing, processing, and consignment of food products more secure. Particular nanodevices may perhaps enable precise tracking and recording of the environmental conditions and shipment history of a specific product (Fonseca et al., 2007; Holland, 2007).Fellman (2008) has developed a method to produce nanoparticles with a triangular prismatic shape that can be used in detecting biological threats. In contrast, the use of nanotechnology for intelligence inks in modified atmosphere packaging (MAP) is gaining popularity.

The novel concept of BioSwitch nano-system contained by package works under the principle that if and when the microbial level reaches a certain level amylase is secreted, which partially degrades the encapsulated antimicrobials (Nachay, 2007). Researchers are optimistic that they can manufacture materials enabled to change properties depending on external or internal conditions. These sensors, often carbon nanotubes, are advantageous (rapid, cost-effective, recycled) over conventional chromatographic, enzymatic, or spectroscopic methods which are expensive and time consuming.

Packaging that incorporates nanomaterials can be "smart/intelligent" which means that it can respond to environmental conditions or repair itself or alert a consumer to contamination and/or the presence of pathogens. Self-healing packaging materials use nano/micro-encapsulated repairing agents. Small amounts of an encapsulated "healing agent" will be released by crack propagation or other triggering mechanism, which have been incorporated into polymeric coatings. The substitution of flexible polyolefin layers with novel, thin, functional polymer coatings in the production of paperboard packaging entails the risk of deteriorated barrier and mechanical properties. However, tiny capsules with a hydrophobic core surrounded by a hydrophobically modified polysaccharide membrane reasonably enhanced the packaging functionality. The results showed a reduced tendency for deteriorated barrier properties and local blockage of cracks formed upon creasing. The self-healing mechanism engages the break of tiny capsules local to the applied stress, with subsequent release of the core material. Fracture propagation is hampered by plasticization of the underlying coating layer, while the augmented hydrophobicity assists in sustaining the barrier characteristics (Andersson et al., 2009). Security of the food supply may possibly be enhanced by making "pathogen-repulsive" surfaces or packaging materials that change color in the incidence of injurious microorganisms or toxins (Moraru et al., 2003). Nanotechnology for food packaging had grown from a $66 million business in 2003 to a $360 million business in 2008, with an average annual increase of 40% (Brody, 2006). There is an increase in the number of nanotechnology developments in either type of packaging, active and intelligent (Nachay, 2007).

It is believed that the first area of nanotechnology will impact upon in the food industry is food packaging. "*GoodFood*" is a multidisciplinary European project designed to raise awareness of the benefits that micro- or nanotechnologies could bring to the food industry. The objective of the project is to help bring

the lab to the foodstuff, from the land to the market, extending the control of the total food chain (Fonseca et al., 2007). This project has developed the new generation of analytical methods based on micro- and nanotechnology solutions for the safety and quality assurance along the food chain in the agro-food industry (http://www.goodfood-project.org/www/results/). Key projects in the long list of solutions include the development of a sensor prototype to detect foodborne pathogens in dairy products, Electrochemical Magneto Immunosensing which is a novel strategy for the detection of pesticides residues, DNA sensing of *Aspergillus carbonarius* and other black *Aspergilli* on grapes with a direct multi-detection DNA chip, rapid PCR screening method for identification of *Aspergillus* species, an innovative e-nose approach for food quality assessment, highly sensitive IR-optical sensor for ethylene-monitoring, miniaturized gas-chromatographic like system for fish quality assessment, and the FID reader method with onboard sensing capability for monitoring fruit quality.

Nanotechnology is likely to be engaged for effective and efficient amendments of food products by bioactive and smart nano-packaging technology. As active nano-packaging is the main theme of our review, a key approach for superior delivery of active ingredients is "micro- and nanoencapsulation." In the following sections, we have attempted to review novel micro- and nanotechnology trends in active food packaging, their selective formulation techniques and overarching concerns over nano-food regulations.

MICRO- AND NANOTECHNOLOGY TRENDS

Currently the customized mode of application of antimicrobial agents is the direct introduction to the food system in free form. However, the problem lies in the fact that the interaction of these compounds with different food components reduces their efficacy against the pathogens and thus high antimicrobial quantities are required to reduce the microbial number within limit. Therefore, to cope with these drastic problems along with an increase in the potential of antimicrobial activity and stability in complex food systems, researchers have devised "encapsulation of antimicrobials." Though encapsulation methods indicated a significant improvement, but real satisfactory results have been achieved by innovative micro- and nanotechnology applications in active packaging.

Different Inhibitors/Active Agents Incorporated at Micro/Nano-Scale

Bacteriocin

The only bacteriocin that has been approved by the World Health Organization as a preservative in food is nisin (FDA, 2008). Nisin exists in two related forms, nisin A and nisin Z. The two forms differ at amino acid position 27; that is, nisin A includes histidine, whereas nisin Z contains asparagine at this position (Mulders et al., 1999). Nisin has an inhibitory effect against a wide variety of Gram positive foodborne pathogens and spoilage microorganisms (Rodriguez, 1996) and can also act on several Gram negative bacteria as a synergistic effect in the presence of some chelating agent (Alvarez et al., 2007; Aymerich et al., 2005). The FDA critically evaluated available information on nisin, confirmed the GRAS status of nisin, and reported that nisin is safe for human consumption at an Acceptable Daily Intake (ADI) of 2.94 mg/per/day. Nisin is proposed for use as an antimicrobial agent on cooked meat and poultry products sold ready-to-eat at 2.5 mg nisin/lb of cooked meat or poultry product, approximately 5.5 mg nisin/kg of food (FDA: GRAS Notice No. GRN 000065).

Excessive nisin amounts are required for guaranteeing effective pathogen growth inhibition because nisin is structurally unstable in food due to its deprivation by interaction with food and cell matrices, and the development of tolerant and resistant *Listeria* strains (Chi-Zhnag et al., 2004). On the other hand, proteolytic enzymes in the food systems, especially in fresh meat products, are responsible for the inactivation of bacteriocin and thus decreasing antimicrobial efficacy (Degnan et al., 1993). In order to improve the bio-availability, the practical way reported is encapsulation of bacteriocin which limits the degree of its degradation in a food model system. The higher stability of encapsulated nisin may be attributed to its maintenance at a high concentration and purity inside nano-vesicles or immobilized on vesicle membranes (Laridi et al., 2003).

Up till now, literature study reveals that relatively low attention has been paid to microencapsulate bacteriocin in foods. In various pharmaceutical and cosmetic applications liposomes have been employed to protect and control the release of active compounds (Benech et al., 2002a; 2002b; Trie et al., 2001). However, the use of liposomes to encapsulate antimicrobials to improve the microbiological stability and safety of foods has received the attention of investigators just a few years ago (Benech et al., 2002a; 2002b).

In terms of encapsulation of food antimicrobials, existing data relating to the bacteriostatic and bacteriolytic capability of liposomes with entrapped antimicrobials is still limited. In this regard a novel work has been done by gathering data relating to liposomes in a fine review (Taylor et al., 2005). The ability of liposomes to withstand exposure to environmental and chemical stresses typically encountered in foods and food processing operations was analysed by the encapsulation efficiency (EE), ζ-potential, and particle size distribution. Liposomes consisting of distearoylphosphatidylcholine and distearoylphosphatidylglycerol, with nisin entrapped, retained 70–90% EE despite exposure to elevated temperatures (25–75°C) and a range of pH (5.5–11.0). Results suggest that liposomes may be an appropriate candidate for nisin entrapment in low- or high-pH foods with light heat treatment tolerance (Taylor et al., 2007).

Benech et al. (2002b) investigated that the encapsulation of nisin Z in liposomes can provide a powerful tool to improve nisin stability and inhibitory action. The inhibition of *Listeria innocua* in cheddar cheese was evaluated during six months of ripening by adding purified nisin Z in liposomes to cheese milk

and compared by in situ production of nisin Z by *Lactococcus lactis* subsp. *lactis biovar diacetylactis* UL719. Immediately after cheese production, 3- and 1.5-log-unit reductions in viable counts of *L. innocua* were obtained in cheeses with encapsulated nisin and the nisinogenic starter, respectively. After 6 months, cheeses made with encapsulated nisin contained less than 10^1 CFU of *L. innocua* per g and 90% of the initial nisin activity, compared with 10^4 CFU/g and only 12% of initial activity in cheeses made with the nisinogenic starter (Benech et al., 2002b). The encapsulation of nisin in phospholipid helps the starter culture against the inhibitory effects of nisin (Benech et al., 2002a).

Different proliposomes (Pro-lipo H, Pro-lipo S, Pro-lipo C, and Pro-lipo DUO) were tested for their capacity to encapsulate nisin Z ranging from 9.5% to 47%. The increase in the cholesterol content in lipid membranes up to 20%, w/w, resulted in a slight reduction in EE. The pH of nisin Z aqueous solution and nisin Z concentration had a significant effect on the amount of the encapsulated nisin (Laridi et al., 2003). Nisin insertion and subsequent perturbations of permeability in lipid membrane are increased with increasing negatively charged lipids and unsaturated phospholipids (El Jastimi et al., 1999). Liposome H may thus be less susceptible to the nisin-membrane destabilizing action compared with the other tested liposomes as 85% of the total phospholipid content is phosphatidylcholine (zwitterionic lipids).

In a meat model system, the entrapment of pediocin AcH in liposomes (18% entrapment efficiency) made from phosphatidylcholine improved the antilisterial activity of pediocin compared with free pediocin. In case of direct incorporation, a decrease in pediocin activity (12–54% recovery of original activity) occurred. Higher pediocin activity (29–62% increase; average over all concentrations) was recovered from the model food system containing encapsulated bacteriocia as compared to free pediocin AcH. The additional recovery of pediocin activity provided by liposomes declined the potential for the direct application of biopreservatives (Degnan and Luchansky, 1992). It has been suggested by the author that encapsulation with higher melting point lipids or different polarity of the capsule might enhance the efficiency for delivering bioactive agents in the food system.

From the dairy industry point of view, the use of nisin in free form is costly and has drawbacks of lower stability, lower activity, and reduced bioavalability (Roberts and Zottola, 1993). The other disadvantages of using free nisin include possible interference with the cheese-making process, inhibition of cheese starter culture, and loss of lactic acid bacteria needed for ripening and flavor development (Buyong et al., 1998). The distinct advantage is that if the encapsulation material is made of natural ingredients it proves beneficial for our health (Huwiler et al., 2000; Lahiri and Futerman, 2007; Thompson and Singh, 2006). This is how researchers has been forced to think and opt for micro- and nano-encapsulation of antimicrobials to avoid the possible defects of using them in free form and on the other hand having the advantage of their effective, stable, and longer bioavailability.

The addition of nisin, free or incorporated in micro-particles, did not influence cheese proteolysis and volatile compound profile. Nisin, free or incorporated in calcium alginate microparticles, was added to pasteurized milk (80% cows' and 20% ewes' milk) used for the manufacture of Hispanico cheese. The efficiency of nisin incorporation in calcium alginate microparticles was calculated to be 94% (Garde et al., 2003). Incorporation of calcium alginate is preferred because of its food-grade additive status, low cost, and simple incorporation process. Another study has revealed that Liposome-encapsulated nisin did not appear to affect cheese proteolysis, rheology, and sensory characteristics. Cheeses with added *Lb. casei* and liposome-encapsulated nisin Z exhibited the highest flavor intensity and were preferred as the best for sensory parameters (Benech et al., 2003).

The mode of application of the antimicrobial agent in the food system is crucial for success. Free nisin has lower accessibility to bacterial cells in cheese milk because a majority of the nisin adheres to fat and protein surfaces. On the other hand, this method is expensive, non-homogeneous, and may result in cheese starter inhibition (Roberts and Zottola, 1993). Similarly the growth, acid, and aroma production of starter cultures might be affected by the addition of nisinogenic strains, which results in an inferior quality product. To avoid such phenomenon, nisin-producing strains should be combined with nisin, a resistant or tolerant starter culture, to ensure a proper balance between lysed and intact cells. The optimum composition of the *Lactococcus lactis* UL 719/commercial flora Danica (FD) mixed culture, 0.6 /1.4%, may offer Gouda cheese with a greater control over undesirable microflora in cheese, both from the perspective of cheese quality as well as safety (Bouksaim et al., 2000). Microencapsulation has a great deal of stability, protection and controlled release in pharmaceuticals, agricultural chemicals, and food ingredients. In this regard three excellent reviews have been published (Champagne and Fustier, 2007; Gouin, 2004; Madene et al., 2006).

Wan claimed that calcium alginate micro-particles containing nisin provide substantial stability against enzymes. The incorporation efficiency of micro-particles smaller than 150 μm was 87–93% and the nisin in the alginate-incorporated form was 100% active against an indicator culture of *Lactobacillus curvatus*. A formulation of 1:9 provides the best barrier against proteolytic inactivation of nisin (Wan et al., 1997). The data reveal that micro-particles of food grade polymers provide a more effective system than liposome encapsulation but to realize the actual potential of this technology requires further research.

A long-lasting antibacterial activity was displayed by Nisin-loaded poly-L-lactide nano-particles produced by gas precipitation which was maintained through 1000 h with the in vitro release results. Due to controlled release these nisin-loaded nano-particles showed a weaker antibacterial activity within the first 3 h incubation as compared to free nisin samples. Interestingly, degraded or cell taken up nisin is replaced by active nisin freshly released by the particles throughout the experiment (Salmaso et al., 2004). It is quite

apparent that protein stabilization and slow release is necessary to yield a long-lasting efficient antibacterial activity.

To give a targeted controlled release is a key functionality for which several techniques have been investigated. Nisin embedded packaging materials or nisin adsorbed solid surfaces such as polyvinilic or polysaccharide films allowed for prolongation of the biological activity (Suppakul et al., 2003; Conte et al., 2006; Cha et al., 2003; Coma et al., 2001; Natrajan and Sheldon, 2000; Siragusa et al., 1999; Ugurlu et al., 2007). Nisin-loaded polymeric micro-/nano-particles seem to be promising formulations to achieve long-lasting antimicrobial activity. These polymeric micro- /nano-colloids are physically stable and can be easily formulated with a variety of materials obtaining the controlled release rate of the active agent (Salmaso et al., 2004).

Mode of Application

In terms of encapsulation of various active agents, a comparison of micro- and nanotechnology data of encapsulation have been gathered with important parameters from the origin till the end use (Table 1).

Lactoferrin

To avoid foodborne illness outbreaks, one of the effective natural antimicrobial for food systems is lactoferrin (LF) which is the main iron-binding glycoprotein present in the milk of mammals that controls bacterial, fungal, or parasitic growth (Franklin et al., 2004; Al-Nabulsi and Holley, 2007; Kim et al., 2004; Pan et al., 2007a; 2007b; Lao et al., 2001; Al-Nabulsi and Holley, 2005). LF enhances the growth of probiotic bacteria like bifidobacterium and meanwhile it also shows antioxidant, antiviral, anti-inflammatory, and anti-cancer qualities (Kim et al., 2004; Lao and Brock, 2001). Lactoferrin occurs naturally in milk and milk-derived ingredients and products. Thus, people who consume milk or milk-derived ingredients already consume lactoferrin. The animal toxicity studies demonstrate that there are no adverse effects related to the consumption of milk-derived lactoferrin at levels up to 2000 milligrams/kilogram/day (FDA: GRAS Notice No. GRN 000042).

Lactoferrin has been used as a potential antimicrobial in coatings but the cations including Na^{+}, Ca^{2},and Mg^{2} interfere with its activity (Franklin et al., 2004; Al-Nabulsi and Holley, 2005). To avoid this microencapsulation and controlled release technology has found broad applications. Paste-like microcapsules were incorporated in edible whey protein isolates (WPI) packaging film to test the antimicrobial activity of LF against a meat spoilage organism *Carnobacterium viridans*. The film was applied to the surface of bologna after its inoculation with the organism and stored under vacuum at 4 or 10°C for 28 d. The growth of *C. viridans* was delayed at both temperatures and microencapsulated LF had greater antimicrobial activity than when unencapsulated (Al-Nabulsi et al., 2006).

Lysozyme

Lysozyme is of interest for use in food systems since it is a naturally occurring enzyme with antimicrobial activity. Lysozyme can be derived from eggs, plants, bacteria, and animal secretions; it is commercially used to inhibit the growth of *Clostridium tyrobutyricum* in cheese (Min et al., 2008). Eggwhite lysozyme is proposed for use as an antimicrobial agent on cooked meat and poultry products sold ready-to-eat at 2.0 mg eggwhite lysozyme/lb of cooked meat or poultry product, approximately 4.4 mg eggwhite lysozyme/kg of food (FDA: GRAS Notice No. GRN 000064). Acceptable Daily Intake "not specified" means that, on the basis of the available data (chemical, biochemical, toxicological, and other), the total daily intake of the substance arising from its use at the levels necessary to achieve the desired effect and from its acceptable background in food does not, in the opinion of the Committee, represent a hazard to health.

Entrapment efficiency, the mean average size, and the stability of the commercially available form that contains 2.5% nisin and lysozyme, are influenced by lipid composition. Encapsulation of commercial nisin extract and lysozyme in PC-, PG-, and cholesterol containing liposomes was achieved and the highest concentration of antimicrobials was encapsulated in 100% PC liposomes but also resulted in higher leakage. The antimicrobial loading was decreased by the addition of cholesterol and PG but cholesterol decreased leakage of PC liposomes (Gregoriadis and Davis, 1979; Hsieh et al., 2002). Application of nisin and lysozyme affected liposome stability; nevertheless, the intact encapsulated liposomes were physically stable for 2 weeks (Were et al., 2003). We can assume that for microbiological stabilization of food products, stable nanoparticulate of polypeptide antimicrobials can be achieved by selecting suitable lipid-antimicrobial combinations.

Silver

Silver compounds have been widely used as broad-spectrum antimicrobials in a variety of applications including dental work, catheters, and burn wounds (Dibrov et al., 2002). Silver ions inactivate vital bacterial enzymes and recent works have shown that silver ions, trapped with zeolites (inorganic ceramic) have the potential for inactivation of vegetative bacterial cells (Cowan et al., 2003; Galeano et al., 2003; Jiang et al., 2004). Silver nitrate is used as an antimicrobial agent, in an aqueous solution with hydrogen peroxide, in bottled water. Silver nitrate is recommended as values NTE (not to exceed) 17 μg/kg in the treated bottled water, and hydrogen peroxide NTE 23 mg/kg (FDA Food Additives Reg. 172.167). FDA is currently evaluating the potential toxic/carcinogenic effects resulting from exposure to dietary supplements and nanoscale-sized particles associated with food containers, food preparation surfaces, and food wraps (www.fda.gov/AboutFDA/WhatWeDo/track/ucm203296.htm). This research will help the

Table 1 Between and within comparison of micro- and nanotechnology systems of encapsulation

Antimicrobial	Encapsulation Method	Carrier/Polymer Carrier/Polymer	Size	Encapsula-tion efficiency EE (%)	Residual activity(%)	Target Organism	Log unit reduction	Medium/Product	Duration of study	Reference
Micro-scale										
Lactoferrin	Paste like microcapsules	WPI	1–4 μm	–	36–40	*Carnobacterium viridans*	2–3	Bologna	28 days	(Al-Nabulsi et al., 2006)
Nisin	Micro-particles	Calcium alginate	< 150 μm	87–93	25–75	*Lactobacillus curvatus*	Below detection limit	MRS, Skim-milk	4 weeks	(Wan et al., 1997)
Lactoseperoxidase system	Acacia gum core and spray drying	Caseinate, paper microbeads				Several	5–30 cm^2 inhibition zone	Soy agar	48 hours	(Jacquot et al., 2004)
Micro- to Nano-scale										
Nisin Z	Liposome	Pro-liposomes (H,S,C & DUO)	140–2400 nm	9.5–47	20–39	*Pediococcus acidilactici UL5*	4–7	Milk, whey, PBS	27 days	(Laridi et al., 2003)
Chitosan Nano-silver Ag-zeolite Montmorillonite	Solvent casting	Chitosan	79 nm-<5 μm	–	–	*Staphylococcus aureus Listeria monocytogenes Salmonella* Typhimurium *Escherichia coli*		Soy agar	8 hours	(Rhim et al., 2006)
Nano-scale										
Nisin Z	Liposome	ProliposomeH	80–120 nm	47	90	*L. innocua*	3	Cheese	6 months	(Benech et al., 2002)
Nisin A	CO_2 anti-solvent ppt	Poly-L-lactide	200–400 nm	78–81	81–84	*Lactobacillus delbrueckeii*	4–5	MRS medium	40 days	(Salmaso et al., 2004)
		PC	144 nm	63		—	—	—	2 weeks	(Were et al., 2003)
		PC/cholesterol (70/30)	223 nm	54						
		PC/PG/cholesterol (50/20/30)	167 nm	59						
Nisin	Liposome									
Nisin Z	Liposome	ProliposomeH	80–120 nm	-	92	*Listeria Lactococcus* and *Lactobacillus*	0–3	Cheese	2–6 months	(Benech et al., 2002)
Nisin Z	Liposome	Prolipo H	app 740 nm	34.6	93	*Lactobacillus casei*	0.5	Cheese	6 months	(Benech et al., 2003)
Nisin	Liposome	PC PC-cholesterol (7:3) PC- PG-cholesterol (5:2:3)	100–200 nm	29–38	—	*L. monocytogenes*	2	Soy agar	30 hours	(Were et al., 2004)
Nisin	Liposome	Disteroylphosphadyl-choline Disteroylphosphadyl-Glycerol	100–240 nm	72–91	70–90	—	—	—	—	(Taylor et al., 2007)
Lysozyme	Liposome	PC PC-cholesterol (7:3) PC- PG-cholesterol (5:2:3)PC	100–200 nm	19–43	—	*L. monocytogenes*	strain dependent -	Soy agar	30 hours	(Were et al., 2004)
Lysozyme	Liposome	PC/cholesterol (70/30)	161 nm	61	—	—	—	—	2 weeks	(Were et al., 2003)
		PC/PG/cholesterol (50/20/30)	162 nm 174 nm	60 61						
Silver	Plasma depositing	Polyethyleneoxide	90 nm	—	—	*Alicyclobacillus acidoterrestris*	2	Apple juice, malt	100 hours	(Del Nobile et al., 2004)
Silver	Plasma	Rubber, steel, paper	nano-particles	—	—	*L. monocytogenes*	4–5	Polmer surface	24 hours	(Jiang et al., 2004)

FDA determine the health effects from exposure to dietary supplements and from nano-particles associated with food preparation materials. A complete analysis of pharmacokinetic studies of nanosilver particles administered by intravenous injection is ongoing.

The use of nanoparticle metallic silver particles as an antimicrobial agent in polyurethane coatings has been achieved which are applied on particular parts of food packaging machines and also on food handling robots in order to reduce the risk of bacterial contamination (Wagener et al., 2006). In the United States, the Food and Drug Administration has added to its list of food contact substances an Ag^+ based system-AgION®. *Alicyclobacillus acidoterrestris* is Gram positive, spore-forming bacteria which is a spoilage agent in acidic beverages. The effectiveness of Ag^+-based antimicrobial film in inhibiting the growth of an *A. acidoterrestris* strain in acidified malt extract broth and apple juice was investigated. The results indicate a 2 log comparative reduction in viable microbial count of thermal resistance microorganism in acidic beverages (Vermeiren et al., 2002). Concerns relating to the amount of silver used in edible films should not be neglected during the production of such active films.

Silver nanoparticle thin layers were deposited onto medical- and food-grade silicone rubber, stainless steel, and paper surfaces. The antimicrobial properties of the silver-coated surfaces were demonstrated by exposing them to *Listeria monocytogenes*. No viable bacteria were detected after 12 to 18 h on silver-coated silicone rubber surfaces thus 4–5 log reduction was achieved (Jiang et al., 2004) These results depict that silver is one of the strongest bactericide and it becomes more effective as nanoparticles.

Chitosan

The uses of renewable resources to produce edible or biodegradable packaging materials that can improve product quality and reduce waste disposal problems are being explored. The literature available for natural biopolymer-based nanocomposite materials is limited, especially concerning antimicrobial films; (See excellent reviews in this regard by Pandey et al., 2005 and Ray and Bousmina, 2005). Chitosan, the second most abundant biopolymer, safe, and non-toxic, holds antibacterial and antifungal properties (Agnihotri et al., 2004; Kim and Kang, 2007). The recommended dose of chitosan as a dietary supplement is 1.5 to 3.0 grams per day. This dosage has been recommended based on the results of clinical trials with human volunteers. It is possible that the consumption of chitosan may provide health benefits from additional dietary fiber intake. Further, no untoward effects have been reported in multiple studies involving chitosan consumption by human volunteers consuming similar amounts of chitosan (FDA: GRAS Notice No. GRN 000073).

A recent study has been done to develop biodegradable antimicrobial bionanocomposite films with acceptable properties for applications in food packaging using biopolymer such as chitosan, as well as Nano-silver, silver zeolite, and nanoscale layered silicates. Tensile strength increased by 7–16%, whereas water vapor permeability decreased by 25–30% depending on the nanoparticle material tested. While the silver containing ones chitosan-based nanocomposite films depicted a potential of antimicrobial activity (Al-Nabulsi and Holley, 2006). The chitosan films containing silver nanocomposites depicted an excellent potential of antimicrobial activity.

Lactoperoxidase System (LP-s)

In developing countries the use of LP-s system as temporary preservation of raw milk is increasing (Kussendrager and Van Hooijdonk, 2000). The major inconvenience is mixing of the components, i.e., lactose peroxidase, hydrogen peroxide oxidoreductase, thiocyanate, and H_2O_2, for which encapsulation as microbeads has been tested. LP-s microbeads are easily incorporated inside protein films and remain active after drying (Jacquot et al., 2004). There are some limitations to the application of this system at nano-scale because it is just not an antimicrobial peptide, enzyme, or metal but a whole system consisting of 4 distinct components. Already it is an excellent effort to design such micro-particles, but on the contrary real success of this system has not been realized as LP-s is approved just for raw milk application by FDA. The EU support the proposal by New Zealand to amend footnote 9 in Appendix A: "Any trade in milk treated by the lactoperoxidase system should only be on the basis of mutual agreement between countries concerned, and without prejudice to trade with other countries." (Codex Alimentarius Commission, 32nd Session, 2009, ALINORM 09/32/9C).

Potential Advantages of Nanotechnology Concerning Active Agent Incorporation in Food

Owing to micro- and nanotechnology numerous advantages can be availed of as these potentials have been the driving force to carry out investigations in the promising field of active food packaging, as shown in Table 2. The research work found in literature proves that the evolution from micro- to nano-scale has amplified the listed benefits.

Eventually micro- and nano-encapsulation has brought a revolution because of the fact that by utilizing these techniques not only the control against foodborne pathogens is more effective but also long-lasting, thus enabling the researchers and industrialists to provide a healthier, safer, and an enhanced shelf-life food to the consumer. It is true that micro- or nano-entrapment in liposomes enhances nisin stability, availability, and distribution which may improve the control of undesirable bacteria in foods stored for long periods. It can be proposed that allergenicity and product labelling concerns are expected to be minimal as liposomes used in micro- and nano-technology are generally formed from lipids that are naturally occurring in various food staples. Therefore it is very crucial to induce further work in the nano-active-packaging, where natural antimicrobials would

Table 2 Potential advantages of micro- or nanotechnology for novel trends

Potential advantages	References
Protection	
To reduce or prohibit bacteriocins affinity to food components	(Laridi et al., 2003)
Safeguard antimicrobial peptide from inhibitors or unfavorable conditions in food matrix	(Laridi et al., 2003)
Stability	
Act as long term preservative in foods for long periods of time	(Laridi et al., 2003)
Avoid interference with lactic starter growth during fermentation	(Laridi et al., 2003)
Relatively stable to pasteurization protocols	(Taylor et al., 2007)
Improved viability of probiotics for future use	(Picot and Lacroix, 2004)
Cost-effective	
Economical as relatively lower dose of antimicrobial is required as compared to free form	(Roberts and Zottola, 1993)
Functionality	
Comparatively enhanced activity and bioavailability	(Roberts and Zottola, 1993)
Isolation/Immobilization	
No or lesser harmful effects on organoleptically important bacteria e.g. LAB for flavor	(Buyong et al., 1998)
Use of active ingredients in low- or hi-pH-foods e.g. nisin is pH-sensitive compound	(Taylor et al., 2007)
Controlled release	
Release of active agents can triggere by physical or chemical stress	(Lee and Rosenberg, 2000)
Effective and easier lactoseperoxidase system provision	(Jacquot et al., 2004)
Controlled release of active ingredients	(Sorrentino et al., 2007)
Nutrition	
Biopolymers for nano-encapsulation are usually edible hence nutritive	(Thompson and Singh, 2006)
Structurization	
High mechanical and barrier properties with nanocomposite	(Rhim et al., 2006)
Nano-particles disperse and act as reservoirs of active ingredients	(Rhim and Ng, 2007)
Senso-textural	
Certain masking effect without destroying texture is possible	(Nachay, 2007)
Optimization	
Integration of nanosensors for detection of pathogen	(Nachay, 2007)
Application of nanotechnology promise to expand the use of edible and biodegradable packaging	(Chen et al., 2006; Rhim and Ng, 2007)

be used with or instead of conventional preservatives utilizing micro- or more preferably nanotechnology to improve the shelf life and safety of perishable foods.

MICRO- AND NANO- ENCAPSULATION TECHNIQUES IN FOOD PACKAGING

Nanoencapsulation is the expertise of wrapping solids, liquids, or gaseous materials in minuscule, conserved capsules that may liberate their contents at controlled rates under particular conditions of the food matrix (Taylor et al., 2005; Champagne and Fustier, 2007; Sukhorukov et al., 2005). With the emergence of nanotechnology, ongoing research has been focused towards nanoencapsulation to attain even more stability, versatility, and effectiveness.

The review of literature reveals a number of excellent review papers on nano- and microencapsulation technologies, which are the applications of nano- and microencapsulated ingredients in the food industry (Gandhi and Chikindas, 2007; Taylor et al., 2005; Gouin, 2004; Madene et al., 2006; Anal and Singh, 2007; Mozafari et al., 2006; Peniche et al., 2003; Yih et al., 2006). The scientific data with respect to active ingredients have been excellently summarized (Champagne and Fustier, 2007; Desai and Park, 2005; Graveland-Bikker and de Kruif, 2006; Lopez-Rubio et al., 2006; Ubbink and Kruger, 2006). The development of innovation lies in the concept of micro- and nanoencapsulation and controlled release of bioactive components or nanocomponents from biodegradable/edible and/or sustainable packaging systems. We attempt to illustrate the principles of different approaches in micro- or nanoencapsulation of active ingredients.

The aim of this review is to give critical perspectives of nano- and microencapsulation with reference to active packaging in the food industry, their strengths/weaknesses, and the mode of release.

Spray drying is the most extensively used microencapsulation technique in the food industry (flavor, vitamins, lipids, etc.); however, the customary aqueous state demand, low solubility, and high water evaporation makes it tiresome. Spray drying makes use of several coating materials such as protein: sodium caseinate, soy protein, whey protein, gelatin; cellulose: hydroxyl propyl methyl cellulose (HPMC), carboxy methyl cellulose (CMC); lipids: fatty acids, cholesterol, wax; carbohydrate: chitosan, etc. (Heurtault et al., 2003; Kolanowski et al., 2004; Loksuwan, 2007).

Nanoparticle suspension might be stabilized by water elimination into re-dispersible dried solid particles in the presence of different water-soluble excipients as drying auxiliaries (Tewa-Tagne et al., 2007). Thus spray drying is a striking technique for improving the nanoparticle conservation. But in the field of antimicrobial packaging, the use of spray drying for nanoencapsulation has not been introduced yet.

In contrast spray drying, spraychilling/cooling does not employ the evaporation of water. A molten medium with low

melting point containing the bioactive compound is atomized through a nozzle into a vessel. The cold air in the vessel enables the solidification of the gel particle into a fine powder particle. The liquid droplet thus solidifies and entraps the bioactive product. Nevertheless, due to little choice of covering material (high melting point), the spray-chilling application is near the ground in food packaging nanoencapsulation. This technology is utilized for lipids such as potassium iodate, retinyl palmitate, and ferric pyrophosphate which were microencapsulated in hydrogenated palm fat by spray cooling for food fortification purposes(Wegmuller et al., 2006).

Extrusion Coating is a relatively new technique of encapsulation but due to a number of limiting draw backs (Table 3), no research work had been carried out using this process for controlled release of antimicrobial agents as core material.

Several controlled release options are feasible with Fluidized Bed Coating because this technology is capable of applying a uniform layer on solid particles with almost every type of coating materials. The cost is also low because of the fact that no energy for evaporation is engaged. Conversely, Desai and Park (2005) have suggested that the food industry should implement a somewhat different approach to this rather costly technology (Desai and Park, 2005). The fluidized coating methods, top-, bottom-, and tangential-spray, are capable of encapsulating very tiny particles as minute as 100 μm. A more ample fluidized bed coating process and application in the food industry had been reviewed (Dewettinck and Huyghebaert, 1999). Up till now, this technique of encapsulation has been used for vitamin, iron, and certain salts for preservation but none of the work refers to micro- or nanoencapsulation of antimicrobial peptides/bacteriocin.

Calcium alginate beads have food-grade additive status, they are low cost, and have a simple incorporation process with high encapsulation efficiency. Successful incorporation (around 90% encapsulation efficiency) of nisin has been done at a micro-scale (150 μm) and further size reduction can be done by grinding and sieving for nano-scale (Garde et al., 2003; Wan et al., 1997). The arresting problem in this system is faster diffusion which may arise due to chelating of calcium with phosphate, citrate, and acetate in the food system. One striking approach may utilize the alginate beads in combination with successful encapsulation material like liposome.

The coacervation nano- or microencapsulation principle is the phase separation of one or more hydrocolloids from the initial solution and the subsequent deposition of the newly formed coacervate phase around the active ingredient suspended or emulsified in the same reaction media (Wu et al., 2005). In simple coacervation, the hydrophilic colloid is deprived of the solvent by "salting out" (addition of a competing hydrophilic substance, such as a salt or alcohol). Complex coacervation is generated by mixing two oppositely charged polyelectrolytes. The polyelectrolyte complex/coacervate complex separates into a polymer rich phase that coexists with a very dilute phase (Peniche et al., 2003).

Capsaicin microcapsules were prepared by the complex coacervation of gelatin, acacia, and tannins and indicated that the capsaicin microcapsules displayed potential antimicrobial applications in food storage (Xing et al., 2006). The problem of the controversial cross-linker formaldehyde/glutaraldehyde was altered by glycerol, a good potential non-toxic cross-linking material for the applications of encapsulation (Huang et al., 2007). In the coming years, coacervation might be widely used because

Table 3 Comparison of different micro- or nanoencapsulation techniques

Encapsulation method	Strengths	Weaknesses
Spray drying	Continuous basis operation	High temperatures use
	Economical, comparative lower cost	Only water-soluble wrap material can be used otherwise expensive.
	Flexible, adjustable to processing equipment	
Spray chilling	Least expensive	High melting point shell material (mostly fat) microcapsules are insoluble in water
Extrusion	True encapsulation (complete wrap)	Payload is low
	Long shelf life	Salute leakage during extrusion large particle formation 500–1000 μm.
		High temperature and pressure may cause degradation of active agents.
Fluidized bed	Broad options of coating material	For effective coating, atomized coating droplets
	Variety of control release possibilities Increased shelf life	should be smaller than encapsulated particle. Coating imperfections
Alginate beads	Easy preparation on small scale	Fast diffusion
	Encapsulate all materials	Very expensive process and a complex system
Coacervation	Very high payloads achievable	Cross-linker has to deal with food regulatory laws
	Sustained release	
Liposome	Simple production methods	Delivery cost of liposome-encapsulated ingredients (aqueous form)
	Stability in high water activity environment	Nisin may disrupt liposomal membrane
	High encapsulation efficiency of large unilammelar vesicles	
	Targeted delivery in food stuff (content, T°)	
	Non-toxic and acceptable for foods	
	Range of size; few nm to several micron	
	Liposome can withstand dairy fermentation cycle	
	Food sensory and rheological properties appear intact	
	Cholesterol addition may improve stability/integrity	
	Phospholipids of natural source is easy on the pocket	

of a very high payload achievable while the only constraint for the moment is the price tag.

Self-assembly of the hydrolyzed milk protein α-lactalbumin leads to long, straight, and stabilized nanotubes containing a special feature "cavity." The characteristics of the alpha-lactalbumin nanotube make it an interesting potential encapsulating agent, like the 8-nm cavity and the controlled disassembly (Raviv et al., 2005). They withstand conditions similar to a pasteurization step in the food industry. As α-lactalbumin is milk protein, it will be fairly easy to apply the nanotubes in food or pharmaceutical applications in the future.

Whether it is nano- or micro-scale, liposome encapsulation has become equally effective and admirable. Recent studies suggest that liposomes are even naturally present in the very first food we take, namely breast milk (Keller et al., 2000; Keller, 2001).

To date, however, in comparison with pharmaceutics and cosmetics, little use of micro- or nanoliposome has been made in the food industry. Regarding encapsulation of antimicrobial compounds (nisin, pediocin, lysozyme), different formulations of lipids has given significant results for inhibiting *Listerial spp.* in dairy and meat food matrix. Thus nanoliposomes are commanding the means to inhibit the growth of pathogenic organism in food matrix, while preventing the harmful effect of antimicrobials on the food's actual senso-textural properties (Laridi et al., 2003; Benech et al., 2002a; 2002b; 2003; Taylor et al., 2005; Degnan and Luchansky, 1992; Were et al., 2003; 2004). For bacterial targeting a new "Mozafari method" for nanoliposome preparation has been devised. This method is based on heating treatment, and the liposomal ingredients were added to a preheated (60°C, 5 min) mixture of nisin and glycerol. The mixture was further heated (60°C) while stirring (approx. 1000 rpm) on a hotplate stirrer for a period of 45–60 min under nitrogen atmosphere. The reaction was performed in a home-made glass vessel specially designed by Mozafari, typically in a total volume of 10 ml ultra pure water (Colas et al., 2007).

One critical aspect is the release of active compounds (Fig. 3) like antimicrobial peptides, from the liposome. The different postulates are (1) liposomes are "kinetically stable" for defined period of time, so extrinsic parameters (pH, T°, ionic strength) and intrinsic parameters (phospholipids concentration, composition, entrapped compound nature, and concentration) influence the functionality of liposome (2) "fusion" between the liposome and the pathogen outer membrane (3) core (antimicrobial) material induced leakage (4) liposome membrane permeability which for instance had been reduced by cholesterol (5) diffusion due to low molecular weight of the antimicrobials (6) opening of liposomal membrane releasing antimicrobial in aqueous phase which results in rapid reduction while membrane-immobilized bacteriocin would be delivered over a longer term (7) certain antimicrobial like nisin-induced leakage by changing the membrane structure (8) anonymous interaction between the liposome and the fat globule membrane may result

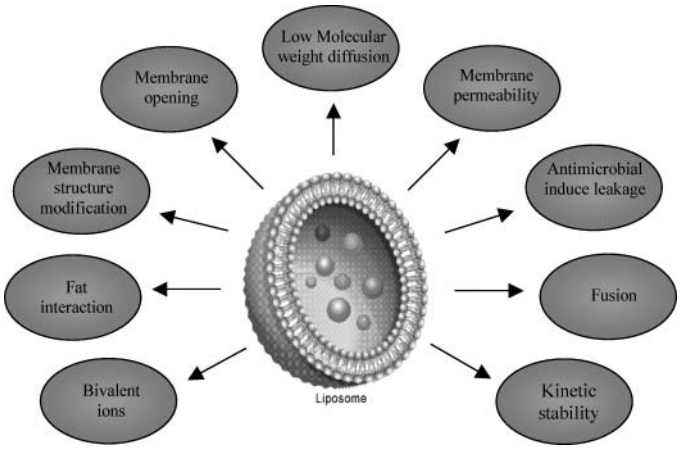


Figure 3 Illustration of active agent release from liposome.

in destabilization of the liposomal membrane and subsequent release of active compounds (9) bivalent ions Ca^{++}/ Mg^{++} induce destability of the liposome especially in the products containing higher bivalent ions concentrations like whey (Laridi et al., 2003; Benech et al., 2002a; Taylor et al., 2005; Were et al., 2003).

Concerning the food industry, the "microencapsulation cost" and the "delivery form of liposome encapsulated components" are key setbacks. The cost issue is most likely resolved by the performance of microfluidization as being cost-effective, having a high encapsulation efficiency, is solvent free, and is a continuous fabrication technique which produces few hundred liters of aqueous liposomes per hour (Maa and Hsu, 1999; Zheng et al., 1999). In our opinion the other issue of delivery, storage, and shipping are quiet non-vital from the food packaging point of view as nano-capsules are incorporated in active coatings of food and delivery conditions and the cost is not supplementary.

The field of nanoencapsulation and release is very promising in terms of potential applications and presents a platform for many applications in food technology, medicine, pharmacy, biotechnology, cosmetics, or detergency (Sukhorukov et al., 2005). Cost considerations in the food industry are much more inflexible than they are for instance in the pharmaceutical or cosmetic industries. The selection of the nanoencapsulation method and coating materials are interdependent. Coating materials can be selected from a wide variety of natural or synthetic polymers, conditional on the material to be coated and features desired in the final nanocapsules. Gouin has roughly estimated a maximum cost for a microencapsulation process in the food industry at 0.1 €/kg considering that the functional ingredients are of minor utilization in foodstuffs (1–5%). Generally the customer will accept a price increase of 0.1 € per portion for a new food product (Gouin, 2004).

In our opinion, for antimicrobial encapsulation, multilamellar vesicles (MLV) liposomes are suitable due to their low

cost (little energy input required), good entrapment efficiency, and maximum storage stability, while for large-scale production high pressure homogenization-microfluidization is considered best because it is fast, efficient, cost-effective, and without organic solvent compulsion. Higher stability, efficient activity of the encapsulated antimicrobial (higher log reduction), controlled release under different physicochemical conditions (% moisture, pH, fat, protein), optimum encapsulation efficiency, long-lasting post-processing protection, natural source and nutritious characteristics, and no regulatory issues are some of the key points which have grabbed the attention of food scientists around the globe and hence there is an emphasis on utilizing liposomes for nano-scale active food packaging.

The amount of material (expensive bioactive agents) required to put forth a specific effect when encapsulated is much less due to improved stability and targeting than the amount required when unencapsulated. A well-timed and targeted release improves the effectiveness of active agents, broadens the application range of food ingredients, and ensures optimal dosage, thereby improving the cost-effectiveness of the product.

SOCIAL, ETHICAL, REGULATORY AND TOXICOLOGICAL ISSUES DEALING WITH NANOTECHNOLOGY

Successful introduction of a new technology requires careful attention to the interactions between the technology and society. These interactions are bi-directional. On the one hand, technology changes and challenges social patterns and, on the other hand, the governance structures and values of the society affect progress in developing the technology (Keller, 2007). Certain scepticism exists in our society between technophiles and technophobes.

Nanotechnology application may raise new challenges in the safety, regulatory, or ethical domains that will require societal debate thus resulting in a sustainable technology which has public acceptance (Helland et al., 2008). General or overview references are provided that will serve as a guide to the literature and indications of the contributions in the field.

Social Issues

All potential applications of nanotechnology significantly affect our lives, our health, our convenience, and our environment; therefore, they tend to trigger major concerns from the public. Unless the concerns related to ethics and social impacts are thoroughly addressed, the progress of nanotechnology could be severely hampered (Sheetz et al., 2005).

Nanotechnology is seen as a transformative technology, which has the potential to stimulate scientific innovation while greatly benefiting society. However, the enthusiasm with which the scientific and technical communities are embracing the technology is being tempered by concerns over possible downsides, including risks to human health (Maynard, 2006). Assuming nanotechnology is a "giga-ideology," a fine review collects the clear standpoints of different members of society (Munshi et al., 2007).

Results of Survey

Even more than scientists and governments, the general public will have the power to shape the direction of nanotechnology development. To avoid a backlash similar to the one created by publicity about genetically modified crops, the nanotechnology community must address and inform the general public; their trust and acceptance must be earned (Sheetz et al., 2005).

A survey conducted at the University of Texas (Fig. 4), including 978 students and staff from non-scientific fields reports that only 17% know what nanotechnology is, while 45% had only heard about nanotechnology, which is larger than the 29% identified by the Royal Society in 2004 (Sheetz et al., 2005).

The results from the study of 1,800 persons in an online survey experiment were released in March 2007 by the Cultural Cognition Project in America. The survey found that over 80% of U.S. respondents had heard "little" or "nothing at all" about

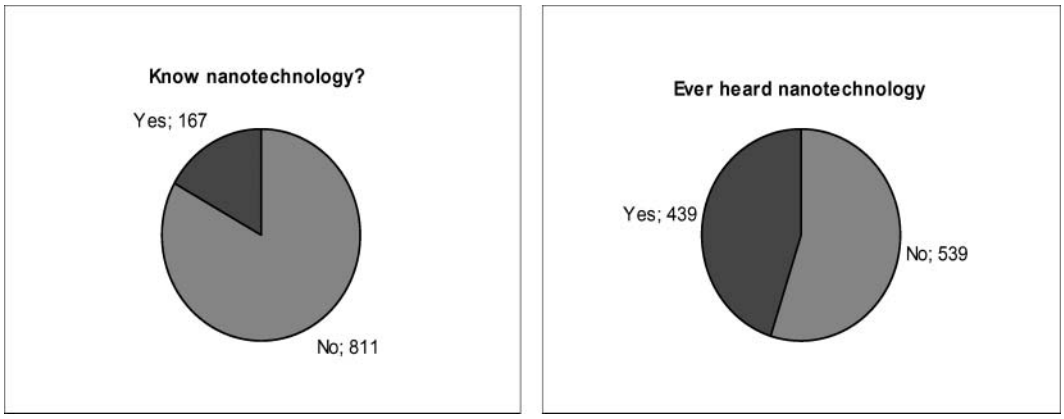


Figure 4 Survey results of nanotechnology knowledge in public (Data from Sheetz et al., 2005).

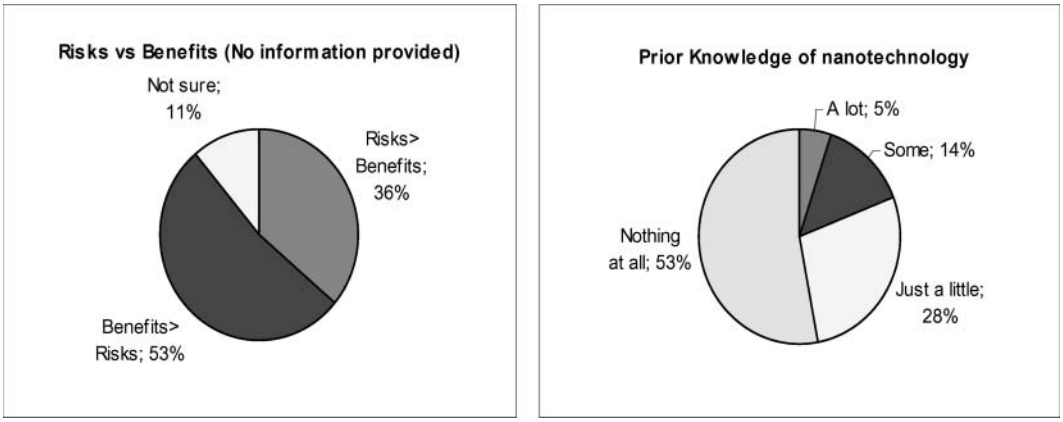


Figure 5 Public opinion of risk vs. benefits of nanotechnology (Data from Kahan et al., 2007).

nanotechnology. Nevertheless, the vast majority of subjects—more than 90%—held an opinion about whether nanotechnology's benefits would outweigh its risks, even when supplied with no additional information (Kahan et al., 2007). A full 81% of the subjects reported having heard either "nothing at all" (53%) or "just a little" (28%) about nanotechnology prior to being surveyed. Only 5% reported having heard "a lot" (Fig. 5).

The perceptions of lay people ($N = 375$) and experts ($N = 46$) in 20 different nanotechnology applications were examined in Germany. The probable dreadfulness of applications and trust in governmental agencies are important factors in determining perceived risks. Lay people perceived greater risks than experts which would diminish if measures were taken to enhance their trust in governmental agencies (Siegrest et al., 2007). One can foresee that the public perception of nanotechnology will be crucial for the realization of technological advances (Roco, 2003).

A survey of American nanotechnology researchers (N = 177) suggests that a wide range of nanotechnologies will likely prove important in the years ahead in a range of areas. Health and technological benefits were identified as more important than environmental benefits and seem to have priority for the need for regulation (Besley et al., 2007).

Perception of Nanotechnology

As always with pioneering science, the pace of social understanding lags behind technological progress (Table 4). The ambiguity of the term nanotechnology is one major problem for policy makers. In recent years the prefix "nano" has come to be associated with almost anything new, small, molecular, atomic, trendy, ominous, or eye-catching. This overhype in order to sell food and entertainment has elicited unreasoned fear in the general public. Food nanotechnology implications are massive and the scientific community should not adopt the policy of wait-and-see. The greater the awareness and understanding of food nanotechnology, the more informed and productive will the decisions be forthcoming from social discussions.

Americans are more optimistic about nanotechnology than Europeans, with almost half of them saying that such technologies will improve the quality of life. Just a quarter of Europeans reported such optimism. Whereas only a quarter of Americans said they did not know anything about the likely impact of nanotechnology, half of the Europeans gave this response (Gaskell et al., 2005). Americans are positive regarding potential health benefits topping the list of key benefits, with invasion of privacy and military uses emerging as key concerns. Many respondents, unsurprisingly, have low trust in business leaders to appropriately manage the technology (MacOubrie, 2006).

The attitude towards actual products has not been studied; instead surveys have been conducted to realize perceptions towards nanotechnology (Sheetz et al., 2005; Besley et al., 2007; Gaskell et al., 2005; MacOubrie, 2006). In the years to come the type of food product/application will influence public perception towards food nanotechnology.

Scientists, futurists, and ethicists agree that nanotechnology has the potential to profoundly shape the human body and the physical and social environments in which humans live because of the extraordinary number of devices, processes, and applications that can be created with this transformative technology (Nickols-Richardson, 2007).

Consumer Education

As with any emerging technology, the full consequences of invasive incorporation into society are currently unknown (Nickols-Richardson, 2007). Policies that acknowledge new risks that may arise from the exploitation of nanotechnology should be designed in order to enjoy benefits. Otherwise, the danger remains that we might stay locked in a "blind spot" where we do not see that we do not see.

Portraying nanotechnology positively by using mass media will be a promising idea as first impression lasts forever. One cannot leave public education in the grip of science fiction movie writers, whose presentation of nanotechnology is misleading. Effective education measures can be taken to spread information. The National Science Foundation has created the "Nano-kid" project for this purpose (Royal Society and Royal Academy of Engineering, 2004). The production of scientists

Table 4 Social and ethical nanoscience and nanotechnology concerns

Nanotech Concern	Strengths	Weaknesses
Nano-divide	If the public in poor countries is well-informed, NT will narrow this divide	NT as every technology is likely to add to this divide between rich and poor
Privacy, safety and security	NT is an economic engine that can redefine the well being nations	If near-invisible devices become available, How one's privacy is protected?
Multi / inter-discipline	NT is capable of enhancing personal safety and security	Who will control access?
	Ubiquitous computing	
Regulation	Combined research from sociologists, scientists, and engineers required	Complex applications realization of NT need coordination & open exchange
Medical concerns	Issues of NT regulation are just beginning to receive attention	Who will regulate it? The question remains open. (Scientists, industry etc.)
		The (existing) gap in healthcare will grow rapidly between rich & poor
Environment issue	Precisely controlled or programmable medical nano-machines and –robots	At what point do we become more machine than human?
Self replication	Drug delivery, cancer treatment, medical imaging, tissue reconstruction. . .	
Media hype	Nanoparticle shell chemotherapy is literally "magic bullet"	Nanoshells for drug delivery may accumulate and cause damage
Economy	Possibility of technological products without dangerous by-products	Nanomaterials (smaller more toxic) may cause tissue and organ damage
Trial-and-error	Mimicking nature	"Grey goo theory," "evil villain"
Work force issues	Movies and television shows are useful to inform public about NT	Mostly the portrayal is inaccurate and is associated with villain not hero e.g. Prey, Xmen, Spiderman, Matrix, Nanobreaker game
Political issues	Enhance production and storage of energy and agricultural products	Destruction of old methods of production and human resource
Intellectual property	Efficient materials and devices with higher strength-to-weight ratios	Difficult to predict the outcomes of experiment for new nanostructures
Human enhancement	Best results in today's practical nanoscience come from this approach	Educated workforce provision from deprived regions
	Training programmes for technicians, undergraduate and master level	Can local politics, ideology and local industries best shape these decisions
	All students study social and ethical issues	Maintain technological leadership
	Share technological development obligation	Rules vary across nations; can they function in nano-oriented economy?
	Laws, regulation and treaties offer protection by patents	Only God should perform such modifications (controversial positive?)
	Enhance the physical and mental capabilities	

Data source: (Lewenstein, 2005; Romig Jr et al., 2007; Sheetz et al., 2005; Uskokovic, 2007).

and engineers who specialize in nanotechnology must increase to meet the demands of the growing industry. Teaching children about nanotechnology is also a great tool to indirectly inform the general public as well (Sheetz et al., 2005).

One of the major concerns in this new field is the development of the necessary manpower. The number of training centers is very low, and greater commitment from the government is needed. Whatever the future, we must ensure that the benefits of nanotechnology research reach all of mankind.

Government Policy and Role

The United States of America has invested billions of dollars in nanotechnology (nearly $3.7 billion for 2005–2008 in the US). Europe has in the meantime an enormously large nanotechnology program (one billion euros, while for the UK alone $45 million per year had been allocated until 2009), billions of Euros worth nanotechnology centers are mushrooming everywhere (Royal Society and Royal Academy of Engineering, 2004). India and The People's Republic of China are investing generously in nanotechnology. The history of technology in general is a history of competition even between friendly neighbors (El Naschie, 2006). These are small investments compared with the $1 trillion return in market shares expected from applications of nanotechnology in the next 6–7 years. On the other hand, it has been extrapolated that nanotechnologies have the potential to create seven million jobs in the global market by 2015 (Roco, 2003). The nanotech areas of greatest scientific uncertainty, such as the toxicology of nanoparticles, should not be exempted from funding (Royal Society and Royal Academy of Engineering, 2004).

Ethical Issues

As a rule, the more prosperous a technology appears to be the more unpredictable the consequences and potential dangers it may entail. Therefore, both sides of a new technology, that

is, its beneficial development and the possible negative consequences, should be investigated in parallel (Table 4). Therefore, the tendency of mainstream science to quickly sell nanotech to the financial community, and to collect money from proposed ideas should be undertaken with extreme care and deliberation (Royal Society and Royal Academy of Engineering, 2004).

Uskokovic has excellently narrated the benefits and risks of nano-biomedicine treatments helping in a variety of useful ways—hyperthermia treatment; drug delivery and targeting; magnetic separation, protein detection, and purification; magnetic field-assisted radionuclide therapy; magneto-relaxometrical diagnostics and eye surgery; the detection of intracellular molecular interactions, with the possibility of developing gene and/or cellular metabolic therapy. Concerns about the possible adverse health effects of nanoparticles include the relative persistence (several months) of nanoparticles in lung tissue; their potential to pass the blood–brain barrier to reach brain tissues and induce damage; and their absorption through the skin into the bloodstream via uptake into lymphatic channels (Uskokovic, 2007). But nanoparticles are ubiquitous in ambient and indoor air; in fact they have been present in the environment since the earliest stages of evolution.

Ethics needs to keep pace with science in order to avoid negative coordination because certain issues are conflicting with religion like trans-humanism (Mnyusiwalla et al., 2003). The ethical debate began with fundamental worries about the place of human beings in the world (playing God, etc.). Indeed, the motto or the hidden agenda of nanotechnology is, "nature does it, why can't we do it"? How can the body be distinguished from a technique which obtains its potential effectiveness exactly from the ability to imitate natural mechanisms and natural functions (Lenk and Biller-Andorno, 2007).

Despite the potential impact of nanotechnology, there is a paucity of published research on its ethical, legal, and social implications. As the science leaps ahead, the ethics lags behind. There is danger of derailing nanotechnology if the study of ethical, legal, and social implications does not catch up with the speed of scientific development (Mnyusiwalla et al., 2003).

Regulatory Issues

One of the ways of gaining the public's confidence is a rigorous regulatory system that assures the safety and efficacy of a new technology. Given the limitations of existing regulatory tools and policies, three distinct initiatives are needed—first, a major increase in nanomaterial risk research; second, rapid development and implementation of voluntary standards of care; and third, development of adequate regulatory policies on risk management (Walsh et al., 2008).

Up to now, there is no international regulation on food nanotechnology or nano-products. Only a few government agencies or organizations from different countries have established standards and regulations to define and regulate the use of nanotechnology. The reader needs to consult the first review published in this field of food nanotechnology regulation (Chau et al., 2007).

FDA states that it regulates "products, not technologies." FDA experts believe that food nanotechnology products present challenges similar to those FDA faces for products of other emerging technologies. FDA experts recognize, however, that product safety and effectiveness can change as the size goes up or down within the nanoscale, adding additional complexity to the product review. The agency expects that many of the nanotechnology products it will regulate will span the regulatory boundaries between drugs, medical devices, and biologics. These, then, would be regulated under the rules established for "combination products" (FDA, 2008). FDA states that particle size is not the issue. If new toxicological risks that are derived from new materials or manufacturing techniques are identified, new safety tests will then be required.

The British Government commissioned the Royal Society and the Royal Academy of Engineers to undertake an independent study about nanotechnologies which was a requirement for public confidence in their safety. The report focused only on the potential health risks from exposure to free engineered nanomaterials (Royal Society and Royal Academy of Engineering, 2004). There are almost no eco-toxicological (including marine) studies with engineered nanomaterials. One of the only studies conducted with aquatic species (fish) suggests that oxidative stress may be a potential mechanism of toxicity associated with free engineered nanoparticles (Oberdorster, 2004). There is a clear need to optimize or develop a range of generic standard test procedures that can be applied to a range of nanomaterials to assess their relative toxicities. Each type of the nanoparticles may need to be assessed one by one because toxicological effects may possess similarities, the exact nature and magnitude of that toxicity may be substance specific, and indeed may vary with size (Owen and Depledge, 2005).

Another excellent review by Nel et al. (2006) concludes that although it is possible that nanoparticles may create toxic effects, there are currently no conclusive data or scenarios that indicate that these effects will become a major problem or that they cannot be addressed by a rational scientific approach. But one should evaluate the safety of nanomaterials and follow a proactive approach to address regulatory issues.

Particle size alone is not a good criterion for differentiating between more or less hazardous materials and technologies but the thing that matters is material structure (Maynard, 2006). As the famous proverb "all things are poison and not without poison; only the dose makes a thing not a poison." The present day regulatory programmes are ineffective because (i) nanomaterials will be far more potent at envisioned low concentrations due to high surface area-to-mass ratio (ii) too little is known to predict nanoproduct toxicity (iii) the nanoproducts are already in the market so the regulatory process lags far behind (Walsh et al., 2008). Voluntary standards are only a temporary expedient; regulatory programs are essential to secure public confidence and support for nanotechnology.

Toxicological Issues

Nanotechnologies cover many aspects, such as disease treatment, food security, new materials for pathogen detection, packaging materials, and delivery systems. As with most new and evolving technologies, potential benefits are emphasized, while little is known about the safety of the application of nanotechnologies in the agro-food sector (Bouwmeester et al., 2009). The concern over the probable adverse effects of nanomaterials on living systems has given rise to "nanotoxicology." However, nanotoxicology has lagged far behind nanotechnology due to a number of experimental challenges and issues faced in designing studies involving toxicological assessment of nanomaterials. The high speed of introduction of nanoparticles-based consumer products observed nowadays urges the need to generate a better understanding about the potential negative impacts that nanoparticles may have on biological systems (Dhawan et al., 2009).

The technology of nanoparticles in the near future involves the incorporation of nano–active agents into packaging materials to increase the barrier properties of packaging materials (e.g., silicate nanoparticles, nanocomposites, and nano-silver, magnesium- and zinc-oxide). When the nanoparticles are applied into the food packaging materials, direct contact with food is only possible following the migration of the nanoparticles. The migration of metals from biodegradable starch/clay nanocomposite films used in packaging materials for its gas barrier properties to vegetable samples, was shown to be minimal (Avella et al., 2005), but more studies are needed to reach a conclusive statement.

It is likely to assume that the use of active packaging releasing nanoparticles with antimicrobial functions into the food (e.g., nano-silver and in rarer cases zinc-oxide nanoparticles), will lead to direct consumer exposure to metals. Hence, this necessitates the need for information on the effects of these nanoparticles to human health. Furthermore, attention should be paid to life-cycle analysis and effects on the environment (Bouwmeester et al., 2009). The EU's approach to nanotechnology is "safe, integrated, and responsible." To that end the EU had demanded its scientific committees and commission services to perform a scientific and legislative review on the suitability of the existing regulation for nanotechnologies. From a number of regulatory reports it became clear that there is currently no nano-specific regulation in the EU. The FDA has no nanotechnology specific regulations, as mentioned before; it regulates "products, not technologies" (Chaudhry et al., 2007).

Human beings are already exposed to a range of natural and man-made nanoparticles in the air, and exposure via the food chain, water supply, and medical applications is likely. Toxicology studies on animals, and cells in vitro, raise the possibility of adverse effects on the immune system, oxidative stress related disorders, lung disease, and inflammation. However, the doses needed to produce these effects are generally high and it remains to be seen if such exposure is possible via the environment or the work place. Data on exposure is also needed for risk calculations (Handy et al., 2007).

Due to lack of methods for the detection of natural/engineered nanoparticles in food matrices, special attention is required for the assessment of nanoscale delivery systems loaded with bioactive compounds. For nanoscale delivery systems, both the amount of bioactive compounds within the capsules as well as the free form in the food matrix has to be determined, because these factors determine the bioavailability and risk associated with it (Bouwmeester et al., 2009).

Optimistic Approach

While the science of nanomaterials and human health impact is maturing, it is still at a stage of raising many more questions than answers. The realization of benefits of nanotechnology should not be hindered by misplaced perceptions of risk to the environment and human health based on poor or no information. Works should not be directed towards erasing the unknown by premature generalizations, but instead towards carefully walking the endless line where the coasts of the known and the sea of the unknown meet. At present, much of nanotechnology constitutes no foreseeable risk to human beings or to the ecosystem (Seaton et al., 2005).

The obvious benefits not only balanced risk against need, but also probably shift public perception of risk. In France and Japan where 80% and 30% electricity is generated respectively from nuclear power, consequently has wider public acceptance than that in the U.S. in spite of the release of activity and waste disposal problems (Keller, 2007).

"How safe is food nanotechnology?" Such a general and unbound question is unlikely to yield useful information on the safety of specific nanotechnologies without further contextual information. Rather, appropriate contexts need to be defined and boundary conditions set if information on the safety of specific nanotechnologies is to be developed (Maynard, 2006). Waiting to address controversial issues until the development is complete may be too late to pacify public concerns.

Food nanotechnology may not be any different than any other area of emerging science and technology. Furthermore, it appears that according to the established methods of quantifying risk in the insurance and risk assessment communities, the fabrication of nanomaterials may present lower risks than those of current activities such as petroleum refining, polyethylene production, and synthetic pharmaceutical production (Robichaud et al., 2005). Studies support the hypothesis that nano-inside (e.g., foods) is perceived as less acceptable than nano-outside (e.g., packaging). Both opponents and proponents of food nanotechnology agree that further research is warranted.

EMERGING CONCEPT OF NANOTECHNOLOGY BLEND WITH 3-BIOS

The next technological revolution in the pasture of food science and nutrition would be 3-BIOS blend with nanotechnology; which refers to Bioactive, Biodegradable, and Bio-

nanocomposite. It is likely to be the smartest development yet to be seen in modern food packaging innovations.

The innovative strength of bioactive packaging lies in the fact that it has a direct impact on the health of the consumer by creating healthier packaged foods. One may conclude, as discussed by Lopez-Rubio et al. (2006) that micro- and nano-encapsulation of the active substances either in packaging and/or within food will make available alternative, more efficient and, in some cases, unique merits to offer food with an improved impact on human health. As the interaction of active compounds with food ingredients influence their efficiency (Devlieghere et al., 2004) nano-encapsulation protection seems one far-reaching solution in food bio-preservation (Teixeira et al., 2008).

Within the scope of natural food preservation for post-production contamination, the application of antimicrobial peptides from lactic acid bacteria (LAB) in bioactive packaging films has received great attention (Cleveland et al., 2001). Despite the broader spectrum of nisin and maintaining its minimum inhibitory concentration (MIC) regarding the probable contaminating pathogens, there is a rising concern about the survival of microorganisms through resistance/immunity resistant against nisin (Naghmouchi et al., 2007). To summarize, there is an immense need for the research of innovative bacteriocins (Rodriguez et al., 2005) and their approval as generally recognized as safe (GRAS) by food regulatory authorities. Thus an ideal and more effective system can be developed for a wide-ranging solution of foodborne illness.

Currently, there is an escalating tendency to employ environmentally-friendly materials with the intention of substituting non-degradable materials, thus reducing the environmental pollution resulting from waste accumulation. To address environmental issues, and concurrently extend the shelf life and food quality, reducing packaging waste has catalyzed the exploration of new bio-based packaging materials such as edible and biodegradable films (Burke, 2006; Tharanathan, 2003). One of the approaches is to use renewable biopolymers such as proteins, polysaccharides, and lipids and their complexes, derived from animals and plants (Ray and Bousmina, 2005). Previous studies show that greater emphasis has been given on safety features associated with biopolymer based antimicrobial packaging (Cha and Chinnan, 2004). Cellulose-based materials are being widely used while animal origin proteins are also increasingly employed. Such biodegradable/edible packaging not only ensures food safety but at the same time is also a good source of nutrition (Khwaldia et al., 2004) but when otherwise used as a food ingredient, neither affects the organoleptic properties of food nor has any regulatory issues (Burdock, 2007).

Unfortunately, as affirmed by Sorrentino et al. (2007) until now the use of biodegradable films for food packaging has been strongly limited on account of the poor barrier and weak mechanical characteristics. To sort out these drawbacks, the application of bio-nanocomposites has emerged as recent technological advancement to reduce the packaging waste and improve the preservation time (Darder et al., 2007; Rhim and Ng, 2007). Nanotechnology carries potential applications in the development of natural biopolymer-based biodegradable packaging material with additional bioactive functions.

Current trends suggest that the next generation of biodegradable/edible and/or bioactive packaging is expected to exploit the substantial possibilities existing for bio-nanocomposite to produce novel packages with superior mechanical, barrier, and thermal performance. The potential benefits of edible films or coatings as carriers of active agents justify continued research in the field of active packaging. Technical challenges exist in incorporating appropriate antimicrobial agents into packaging systems.

CONCLUDING REMARKS AND 21ST CENTURY SHOW

To believe that every technology is dangerous is one thing but to not believe in technology can be very hazardous. The scientific breakthroughs of information technology, the discovery of DNA, nuclear technology, and molecular biology have changed the world we live in. Here in the first decade of the 21st century we now stand on the brink of another scientific innovation—nanotechnology.

In our opinion, owing to nanotechnology application in the food system and especially to active packaging, the probabilities of enhancing food quality, safety, stability, and efficiency as an innovative active packaging are just touching pores of our fingers and in the near future it might be under our grip. As a consequence of nano-active-packaging, a far less amount of material would be required to exert a specific effect which is particularly useful when dealing with expensive bioactive agents. A timely and targeted release may improve the effectiveness of micronutrients, broaden the application range of food ingredients, and ensure optimal dosage, thereby improving the cost-effectiveness of the product.

Nanoencapsulation processes that have been developed for pharmaceutical and chemical applications could easily be adopted in the food industry by substituting food grade coating material for the encapsulation of food ingredients. Future research is expected to focus on aspects of delivery and the prospective use of co-encapsulation concept at nano-scale, where two or more bioactive ingredients can be combined to give a synergistic effect. Migration or diffusion kinetics of the active agents towards the food system will be the prime challenge in the future for food science researchers. The authoritarian permission of novel active compounds and integration of 3-BIOS concept blended with nanotechnology may open the doors to the world of germ-free healthy foods.

REFERENCES

Agnihotri, S.A., Mallikarjuna, N.N., and Aminabhavi, T.M. (2004). Recent advances on chitosan-based micro- and nanoparticles in drug delivery. *J. Control. Release.* **100**: 5–28.

Al-Nabulsi, A.A., and Holley, R.A. (2005). Effect of bovine lactoferrin against *Carnobacterium viridans*. *Food Microbiol.* **22**: 179–187.

Al-Nabulsi, A.A., and Holley, R.A. (2006). Enhancing the antimicrobial effects of bovine lactoferrin against *Escherichia coli* O157:H7 by cation chelation, NaCl and temperature. *J. Appl. Microbiol.* **100**: 244–255.

Al-Nabulsi, A.A., and Holley, R.A. (2007). Effects on *Escherichia coli* O157:H7 and meat starter cultures of bovine lactoferrin in broth and microencapsulated lactoferrin in dry sausage batters. *Int. J. Food Microbiol.* **113**: 84–91.

Al-Nabulsi, A.A., Han, J.H., Liu, Z., Rodrigues-Vieira, E.T., and Holley, R. A. (2006). Temperature-sensitive microcapsules containing lactoferrin and their action against *Carnobacterium viridans* on Bologna. *J. Food Sci.* **71**: 208–214.

Alvarez, I., Niemira, B.A., Fan, X., and Sommers, C.H. (2007). Inactivation of *Salmonella Enteritidis* and *Salmonella Senftenberg* in liquid whole egg using generally recognized as safe additives, ionizing radiation, and heat. *J. Food Protect.* **70**: 1402–1409.

Anal, A.K., and Singh, H. (2007). Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. *Trends Food Sci. Tech.* **18**: 240–251.

Andersson, C., Järnström, L., Fogden, A., Mira, I., Voit, W., Zywicki, S., and Bartkowiak, A. (2009). Preparation and incorporation of microcapsules in functional coatings for self-healing of packaging board. *Pack. Technol. Sci*. **22**: 275–291.

Avella, M., De Vlieger, J.J., Errico, M.E., Fischer, S., Vacca, P., and Volpe, M.G. (2005). Biodegradable starch/clay nanocomposite films for food packaging applications. *Food Chem.* **93**: 467–474.

Aymerich, T., Jofre, A., Garriga, M., and Hugas, M. (2005). Inhibition of *Listeria monocytogenes* and *Salmonella* by natural antimicrobials and high hydrostatic pressure in sliced cooked ham. *J. Food Protect.* **68**: 173–177.

Baer, D.R., Burrows, P.E., and El-Azab, A.A. (2003). Enhancing coating functionality using nanoscience and nanotechnology. *Prog. Org. Coat.* **47**: 342–356.

Benech, R.O., Kheadr, E.E., Lacroix, C., and Fliss, I. (2002a). Antibacterial activities of nisin Z encapsulated in liposomes or produced *in situ* by mixed culture during Cheddar cheese ripening. *Appl. Environ. Microb.* **68**: 5607–5619.

Benech, R.O., Kheadr, E.E., Lacroix, C., and Fliss, I. (2003). Impact of nisin producing culture and liposome-encapsulated nisin on ripening of *Lactobacillus* added-Cheddar cheese. *J. Dairy Sci.* **86**: 1895–1909.

Benech, R.O., Kheadr, E.E., Laridi, R., Lacroix, C., and Fliss, I. (2002b). Inhibition of *Listeria innocua* in cheddar cheese by addition of nisin Z in liposomes or by *in situ* production in mixed culture. *Appl. Environ. Microb.* **68**: 3683–3690.

Besley, J.C., Kramer, V.L., and Priest, S.H. (2007). Expert opinion on nanotechnology: risks, benefits, and regulation. *J. Nanopartic. Res.* **10**: 549–588.

Bouksaim, M., Lacroix, C., Audet, P., and Simard, R.E. (2000). Effects of mixed starter composition on nisin Z production by *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* UL 719 during production and ripening of Gouda cheese. *Int. J. Food Microbiol.* **59**: 141–156.

Bouwmeester, H., Dekkers, S., Noordam, M.Y., Hagens, W.I., Bulder, A.S., Heer, C.D., Voorde, S.E.C.G., Wijnhoven, S.W.P., Marvin, H.J.P., and Sips, A.J.A.M. (2009). Review of health safety aspects of nanotechnologies in food production. *Reg. Toxicol. Pharmacol.* **53**: 52–62.

Brody, A.L. (2006). Nano and food packaging technologies converge. *Food Technol.* **60**: 92–94.

Bugusu, B., and Lubran, M. (2007). International food nanoscience conference. *Food Technol.* **61**: 121–124.

Burdock, G.A. (2007). Safety assessment of hydroxypropyl methylcellulose as a food ingredient. *Food Chem. Toxicol.* **45**: 2341–2351.

Burke, J.R. (2006). Biodegradeable-compostable packaging: The promise and the problems. *Paper Packag.* **47**: 20–26

Buyong, N., Kok, J., and Luchansky, J. B. (1998). Use of a genetically enhanced, pediocin-producing starter culture, *Lactococcus lactis* subsp. *lactis* MM217, to control *Listeria monocytogenes* in cheddar cheese. *Appl. Environ. Microb.* **64**: 4842–4845.

Cagri, A., Ustunol, Z., and Ryser, E.T. (2004). Antimicrobial edible films and coatings. *J. Food Protect.* **67**: 833–848.

Cha, D.S., and Chinnan, M.S. (2004). Biopolymer-based antimicrobial packaging: A review. *Cr. Rev. Food Sci.* **44**: 223–237.

Cha, D.S., Cooksey, K., Chinnan, M.S., and Park, H.J. (2003). Release of nisin from various heat-pressed and cast films. *LWT - Food Sci. Technol.* **36**: 209–213.

Champagne, C.P., and Fustier, P. (2007). Microencapsulation for the improved delivery of bioactive compounds into foods. *Curr. Opin. Biotech.* **18**: 184–190.

Chau, C.F., Wu, S.H., and Yen, G.C. (2007). The development of regulations for food nanotechnology. *Trends Food Sci. Tech.* **18**: 269–280.

Chaudhry, Q., George, C., and Watkins, R. (2007). Nanotechnology regulation and developments in the United Kingdom. **In:** New Global Frontiers in Regulation: The Age of Nanotechnology. pp. 212–238. Hodge, G.A., Bowman, D.M., Ludlow, K., Eds. Edward Elgar Publishing, Cheltam, U.K.

Chen, H., Weiss, J., and Shahidi, F. (2006). Nanotechnology in nutraceuticals and functional foods. *Food Technol.* **60**: 30–36.

Chi-Zhang, Y., Yam, K.L., and Chikindas, M.L. (2004). Effective control of *Listeria monocytogenes* by combination of nisin formulated and slowly released into a broth system. *Int. J. Food Microbiol.* **90**: 15–22.

Cleveland, J., Montville, T.J., Nes, I.F., and Chikindas, M. L. (2001). Bacteriocins: Safe, natural antimicrobials for food preservation. *Int. J. Food Microbiol.* **71**: 1–20.

Colas, J.C., Shi, W., Rao, V.S.N.M., Omri, A., Mozafari, M.R., and Singh, H. (2007). Microscopical investigations of nisin-loaded nanoliposomes prepared by Mozafari method and their bacterial targeting. *Micron.* **38**: 841–847.

Coma, V. (2008). Bioactive packaging technologies for extended shelf life of meat-based products. *Meat Sci.* **78**: 90–103.

Coma, V., Sebti, I., Pardon, P., Deschamps, A., and Pichavant, F.H. (2001). Antimicrobial edible packaging based on cellulosic ethers, fatty acids, and nisin incorporation to inhibit *Listeria innocua* and *Staphylococcus aureus*. *J. Food Protect.* **64**: 470–475.

Conte, A., Sinigaglia, M., and Del Nobile, M.A. (2006). Antimicrobial effectiveness of lysozyme immobilized on polyvinylalcohol-based film against *Alicyclobacillus acidoterrestris*. *J. Food Protect.* **69**: 861–865.

Court, E.B., Daar, A.S., Persad, D.L., Salamanca-Buentello, F., and Singer, P.A., Tiny technologies for the global good. *Mater. Today.*, 2005; **8**: 14–15.

Cowan, M.M., Abshire, K.Z., Houk, S.L., and Evans, S.M. (2003). Antimicrobial efficacy of a silver-zeolite matrix coating on stainless steel. *J. Ind. Microbiol Biot.* **30**: 102–106.

Darder, M., Aranda, P., and Ruiz-Hitzky, E. (2007). Bionanocomposites: A new concept of ecological, bioinspired, and functional hybrid materials. *Adv. Mater.* **19**: 1309–1319.

Darnton-Hill, I., and Nalubola, R. (2002). Fortification strategies to meet micronutrient needs: Successes and failures. *P. Nutr. Soc.* **61**: 231–241.

De Jong, A.R., Boumans, H., Slaghek, T., Van Veen, J., Rijk, R., and Van Zandvoort, M. (2005). Active and intelligent packaging for food: Is it the future? *Food Addit. Contam.* **22**: 975–979.

De Kruijf, N., van Beest, M., Rijk, R., Sipilainen-Malm, T., Paseiro Losada, P., and De Meulenaer, B. (2002). Active and intelligent packaging: Applications and regulatory aspects. *Food Addit. Contam.* **19**: 144–162.

Degnan, A.J., and Luchansky, J.B. (1992). Influence of beef tallow and muscle on the antilisterial activity of pediocin AcH and liposome-encapsulated pediocin AcH. *J. Food Protect.* **55**: 552–554.

Degnan, A.J., Buyong, N., and Luchansky, J.B. (1993). Antilisterial activity of pediocin AcH in model food systems in the presence of an emulsifier or encapsulated within liposomes. *Int. J. Food Microbiol.* **18**: 127–138.

Del Nobile, M.A., Cannarsi, M., Altieri, C., Sinigaglia, M., Favia, P., Iacoviello, G., and D'Agostino, R. (2004). Effect of Ag-containing nano-composite active packaging system on survival of *Alicyclobacillus acidoterrestris*. *J. Food Sci.* **69**: 379–383.

Desai, K.G.H., and Park, H.J. (2005). Recent developments in microencapsulation of food ingredients. *Dry. Technol.* **23**: 1361–1394.

Devlieghere, F., Vermeiren, L., and Debevere, J. (2004). New preservation technologies: Possibilities and limitations. *Int. Dairy J.* **14**: 273–285.

Dewettinck, K., and Huyghebaert, A. (1999). Fluidized bed coating in food technology. *Trends Food Sci Tech.* **10**: 163–168.

Dhawan, A., Sharma, V., and Parmar, D. (2009). Nanomaterials: A challenge for toxicologists. *Nanotoxicol*. **1**: 1–9.

Dibrov, P., Dzioba, J., Gosink, K.K., and Hase, C.C. (2002). Chemiosmotic mechanism of antimicrobial activity of Ag+ in *Vibrio cholerae*. *Antimicrob. Agents Ch.* **46**: 2668–2670.

El Jastimi, R., Edwards, K., and Lafleur, M. (1999). Characterization of permeability and morphological perturbations induced by nisin on phosphatidylcholine membranes. *Biophys. J.* **77**: 842–852.

El Naschie, M.S. (2006). Nanotechnology for the developing world. *Chaos Soliton Fract.* **30**: 769–773.

EU. (2004). Regulation (EC) No. 1935/2004 European Parliament and the Council of 27 October 2004 on materials and articles intended to come into contact with food repealing. *Off. J. Eur. Union*. **338**: 4–16.

FDA (2008). Food and drug authority. FDA regulation of nanotechnology product. 2008, Available at (www.fda.gov/cder/regulatory/applications/ind_page_1.htm)., Accessed January 14, 2008.

FDA (2008). Food and drug authority., FDA/CFSAN/OPA: Agency Response Letter: GRAS Notice No. GRN 000065, Available at (http://www.cfsan.fda.gov/∼rdb/opa-g065.html). Accessed January 14, 2008.

Fellman, M. (2008). Nanoparticle prism could serve as bioterror detector, Available at (http://unisci.com/stories/20014/1204011.htm). Accessed Feb 11, 2008.

Fernandez, A., Cava, D., Ocio, M.J., and Lagaron, J.M. (2008). Perspectives for biocatalysts in food packaging. *Trends Food Sci. Tech.* **19**: 198–206.

Fonseca, L., Cane, C., and Mazzolai, B. (2007). Application of micro and nanotechnologies to food safety and quality monitoring. *Meas. Control.* **40**: 116–119.

Franklin, N.B., Cooksey, K.D., and Getty, K.J.K. (2004). Inhibition of *Listeria monocytogenes* on the surface of individually packaged hot dogs with a packaging film coating containing nisin. *J. Food Protect.* **67**: 480–485.

Galeano, B., Korff, E., and Nicholson, W.L. (2003). Inactivation of vegetative cells, but not spores, of *Bacillus anthracis*, *B. cereus*, and *B. subtilis* on stainless steel surfaces coated with an antimicrobial silver- and zinc-containing zeolite formulation. *Appl. Environ. Microb.* **69**: 4329–4331.

Gandhi, M., and Chikindas, M.L. (2007). *Listeria*: A foodborne pathogen that knows how to survive. *Int. J. Food Microbiol.* **113**: 1–15.

Garde, S., Gaya, P., Fernandez-Garcia, E., Medina, M., and Nunez, M. (2003). Proteolysis, volatile compounds, and sensory evaluation in Hispanico cheese manufactured with the addition of a thermophilic adjunct culture, nisin, and calcium alginate-nisin microparticles. *J. Dairy Sci.* **86**: 3038–3047.

Gaskell, G., Eyck, T.T., Jackson, J., and Veltri, G. (2005). Imagining nanotechnology: Cultural support for technological innovation in Europe and the United States. *Public Underst. Sci.* **14**: 81–90.

Gouin, S. (2004). Microencapsulation: Industrial appraisal of existing technologies and trends. *Trends Food Sci. Tech.* **15**: 330–347.

Gounadaki, A.S., Skandamis, P.N., Drosinos, E.H., and Nychas, G.J.E. (2007). Effect of packaging and storage temperature on the survival of *Listeria monocytogenes* inoculated postprocessing on sliced salami. *J. Food Protect.* **70**: 2313–2320.

Graveland-Bikker, J.F., and de Kruif, C.G. (2006). Unique milk protein based nanotubes: Food and nanotechnology meet. *Trends Food Sci. Tech.* **17**: 196–203.

Gregoriadis, G., and Davis, C. (1979). Stability of liposomes *in vivo* and *in vitro* is promoted by their cholesterol content and the presence of blood cells. *Biochem. Bioph. Res. Co.* **89**: 1287–1293.

Greig, J.D., Todd, E.C.D., Bartleson, C.A., and Michaels, B.S. (2007). Outbreaks where food workers have been implicated in the spread of foodborne disease. Part 1. Description of the problem, methods, and agents involved. *J. Food Protect.* **70**: 1752–1761.

Handy, R.D., and Shaw, B.G. (2007). Toxic effects of nanoparticles and nanomaterials: Implications for public health, risk assessment and the public perception of nanotechnology. *Health Risk Society*. **9**: 125–144.

Heckman, J.H. (2005). Food packaging regulation in the United States and the European Union. *Regul. Toxicol. Pharm.* **42**: 96–122.

Helland, A., and Kastenholz, H. (2008). Development of nanotechnology in light of sustainability. *J. Cleaner Prod.* **16**: 885–888.

Heurtault, B., Saulnier, P., Pech, B., Proust, J.E., and Benoit, J.P. (2003). Physico-chemical stability of colloidal lipid particles. *Biomaterials.* **24**: 4283–4300.

Hoffman, K.L., Han, I.Y., and Dawson, P.L. (2001). Antimicrobial effects of corn zein films impregnated with nisin, lauric acid, and EDTA. *J. Food Protect.* **64**: 885–889.

Holland, C. (2007). A small world with a big future. *Converting Today.* **21**: 17–19.

Hsieh, Y.F., Chen, T.L., Wang, Y.T., Chang, J.H., and Chang, H.M. (2002). Properties of liposomes prepared with various lipids. *J. Food Sci.* **67**: 2808–2813.

Huang, Y.I., Cheng, Y.H., Yu, C.C., Tsai, T.R., and Cham, T.M. (2007). Microencapsulation of extract containing shikonin using gelatin-acacia coacervation method: A formaldehyde-free approach. *Colloid Surface B.* **58**: 290–297.

Huwiler, A., Kolter, T., Pfeilschifter, J., and Sandhoff, K. (2000). Physiology and pathophysiology of sphingolipid metabolism and signalling, *BBA – Mol. Cell Biol. L.* **1485**: 63–99.

Jacquot, M., Revol-Junelles, A.M., and Desobry, S. (2004). Use of LPS microbeads as antibacterial agent in food packaging, 2004. Available at (www.bioencapsulation.net/ XII_IWB/management/fulltext/P-52.pdf)., Accessed January 18, 2008.

Janes, M.E., Kooshesh, S., and Johnson, M.G. (2002). Control of *Listeria monocytogenes* on the surface of refrigerated, ready-to-eat chicken coated with edible zein film coatings containing nisin and/or calcium propionate. *J. Food Sci.* **67**: 2754–2757.

Jevsnik, M., Hlebec, V., and Raspor, P. (2008). Consumers awareness of food safety from shopping to eating. *Food Control.* **19**: 737–745.

Jiang, H., Manolache, S., Wong, A.C.L., and Denes, F.S. (2004). Plasma-enhanced deposition of silver nanoparticles onto polymer and metal surfaces for the generation of antimicrobial characteristics. *J. Appl. Polym. Sci.* **93**: 1411–1422.

Joerger, R.D. (2007). Antimicrobial films for food applications: A quantitative analysis of their effectiveness. *Packag. Technol. Sci.* **20**: 231–273.

Kahan, D.M., Sloric, P., Braman, D., Gastil, J., and Nohan, G.L. (2007). Affect, values, and nanotechnology risk perceptions: an experimental investigation. 2007, Available at (http://papers.ssrn.com). Accessed December 13, 2007.

Keller, B.C. (2001). Liposomes in nutrition. *Trends Food Sci. Tech.* **12**: 25–31.

Keller, B.C., Faulkner, G., and Lasic, D.D. (2000). Liposomes in breast-milk. *Agro Food Ind. Hi Tec.* **11**: 6–8.

Keller, K.H. (2007). Nanotechnology and society. *J. Nanopartic. Res.* **9**: 5–10.

Khosravi-Darani, K., Pardakhty, A., Honarpisheh, H., Rao, V.S.N.M., and Mozafari, M.R. (2007). The role of high-resolution imaging in the evaluation of nanosystems for bioactive encapsulation and targeted nanotherapy. *Micron.* **38**: 804–818.

Khwaldia, K., Ferez, C., Banon, S., Desobry, S., and Hardy, J. (2004). Milk proteins for edible films and coatings. *Cr. Rev. Food Sci. Nutr.* **44**: 239–251.

Kim, B.G., and Kang, I.J. (2007). Biodegradable nanoparticles effect on drug delivery system. NSTI Nanotechnology Conference and Trade Show - NSTI Nanotech, Santa Clara, CA. *Technical Proceedings.*, 2007; pp 390–393.

Kim, W.S., Ohashi, M., Tanaka, T., Kumura, H., Kim, G.Y., Kwon, I.K., Goh, J.S., and Shimazaki, K.I. (2004). Growth-promoting effects of lactoferrin on *L. acidophilus* and Bifidobacterium spp. *BioMetals.* **17**: 279–283.

Kolanowski, W., Jaworska, D., Laufenberg, G., and Weibrodt, J. (2007). Evaluation of sensory quality of instant foods fortified with omega-3 PUFA by addition of fish oil powder. *Eur. Food Res. Technol.* **225**: 715–721.

Kolanowski, W., Laufenberg, G., and Kunz, B. (2004). Fish oil stabilisation by microencapsulation with modified cellulose. *Int. J. Food Sci. Nutr.* **55**: 333–343.

Kolanowski, W., Ziolkowski, M., Weißbrodt, J., Kunz, B., and Laufenberg, G. (2006). Microencapsulation of fish oil by spray drying - Impact on oxidative stability. Part 1. *Eur. Food Res. Technol.* **222**: 336–342.

Kristo, E., Koutsoumanis, K.P., and Biliaderis, C.G. (2008). Thermal, mechanical and water vapor barrier properties of sodium caseinate films containing antimicrobials and their inhibitory action on *Listeria monocytogenes*. *Food Hydrocolloid.* **22**: 373–386.

Kussendrager, K.D., and Van Hooijdonk, A.C.M. (2000). Lactoperoxidase: Physico-chemical properties, occurrence, mechanism of action and applications. *Brit. J. Nutr.* **84**: 19–25.

Labuza, T.P., and Breene, W. (1989). Application of 'active packaging' technologies for the improvement of shelf-life and nutritional quality of fresh and extended shelf-life foods. *Bibl. Nutr. Diet.* **43**: 252–259.

Lahiri, S., and Futerman, A.H. (2007). The metabolism and function of sphingolipids and glycosphingolipids. *Cell. Mol. Life Sci.* **64**: 2270–2284.

Lao, A.A., and Brock, J.H. (2001). Lactoferrin: Antimicrobial and diagnostic properties. *Biotecnologia Aplicada.* **18**: 76–83.

Laridi, R., Kheadr, E.E., Benech, R.O., Vuillemard, J.C., Lacroix, C., and Fliss, I. (2003). Liposome encapsulated nisin Z: Optimization, stability and release during milk fermentation. *Int. Dairy J.* **13**: 325–336.

Lee, S.J., and Rosenberg, M. (2000). Whey protein-based microcapsules prepared by double emulsification and heat gelation. *LWT — Food Sci. Tech.* **33**: 80–88.

Lenk, C., and Biller-Andorno, N. (2007). Nanomedicine-emerging or re-emerging ethical issues? A discussion of four ethical themes. *Med. Healthcare Philos.* **10**: 173–184.

Lewenstein, B.V. (2005). What counts as a "social and ethical issue" in nanotechnology? *Hyle.* **11**: 5–18.

Loksuwan, J. (2007). Characteristics of microencapsulated beta-carotene formed by spray drying with modified tapioca starch, native tapioca starch and maltodextrin. *Food Hydrocolloid.* **21**: 928–935.

Lopez-Rubio, A., Gavara, R., and Lagaron, J. M. (2006). Bioactive packaging: turning foods into healthier foods through biomaterials. *Trends Food Sci. Tech.* **17**: 567–575.

Luff, J. (2007). Omega-3 and micro-encapsulation technology - Making functional foods taste better for longer. *Food Sci. Technol.* **21**: 30–31.

Maa, Y.F., and Hsu, C.C. (1999). Performance of sonication and microfluidization for liquid-liquid emulsification. *Pharm. Dev. Technol.* **4**: 233–240.

MacOubrie, J. (2006). Nanotechnology: Public concerns, reasoning and trust in government, *Public Underst. Sci.* **15**: 221–241.

Madene, A., Jacquot, M., Scher, J., and Desobry, S. (2006). Flavor encapsulation and controlled release - A review. *Int. J. Food Sci. Tech.* **41**: 1–21.

Mauriello, G., De Luca, E., La Storia, A., Villani, F., and Ercolini, D. (2005). Antimicrobial activity of a nisin-activated plastic film for food packaging. *Lett. Appl. Microbiol.* **41**: 464–469.

Mauriello, G., Ercolini, D., La Storia, A., Casaburi, A., and Villani, F. (2004). Development of polythene films for food packaging activated with an antilisterial bacteriocin from *Lactobacillus curvatus* 32Y. *J. Appl. Microbiol.* **97**: 314–322.

Maynard, A.D (2006). Nanotechnology: Assessing the risks. *Nano Today.* **1**: 22–33.

McCabe-Sellers, B.J., and Beattie, S.E. (2004). Food safety: Emerging trends in foodborne illness surveillance and prevention. *J. Am. Diet. Assoc.* **104**: 1708–1717.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., and Tauxe, R.V. (1999). Food-related illness and death in the United States. *Emerg. Infect. Dis.* **5**: 607–625.

Millette, M., Le Tien, C., Smoragiewicz, W., and Lacroix, M. (2007). Inhibition of *Staphylococcus aureus* on beef by nisin-containing modified alginate films and beads. *Food Control.* **18**: 878–884.

Min, S., Rumsey, T.R., and Krochta, J.M. (2008). Diffusion of the antimicrobial lysozyme from a whey protein coating on smoked salmon. *J. Food Eng.* **84**: 39–47.

Mnyusiwalla, A., Daar, A.S., and Singer, P.A. (2003). 'Mind the gap': Science and ethics in nanotechnology. *Nanotechnology* **14**: 9–13.

Moraru, C.I., Panchapakesan, C.P., Huang, Q., Takhistov, P., Liu, S., and Kokini, J.L. (2003). Nanotechnology: A new frontier in food science. *Food Technol*. **57**: 24–29.

Mozafari, M.R., Flanagan, J., Matia-Merino, L., Awati, A., Omri, A., Suntres, Z.E., and Singh, H. (2006). Recent trends in the lipid-based nanoencapsulation of antioxidants and their role in foods. *J. Sci. Food Agr.* **86**: 2038–2045.

Mulders, J.W.M., Boerrigter, I.J., Rollema, H.S., Siezen, R.J., and De Vos, W.M. (1991). Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. *Eur. J. Biochem.* **201**: 581–584.

Munshi, D., Kurian, P., Bartlett, R.V., and Lakhtakia, A. (2007). A map of the nanoworld: Sizing up the science, politics, and business of the infinitesimal. *Futures.* **39**: 432–452.

Nachay, K. (2007). Analyzing nanotechnology. *Food Technol.* **61**: 34–36.

Naghmouchi, K., Kheadr, E., Lacroix, C., and Fliss, I. (2007). Class I/Class IIa bacteriocin cross-resistance phenomenon in *Listeria monocytogenes*. *Food Microbiol.* **24**: 718–727.

Natrajan, N., and Sheldon, B.W. (2000). Efficacy of nisin-coated polymer films to inactivate *Salmonella* Typhimurium on fresh broiler skin. *J Food Protect.* **63**: 1189–1196.

Nel, A., Xia, T., Madler, L., and Li, N. (2006).Toxic potential of materials at the nanolevel. *Science* **311**: 622–627.

Nickols-Richardson, S.M. (2007). Nanotechnology: Implications for food and nutrition professionals. *J. Am. Diet. Assoc*. **107**: 1494–1497.

NNI. National Nanotechnology Initiative (2001). Available at (http://www.nano.gov/)., Accessed January 14, 2008.

Oberdorster, E. (2004). Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass. *Environ. Health Persp*. **112**: 1058–1062.

Owen, R., and Depledge, M. (2005). Nanotechnology and the environment: Risks and rewards. *Mar. Pollut. Bull.* **50**: 609–612.

Ozdemir, M., and Floros, J.D. (2004). Active food packaging technologies. *Cr. Rev. Food Sci.* **44**: 185–193.

Pan, Y., Rowney, M., Guo, P., and Hobman, P. (2007a). Biological properties of lactoferrin: An overview. *Aus. J. Dairy Technol.* **62**: 31–42.

Pan, Y., Shiell, B., Wan, J., Coventry, M.J., Roginski, H., Lee, A., and Michalski, W.P. (2007b). The molecular characterisation and antimicrobial activity of amidated bovine lactoferrin. *Int. Dairy J.* **17**: 606–616.

Pandey, J.K., Pratheep Kumar, A., Misra, M., Mohanty, A.K., Drzal, L.T., and Singh, R.P. (2005). Recent advances in biodegradable nanocomposites. *J. Nanosci. Nanotech.* **5**: 497–526.

Peniche, C., Arguelles-Monal, W., Peniche, H., and Acosta, N. (2003). Chitosan: An attractive biocompatible polymer for microencapsulation. *Macromol. Biosci.* **3**: 511–520.

Picot, A., and Lacroix, C. (2000). Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. *Int. Dairy J.* **14**: 505–515.

Quintavalla, S., and Vicini, L. (2002). Antimicrobial food packaging in meat industry. *Meat Sci.* **62**: 373–380.

Rasmussen, K., and MacLellan, M.A. (2001). The control of active substances used in biocides in the European Union by means of a review regulation. *Environ. Sci. Policy.* **4**: 137–146.

Raviv, U., Needleman, D.J., Li, Y., Miller, H.P., Wilson, L., and Safinya, C.R. (2005). Cationic liposome-microtubule complexes: Pathways to the formation of two-state lipid-protein nanotubes with open or closed ends. *P. Natl. Acad. Sci. USA.* **102**: 11167–11172.

Ray, S.S., and Bousmina, M. (2005). Biodegradable polymers and their layered silicate nanocomposites: In greening the 21st century materials world. *Prog. Mater. Sci.* **50**: 962–1079.

Rektor, A., and Vatai, G. (2004). Membrane filtration of Mozzarella whey. *Desalination.* **162**: 279–286.

Rhim, J.W., and Ng, P.K.W. (2007). Natural biopolymer-based nanocomposite films for packaging applications. *Cr. Rev. Food Sci.* **47**: 411–433.

Rhim, J.W., Hong, S.I., Park, H.M., and Ng, P.K.W. (2006). Preparation and characterization of chitosan-based nanocomposite films with antimicrobial activity. *J. Agr. Food Chem.* **54**: 5814–5822.

Roberts, R.F., and Zottola, E.A. (1993). Shelf-life of pasteurized process cheese spreads made from cheddar cheese manufactured with a nisin-producing starter culture. *J. Dairy Sci.* **76**: 1829–1836.

Robichaud, C.O., Tanzie, D., Weilenmann, U., and Wiesner, M.R. (2005). Relative risk analysis of several manufactured nanomaterials: An insurance industry context. *Environ. Sci. Technol.* **39**: 8985–8994.

Roco, M.C. (2003). Broader societal issues of nanotechnology. *J. Nanopartic. Res.* **5**: 181–189.

Rodriguez, E., Calzada, J., Arques, J.L., Rodriguez, J.M., Nunez, M., and Medina, M. (2005). Antimicrobial activity of pediocin-producing *Lactococcus lactis* on *Listeria monocytogenes, Staphylococcus aureus* and *Escherichia coli* O157:H7 in cheese. *Int. Dairy J.* **15**: 51–57.

Rodriguez, J.M. (1996). Review: Antimicrobial spectrum, structure, properties and mode of action of nisin, a bacteriocin produced by *Lactococcus lactis*. *Food Sci. Tech.* **2**: 61–68.

Rohner, F., Ernst, F.O., Arnold, M., Hilbe, M., Biebinger, R., Ehrensperger, F., Pratsinis, S.E., Langhans, W., Hurrell, R. F., and Zimmermann, M. B. (2007). Synthesis, characterization, and bioavailability in rats of ferric phosphate nanoparticles. *J. Nutr.* **137**: 614–619.

Royal Society and Royal Academy of Engineering. (2004). Nanoscience and nanotechnologies: opportunities and uncertainties 2004, Available at (http://www.nanotec.org.uk/finalreport. pdf)., Accessed January 14, 2008.

Salmaso, S., Elvassore, N., Bertucco, A., Lante, A., and Caliceti, P. (2004). Nisin-loaded poly-L-lactide nano-particles produced by CO_2 anti-solvent precipitation for sustained antimicrobial activity. *Int. J. Pharm.* **287**: 163–173.

Sanguansri, P., and Augustin, M.A. (2006). Nanoscale materials development - a food industry perspective. *Trends Food Sci. Tech.* **17**: 547–556.

Scannell, A.G.M., Hill, C., Ross, R.P., Marx, S., Hartmeier, W., and Arendt, E.K. (2000). Development of bioactive food packaging materials using immobilised bacteriocins Lacticin 3147 and Nisaplin. *Int. J. Food Microbiol.* **60**: 241–249.

Scott, N.R., and Chen, H. (2008). Nanoscale Science and Engineering for Agriculture and Food Systems, National Planning Workshop USA., Available at (http://www.csrees.usda.gov/nea/technology/pdfs/nanoscale_10–30–03.pdf)., Accessed January 17, 2008.

Seaton, A., and Donaldson, K. (2005). Nanoscience, nanotoxicology, and the need to think small. *Lancet.* **365**: 923–924.

Shahidi, F. (2000). Antioxidants in food and food antioxidants. *Nahrung.* **44**: 158–163.

Sheetz, T., Vidal, J., Pearson, T.D., and Lozano, K. (2005). Nanotechnology: Awareness and societal concerns. *Technol. Soc.* **27**: 329–345.

Siegrist, M., Keller, C., Kastenholz, H., Frey, S., and Wiek, A. (2007). Laypeople's and experts' perception of nanotechnology hazards. *Risk Anal.* **27**: 59–69.

Siragusa, G.R., Cutter, C.N., and Willett, J.L. (1999). Incorporation of bacteriocin in plastic retains activity and inhibits surface growth of bacteria on meat. *Food Microbiol.* **16**: 229–235.

Sobrino-Lopez, A., and Martin-Belloso, O. (2008). Use of nisin and other bacteriocins for preservation of dairy products. *Int. Dairy J.* **18**: 329–343.

Sofos, J.N. (2008). Challenges to meat safety in the 21st century. *Meat Sci.* 7**8**: 3–13.

Sorrentino, A., Gorrasi, G., and Vittoria, V. (2007). Potential perspectives of bio-nanocomposites for food packaging applications. *Trends Food Sci. Tech.* **18**: 84–95.

Sukhorukov, G., Fery, A., and Mohwald, H. (2005). Intelligent micro- and nanocapsules. *Prog. Polym. Sci.* **30**: 885–897.

Suppakul, P., Miltz, J., Sonneveld, K., and Bigger, S. W. (2003). Active packaging technologies with an emphasis on antimicrobial packaging and its applications. *J. Food Sci.* **68**: 408–420.

Tan, C.P., and Nakajima, M. (2005). Beta-Carotene nanodispersions: preparation, characterization and stability evaluation. *Food Chem.* **92**: 661–671.

Taylor, T.M., Davidson, P.M., Bruce, B.D., and Weiss, J. (2005). Liposomal nanocapsules in food science and agriculture. *Cr. Rev. Food Sci.* **45**: 587–605.

Taylor, T.M., Gaysinsky, S., Davidson, P.M., Bruce, B.D., and Weiss, J. (2007). Characterization of antimicrobial-bearing liposomes by zeta-potential, vesicle size, and encapsulation efficiency. *Food Biophys.* **2**: 1–9.

Teixeira, M.L., dos Santos, J., Silveira, N.P., and Brandelli, A. (2008). Phospholipid nanovesicles containing a bacteriocin-like substance for control of *Listeria monocytogenes*. *Innov. Food Sci. Emerg. Technol.* **9**: 49–53.

Tewa-Tagne, P., Briancon, S., and Fessi, H. (2007). Preparation of redispersible dry nanocapsules by means of spray-drying: Development and characterisation. *Eur. J. Pharm. Sci.* **30**: 124–135.

Tharanathan, R.N. (2003). Biodegradable films and composite coatings: Past, present and future. *Trends Food Sci. Tech.* **14**: 71–78.

Thompson, A.K., and Singh, H. (2006). Preparation of liposomes from milk fat globule membrane phospholipids using a microfluidizer. *J. Dairy Sci.* **89**: 410–419.

Trie, M., Guillen, C., Vaughan, D.M., Telfer, J.M., Brewer, J.M., Roseanu, A., and Brock, J.H. (2001). Liposomes as possible carriers for lactoferrin in the local treatment of inflammatory diseases. *Exp. Biol. Med.* **226**: 559–564.

Ubbink, J., and Kruger, J. (2006). Physical approaches for the delivery of active ingredients in foods. *Trends Food Sci. Tech.* **17**: 244–254.

Ugurlu, T., Turkoglu, M., Gurer, U.S., and Akarsu, B.G. (2007). Colonic delivery of compression coated nisin tablets using pectin/HPMC polymer mixture. *Eur. J. Pharm. Biopharm.* **67**: 202–210.

Un, S., and Price, N. (2007). Bridging the gap between technological possibilities and people:. Involving people in the early phases of technology development. *Technol. Forecast. Soc.* **74**: 1758–1772.

Uskokovic, V. (2007). Nanotechnologies: What we do not know. *Technol. Soc.* **29**: 43–61.

Vermeiren, L., Devlieghere, F., and Debevere, J. (2002). Effectiveness of some recent antimicrobial packaging concepts. *Food Addit. Contam.* **19**: 163–171.

Vermeiren, L., Devlieghere, F., Van Beest, M., De Kruijf, N., and Debevere, J. (1999). Developments in the active packaging of foods. *Trends Food Sci. Tech.*, **10**: 77–86.

Villalobos, R., Hernandez-Munoz, P., and Chiralt, A. (2006). Effect of surfactants on water sorption and barrier properties of hydroxypropyl methylcellulose films. *Food Hydrocolloid* **20**: 502–509.

Vincze, I., and Vatai, G. (2004). Application of nanofiltration for coffee extract concentration. *Desalination.* **162**: 287–294.

Vugia, D., Cronquist, A., Hadler, J., Tobin-D'Angelo, M., Blythe, D., Smith, K., Lathrop, S., Morse, D., Cieslak, P., Dunn, J., White, P.L., Guzewich, J.J., Henao, O.L., Hoekstra, R.M., Scallan, E., Angulo, F.J., Griffin, P.M., Tauxe, R.V., and Barton Behravesh, C. (2008). Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food - 10 States, 2007. *Morbidity and Mortality Weekly Report.* **57**: 366–370.

Wagener, M. (2006). Antimicrobial coatings. *Polym. Paint Colour J.* **196**: 34–37.

Walsh, S., Balbus, J.M., Denison, R., and Florini, K. (2008). Nanotechnology: Getting it right the first time. *J. Cleaner Prod.* **16**: 1018–1020.

Wan, J., Gordon, J.B., Muirhead, K., Hickey, M.W., and Coventry, M.J. (1997). Incorporation of nisin in micro-particles of calcium alginate. *Lett. Appl. Microbiol.* **24**: 153–158.

Weber, C.J., Haugaard, V., Festersen, R., and Bertelsen, G. (2002). Production and applications of biobased packaging materials for the food industry. *Food Addit. Contam.* **19**: 172–177.

Wegmuller, R., Zimmermann, M.B., Buhr, V.G., Windhab, E.J., and Hurrell, R.F. (2006). Development, stability, and sensory testing of microcapsules containing iron, iodine, and vitamin A for use in food fortification. *J. Food Sci.* **71**: 181–187.

Were, L.M., Bruce, B.D., Davidson, P.M., and Weiss, J. (2003). Size, stability, and entrapment efficiency of phospholipid nanocapsules containing polypeptide antimicrobials. *J. Agr. Food Chem.* **51**: 8073–8079.

Were, L.M., Bruce, B., Davidson, P.M., and Weiss, J. (2004). Encapsulation of nisin and lysozyme in liposomes enhances efficacy against *Listeria monocytogenes*. *J. Food Protect.* **67**: 922–927.

Wu, K.G., Chai, X.H., and Chen, Y. (2005). Microencapsulation of fish oil by simple coacervation of hydroxypropyl methylcellulose. *Chinese J. Chem.* **23**: 1569–1572.

Xing, F., Cheng, G., and Yi, K. (2006). Study on the antimicrobial activities of the capsaicin microcapsules. *Journal of Applied Polymer Science.* **102**: 1318–1321.

Yep, Y.L., Li, D., Mann, N.J., Bode, O., and Sinclair, A.J. (2002). Bread enriched with microencapsulated tuna oil increases plasma docosahexaenoic acid and total omega-3 fatty acids in humans. *Asia Pac. J. Clin. Nutr.* **11**: 285–291.

Yih, T.C., and Al-Fandi, M. (2006). Engineered nanoparticles as precise drug delivery systems. *J. Cell. Biochem.* **97**: 1184–1190.

Zheng, S., Alkan-Onyuksel, H., Beissinger, R.L., and Wasan, D.T. (1999). Liposome microencapsulations without using any organic solvent. *J. Disper. Sci. Technol.* **20**: 1189–1203.

Chapitre 2

Etudes de libération contrôlée et les applications aux nanocomposites

Majid Jamshidian, Elmira Arab Tehrany, Muhammad Imran, Muriel Jacquot, and Stéphane Desobry

Laboratoire d'Ingénierie des Biomolécules, ENSAIA–INPL, Nancy Université, 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France

(Comprehensive Reviews in Food Science and Food Safety (9), 552–571. 2010)

Comprehensive REVIEWS in Food Science and Food Safety

Poly-Lactic Acid: Production, Applications, Nanocomposites, and Release Studies

Majid Jamshidian, Elmira Arab Tehrany, Muhammad Imran, Muriel Jacquot, and Stéphane Desobry

Abstract: Environmental, economic, and safety challenges have provoked packaging scientists and producers to partially substitute petrochemical-based polymers with biodegradable ones. The general purpose of this review is to introduce poly-lactic acid (PLA), a compostable, biodegradable thermoplastic made from renewable sources. PLA properties and modifications via different methods, like using modifiers, blending, copolymerizing, and physical treatments, are mentioned; these are rarely discussed together in other reviews. Industrial processing methods for producing different PLA films, wrappings, laminates, containers (bottles and cups), are presented. The capabilities of PLA for being a strong active packaging material in different areas requiring antimicrobial and antioxidant characteristics are discussed. Consequently, applications of nanomaterials in combination with PLA structures for creating new PLA nanocomposites with greater abilities are also covered. These approaches may modify PLA weaknesses for some food packaging applications. Nanotechnology approaches are being broadened in food science, especially in packaging material science with high performances and low concentrations and prices, so this category of nano-research is estimated to be revolutionary in food packaging science in the near future. The linkage of a 100% bio-originated material and nanomaterials opens new windows for becoming independent, primarily, of petrochemical-based polymers and, secondarily, for answering environmental and health concerns will undoubtedly be growing with time.

PLA as an Active Packaging Material

Release studies from PLA

Active packaging is defined as an intelligent or smart system that involves interactions between package or package components and food or internal gas atmosphere and complies with consumer demands for high quality, fresh-like, and safe products (Labuza and Breene 1989).

Active packaging is an innovative approach to change the condition of the packaging to extend shelf-life or improve safety or sensory properties while maintaining the quality of the food. Traditional packaging concepts are limited in their ability to prolong shelf-life of food products. The most important active packaging concepts are O_2 and ethylene scavenging, CO_2 scavengers and emitters, moisture regulators, antimicrobial packaging concepts, antioxidant release, release or adsorption of taste, and aroma molecules (Vermeiren and others 1999; Lopez-Rubio and others 2004; Kerry and others 2006).

As a GRAS and biodegradable material, and also because of its biosorbability and biocompatible properties in the human body, PLA and its copolymers (especially poly-glycolic acid) is attractive to pharmaceutical and medical scientists as a carrier for releasing various drugs and agents like bupivacaine (Sokolsky-Papkov and others 2009), rapamycin (Miao and others 2008), melittin (Cun and others 2008), 5-fluorouracil (Liu and others 2008), amoxicillin (Xu and Czernuszka 2008), human nerve growth factor (rhNGF) (Gu and others 2007), and gentamicin (Schnieders and others 2006) and many others.

In food domains, little research has been done studying the ability of PLA as an active packaging material. PLA is a relatively new polymer and needs time to become an acceptable and an effective active packaging in the market.

Antioxidants have been added to food packaging material for the intentional purpose of migration into food, because prooxidant effects are often seen to a high extent and could be reduced by antioxidants. Van Aardt and others (2007) studied the release of antioxidants from loaded poly (lactide-co-glycolide) (PLGA) (50:50) films, with 2% α-tocopherol, and a combination of 1% butylated hydroxytoluene (BHT) and 1% butylated hydroxyanisole (BHA), into water, oil (food simulant: Miglyol 812), and milk products at 4 and 25 °C in the presence and absence of light. They concluded that in water medium PLGA (50:50) showed hydrolytic degradation of the polymer and release of BHT into water. In Miglyol 812, no degradation or antioxidant release took place, even after 8 wk at 25 °C. Milk fat was stabilized to some extent when light-exposed dry whole milk and dry buttermilk were exposed to antioxidant-loaded PLGA (50:50). The authors also suggested potential use of degradable polymers as a unique active packaging option for sustained delivery of antioxidants, which could be a benefit to the dairy industry by limiting the oxidation of high-fat dairy products, such as ice cream mixes.

PLA and antimicrobial packaging trends

The innovative strength of PLA antimicrobial packaging has a direct impact on consumer health by creating safer and more wholesome packaged foods. Active packaging realizes certain extraordinary and vital functions other than providing an inert barrier between product and external conditions.

Active substances that are important and considered for novel bioactive packaging include antimicrobials, vitamins, phytochemicals, prebiotics, marine oils, and immobilized enzymes (Lopez-Rubio and others 2006).

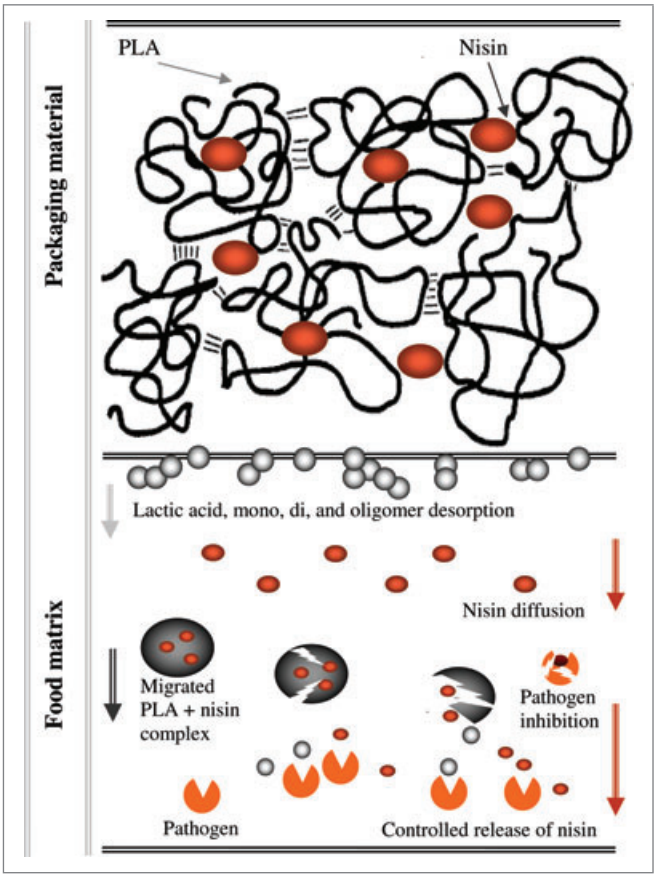


Figure 3–Schematic representation of PLA film with nisin as an active agent incorporated and release thereof.

A whole range of active additives, including silver-substituted zeolite, organic acids and their salts, bacteriocins such as nisin and pediocin, enzymes such as lysozyme, a chelator like ethylenediaminetetraacetic acid (EDTA), lactoferrin, and plant extracts have already been successfully incorporated in antimicrobial active packaging (Joerger 2007).

The most widely used bacteriocin in active food packaging is nisin due to its GRAS status (FDA 2001). Successful introduction of a new active packaging requires careful attention to the interactions in the active agent, packaging, and food triangle.

Notion of controlled release. In particular for active packaging, the major complexity emerges for migration/diffusion as either slow release of package component itself or as an active agent being incorporated. In both contexts, the evaluation of materials compliance with regulations includes migration monitoring for package component (monomer) and additives (active agent). A schematic representation is showed in Figure 3 for release of an active agent (nisin) alone and in conjunction with packaging material. Recently, the predictive mathematical modeling for active agent-controlled release and its various approaches were excellently reviewed by Poças and others (2008).

On the contrary, to study an additive's release from a package, active agent desorption from the multilayer biodegradable film and diffusion in agarose gels were monitored. The data attained after 2 or 6 d of contact between antimicrobial films and agarose gels were employed to find out nisin mass transfer by numerical modeling following Fick's 2nd law. The values were in the range from 0.87×10^{-3} m/s to 4.30×10^{-3} m/s and 6.5×10^{-11} m^2/s to 3.3×10^{-10} m^2/s, for nisin apparent desorption and diffusion

coefficients, respectively. The diffusion process was governed by interactions between nisin, package, and food matrix simulant (Chollet and others 2009).

Mode of incorporation. The customized direct incorporation of active agents may result in a loss of activity due to interactions with food components, thus showing from a diminution of active concentration and dilution into bulk foods (Kim and others 2002; Coma 2008). The incorporation of antimicrobial agents into PLA packaging material slows down their release and helps to maintain high concentrations of the active compounds against pathogenic bacteria like *Listeria monocytogenes* (Jin and others 2009).

In the last decade, the above-mentioned slow release approach has been used for PLA. In this regard, major antimicrobial agents include bacteriocins, predominantly nisin (Ariyapitipun and others 1999; Jin and others 2009), lactic acid (Ariyapitipun and others 1999), lysozyme (Del Nobile and others 2009), and chitosan (Torres-Giner and others 2008).

Novel PLA active packaging potential approaches

Although the above-mentioned PLA systems reduced resistant bacterial strain development and guaranteed a higher level of microbial protection for certain food products, their casting and preparation was complicated and inactivation of the active proteins was observed. Active agent modification by attachment to a polymer did not yield biologically active derivatives. Up till now, a literature study reveals that relatively low attention has been given to micro-encapsulated active agents in foods. Active agent-loaded polymeric micro-/nanoparticles give the impression of being promising formulations to achieve long-lasting antimicrobial activity (Salmaso and others 2004; Sanchez-Garcia and others 2007).

Thus, this particular controlled release concept can be enlarged to the applications of other active agents like antioxidants for oil-rich foods and antisticking/antifogging agents for cheese slices and fresh fruits, respectively.

PLA Modifications

The special characteristics of PLA can make it a good fit for some applications but may also require modifications for some others. For example, the oxygen and moisture permeability of PLA is much higher than for most other plastics, such as PE, PP, and even PET. However, the applications of PLA are limited by several factors such as low glass transition temperature, weak thermal stability, and low toughness and ductility (Harada and others 2007).

For extending PLA applications, the properties like impact strength or flexibility, stiffness, barrier properties, thermal stability, and production costs must be improved. Generally, modifiers have been studied to improve stiffness at elevated temperatures, reduce cost, or increase the degradation rate of PLA.

Some efforts of PLA modifications in the field of packaging are presented in Table 10.

A large number of investigations have been performed on the blending of PLA with various polymers, for example, thermoplastic starch, poly (ethylene oxide), poly (ethylene glycol), poly (ε-caprolactone), poly (vinyl acetate), poly (hydroxy butyrate), cellulose acetate, poly (butylene succinate), and poly (hexamethylene succinate). Low molecular weight compounds have also been used as plasticizers for PLA, for example, oligomeric lactic acid, glycerol, triacetine, and low molecular weight citrates (Ljungberg and others 2005).

The choice of polymers or plasticizers to be used as modifiers for PLA is limited by the requirements of the application. For packaging and hygiene applications, only nontoxic substances approved for food contact and personal care can be considered as plasticizing agents. The plasticizer should be miscible with PLA, thus creating a homogeneous blend. The plasticizer should not be too volatile, because this would cause evaporation to occur at the high temperature used during processing. Furthermore, the plasticizer should not be prone to migration into the materials in contact with the plasticized PLA. It would also cause the blended materials to regain the brittleness of pure PLA (Ren and others 2006).

There is a tendency for plasticizers to migrate to the surface of a polymer. A possible way to prevent this migration would be to increase the molecular weight of the plasticizers.

However, increasing the molecular weight too much would eventually decrease the solubility causing phase separation and formation of a 2-phase system.

The final properties of these blends depend on the chemical structure of the original components, the mixing ratio of the constituent polymers, the interaction between the components, and the processing steps to which they are then subjected.

Amorphous PLA exhibits lower modulus above the glass transition temperature and poor heat resistance, which limits the wide application of PLA in the general plastic use. Thus, how to improve the crystallization behavior or enhance the degree of crystallinity (% Xc) of PLA becomes the main problem that must be solved. Decreasing the cooling rate of PLA from melt and providing an annealing process for PLA articles is believed to be the most efficient way to enhance the (% Xc) of PLA. It has been reported that the smaller the cooling rate, the higher the (% Xc). Annealing endows PLA chain segments enough activation energy and promotes the crystallization through the reorganization process. Especially, in a certain condition, the annealing process also induces the polymorphic transition in PLA (Li and others 2009).

Other kinds of modifications, such as surface modifications, are being applied in biomedical uses for improving polymer release properties (Janorkar and Hirt 2004; Koo and Jang 2008).

Nanotechnology and PLA Food Packaging

Nanotechnology and its applications in food science have recently been studied by several researchers. The use of nanoparticles, such as micelles, liposomes, nanoemulsions, biopolymeric nanoparticles, and cubosomes, as well as the development of nanosensors aimed at ensuring food safety, are some novel nano-food applications.

Nanoparticles can be used as bioactive compounds in functional foods. Bioactive compounds that can be found naturally in certain foods have physiological benefits and might help to reduce the risk of certain diseases, including cancer. Omega-3 and omega-6 fatty acids, probiotics, prebiotics, vitamins, and minerals have found their applications in food nanotechnology as bioactive compounds (Sozer and Kokini 2009).

Nanotechnology is also applicable in food packaging in the form of elementary components of food packaging. This approach includes improving packaging performances like its gas, moisture, ultraviolet, and volatile barriers, increasing mechanical strength, decreasing weight, and increasing the heat resistance and flame retardancy of the packaging material. Nanoadditives, intelligent packaging (using nanosensors), delivery and controlled release of neutraceuticals, antibacterial agents, self-cleaning packaging, and systems to monitor product conditions during transportation are other novel nano-approaches in food packaging (Ray and Bousmina 2005; Sozer and Kokini 2009).

Table 10–Summary of PLA modifications for packaging applications.

Type of modification	Treatment or added material	Effect	Reference
Modifier	Citrate esters	Lowering the Tg and improving the elongation at break	Labrecque and others (1997)
	Triacetine or tributyl citrate	Decrease in Tg and increase in crystallinity	Ljungberg and Wesslén (2002)
	Oligomeric malonate esteramides	Decrease in Tg and improvement of the strain at break	Ljungberg and others (2005)
	4,4-Methylene diphenyl diisocyanate	Tg value increased to 64 °C, tensile strength increased from 4.9 to 5.8 MPa good nucleating agent for PLA crystallization	Li and Yang (2006)
	Polyglycerol esters	Improving the elongation at break	Uyama and others (2006)
	Polyethylene glycol and acetyl triethyl citrate	Decrease in Tg and increase in crystallization rate	Li and Huneault (2007)
	Talc	Increase the ductility at more than 10%	Li and Huneault (2007)
	Bifunctional cyclic ester	Enhance PLA toughness	Jing and Hillmyer (2008)
	Poly(1,3-butylene adipate)	Decrease in storage modulus and glass transition temperature but increase in elongation at break	Wang and others (2008)
	Polycarbodiimide	Improve the thermal stability at 210 °C for up to 30 min	Yang and others (2008)
Blending with:	Polyvinyl acetate	Increase in tensile strength and percent elongation	Gajria and others (1996)
	Poly ethylene oxide (PEO)	Elongation at break of more than 500%	Nijenhuis and others (1996)
	Poly ε-caprolactone (PCL)	High improvement in mechanical properties	Tsuji and Ikada (1996)
	Poly ethylene glycol (PEG)	Enhance the crystallinity of PLA and biodegradability	Sheth and others (1997)
	Starch with different plasticizers	Lowering the price, decreasing Tg, and increasing crystallinity and biodegradability	Ke and Sun (2001); Jacobsen and Fritz (1996); Ke and others (2003)
	Polyvinyl alcohol and starch	Increase in tensile strength	Ke and Sun (2003a)
	Ethylene vinyl alcohol (EVOH)	Improvement of mechanical, thermal, and biodegradability properties	Lee and others (2005)
	Polycarbonate	Improvement of mechanical properties and biodegradation rate	Wang and others (2007)
	Poly ethylene glycidyl methacrylate (PEGMA)	Production of super-tough PLA materials	Oyama (2009)
Copolymerization of PLA and:	DL-mandelic acid	Increasing Tg and improving mechanical properties	Kylmä and others (1997)
	ε-Caprolactone	Improving the decomposition temperatures and crystallinity	Park and others (1998)
	Polyvinyl chloride	Improving strength and toughness	Lu and others (2008)
	Acrylonitrile–butadiene–styrene	Improved impact strength and elongation at break with a slight loss in modulus and tensile strength	Li and Shimizu (2009)
Physical treatment	Vacuum compression-molding and solid-state extrusion techniques	Flexural strength and flexural modulus were improved up to 221 MPa and 8.4 GPa, respectively	Lim and others (2001)
	Orientation	Significant improvement in tensile and impact properties	Grijpma and others (2002)
	Annealing	Increasing the toughness	Park and others (2004)
	Aging	Increasing the Tg	Quan and others (2004)
	Drawing	Improvement in tensile and fracture properties	Todo (2007)

Addition of different fillers to polymers for improving their performances like their strength and stiffness, barrier properties, resistance to fire and ignition, and also decreasing their price has always been a common objective in packaging technology. Traditionally, mineral fillers such as clay, silica, and talc are incorporated in film preparations in the range of 10% to 50% by weight to reduce film cost or to improve its performance in some way. However, mechanical strength of such films, in general, decreases when fillers are present. Recently, nanocomposites have received significant attention as an alternative to conventional filled polymers (Rhim 2007).

Nanocomposites

Nanocomposites are a new class of composites that are particle-filled polymers for which at least 1 dimension of the dispersed particles is in the nanometer range. Three types of nanocomposites include isodimensional nanoparticles (with 3 nano dimensions), nanotubes or whiskers (with 2 nano dimensions), and polymer-layered crystal nanocomposites (with 1 nano dimension) (Alexandre and Dubois 2000).

Although several nanoparticles have been recognized as possible additives to enhance polymer performance, the packaging industry has focused its attention mainly on layered inorganic solids like clays and silicates, due to their availability, low cost, significant enhancements, and relative simple processability (Azeredo 2009). These nanocomposites exhibit markedly improved mechanical, thermal, optical, and physicochemical properties when compared with the pure polymer or conventional (microscale) composites. The layered silicates commonly used in nanocomposites consist of 2-dimensional layers, which are 1-nm thick and several microns long depending on the particular silicate (Alexandre and Dubois 2000).

The commonly used layered silicates for the preparation of polymer-layered silicate (PLS) nanocomposites are montmorillonite (MMT), hectorite, and saponite (Sinha Ray and Okamoto 2003).

The matrix of polymer/clay nanocomposites consists mainly of synthetic polymers including thermosets such as epoxy, thermoplastics like poly (methyl methacrylate), nonpolar polymers like polyethylene and polypropylene, polar polymers like nylon, and conductive polymers like polyaniline. In addition, biodegradable polymers such as PLA and polycaprolactone (PCL) have also been tested for the manufacture of nanocomposites with layered silicate (Rhim 2007).

PLA nanocomposites

The combination of PLA and montmorillonite-layered silicate may result in a nanocomposite with good barrier properties that is suitable for film packaging material. The modulus of PLA would be increased by the addition of montmorillonite. However, the incorporation of the montmorillonite clay into PLA could decrease the toughness of the PLA composites. There are various technical approaches to achieve a balance of good strength and toughness for PLA nanocomposites. The addition of poly ethylene glycol could act as a good plasticizer in a PLA/clay systems (Shibata and others 2006).

A comprehensive review is provided by Sinha Ray and Okamoto (2003) for the preparation, characterization, materials properties, crystallization behavior, melt rheology, and foam processing of pure polylactide (PLA) and PLA/layered silicate nanocomposites. They concluded this new family of composite materials frequently exhibits remarkable improvements in its material properties when compared with those of virgin PLA. Improved properties can include a high storage modulus both in the solid and melt states, increased tensile and flexural properties, decreased gas permeability, increased heat distortion temperature, and increased rate of biodegradability of pure PLA.

In a complementary review, Sinha Ray and Bousmina (2005) presented recent developments on the above-mentioned properties for many biodegradable polymers' nanocomposites. They described 2 types of biodegradable polymers: (a) originating from renewable sources like PLA, poly (3-hydroxybutyrate), thermoplastic starch (TPS), plant-based polymers, cellulose, gelatin, or chitosan; and (b) originating from petroleum sources like poly (butylene succinate), aliphatic polyesters, poly(ε-caprolactone), or poly (vinyl alcohol).

Recent research on PLA nanocomposites

The potential applications of PLA-based nanocomposites are in food packaging, medical applications, and tissue cultures. Some research conducted on PLA nanocomposites in the field of food packaging after the year 2005 is presented here.

Biodegradability of polymers through photodegradation has been studied by using TiO_2 nanoparticles as photocatalysts that decompose various organic chemicals like aldehyde, toluene, and polymers such as PE, PP, PVC, and PS.

In a study done by Nakayama and Hayashi (2007), TiO_2 nanoparticles were prepared and the surface of TiO_2 was modified using propionic acid and n-hexylamine, with the modified TiO_2 uniformly dispersed into PLA matrixes without aggregation. They studied the PLA-TiO_2 nanocomposite's photodegradation under UV light and concluded photodegradability of nanocomposites can be efficiently promoted.

Melt intercalation is a method where the blending of polymer and silicate layers is followed by molding to form a polymer-layered silicate nanocomposite. In general, for intercalation, polymers and layered hosts are annealed above the softening point of the polymer. Chow and Lok (2009) used this method for studying the effect of maleic anhydride-grafted ethylene propylene rubber (EPMgMA) on the thermal properties of PLA/organo-montmorillonite nanocomposites. They concluded that the addition of OMMT (Organo-montmorillonite) and EPMgMA did not influence much the T_g and T_m (melting temperature) of PLA nanocomposites. The degree of crystallinity of PLA increased slightly in the presence of OMMT; it had been supposed that OMMT could act as a nucleating agent to increase the crystallinity of PLA. In contrast, the addition of EPMgMA may restrict the crystallization process and crystal formation of PLA, which subsequently reduces the degree of crystallinity of PLA/OMMT nanocomposites. Finally, they claimed that the thermal stability of PLA/OMMT was greatly enhanced by the addition of EPMgMA.

Kim and others (2009) studied the effect of bacterial cellulose on the transparency of PLA/bacterial nanocomposites, since bacterial cellulose had shown good potential as reinforcement or preparing optically transparent materials due to its structure, which consists of ribbon-shaped fibrils with diameters in the range from 10 to 50 nm. They found that light transmission of the PLA/bacterial cellulose nanocomposite was quite high due to the size effect of the nanofibrillar bacterial cellulose. Additionally, the tensile strength and Young's modulus of the PLA/bacterial cellulose nanocomposite were increased by 203% and 146%, respectively, compared with those of the PLA.

Carbon nanotubes (CNTs) have been the subject of much attention because of their outstanding performance including excellent mechanical, electrical, and thermal properties. The most promising area of nanocomposite research involves the reinforcement of polymers using CNTs as reinforcing filler (Kim and others 2007).

Li and others (2009) introduced functionalized multiwalled carbon nanotubes (f-MWCNTs) into PLA to investigate the effect of such filler on the crystallization behavior of PLA. They concluded that the addition of f-MWCNTs accelerates the crystallization of PLA dramatically and induces formation of homogeneous and very small spherulites. The results of polarized optical microscopy showed that the average spherulite diameter is about 200 μm, but for nanocomposites it was very difficult to differentiate the spherulites one by one.

Numerous studies have also been done on PLA nanocomposites in medical science regarding drug delivery systems, tissue engineering, and bone fixation (Jo and others 2004; Sakata and others 2006; Chen and others 2007).

PLA Degradability, Biodegradability, and Recyclability

Almost all the conventional plastics such as PE, PP, PS, and PVC are resistant to microbial attack; on the contrary aliphatic polyesters like PLA are readily degraded by microorganisms present in the environment. According to ASTM D6400–04, a biodegradable plastic is "a plastic that degrades because of the action of naturally occurring microorganisms such as bacteria, fungi, and algae," and a compostable plastic is "a plastic that undergoes degradation by biological processes during composting to yield carbon dioxide, water, inorganic compounds, and biomass at a rate consistent with other known compostable materials and leaves no visually distinguishable or toxic residues.

PLA degradation was studied in animal and human bodies for medical applications like implants, surgical sutures, and drug delivery materials (Vainionpaa and others 1989). In these environments, PLA is initially degraded by hydrolysis and the soluble oligomers formed are metabolized by cells. PLA degradation upon disposal in the environment is more challenging because PLA is largely resistant to attack by microorganisms in soil or sewage under

ambient conditions. The polymer must 1st be hydrolyzed at elevated temperatures (about 58 °C) to reduce the molecular weight before biodegradation can commence. No degradation was observed on PLA sheets after 6 wk in soil, thus PLA will not degrade in typical garden compost (Brandrup and others 1999; Ohkita and Lee 2006). Urayama and others (2002) reported that the molecular weight of PLA films with different optical purity of the lactate units (100% L and 70% L) decreased by 20% and 75%, respectively, after 20 mo in soil.

Kale and others (2007) studied the degradation of PLA bottles in a real composting condition (compost containing cow manure, wood shavings, and waste feed) at 65 °C for 30 d. They observed major fragmentation, which produces decomposition of the polymer chain into shorter oligomer chains and monomers since the 4th day, and on the15th day, the bottles were already in pieces and mostly consisted of parts from cap threads, and neck (bottle parts having higher thickness) and finally on the 30th day the bottles were completely degraded.

Microbial and enzymatic degradation of PLA have recently been studied by many researchers because these types of degradations usually do not need the high temperatures to be accomplished. Williams (1981) 1st reported the degradation for PLLA by proteinase K from *Tritirachium.album.,* afterward many studies were done for finding different enzymes corresponding PLA degradation. Reported enzymes that enable to degrade PLA in different scale include, alkaline protease (Oda and others 2000), serine proteases such as subtilisin, trypsin, elastase, and α-chymotrypsin (Lim and others 2005), Cutinase-like enzyme (Masaki and others 2005). Lipase could hydrolyze low molecular weight PLLA and some copolymers such as PDLLA (poly D,L-lactic acid) and, poly(D-lactid-co-glycolide) but not PDLA (poly D-lactic acid) and high molecular weight PLLA (Fukuzaki and others 1989). Pranamuda and others (2001) found an enzyme from *Amycolatopsis* sp. cultures and named it PLLA depolymerase. The optimum pH and temperature for this enzyme were 6.0 and 37 to 45 °C, respectively. PLLA depolymerase can also hydrolyze casein, silk fibroin, succinyl-*p*-nitroanilide, but not PHB and PCL. The enzymatic degradation of aliphatic polyesters by hydrolysis is a 2-step process. The 1st step is adsorption of the enzyme on the surface of the substrate through surface-binding and the 2nd step is hydrolysis of the ester bond (Tokiwa and Calabia 2006).

Pranamuda and others (2001) 1st isolated a PLA-degrading microorganism of *Amycolatopsis* strain from soil environment, which was capable of degrading 60% of the PLA film after 14 d. Suyama and others (1998) reported that PLA-degrading microorganisms are not widely distributed in the natural environment and, thus, PLA is less susceptible to microbial attack in the natural environment than other synthetic aliphatic polyesters like PHB, PCL, and Poly(butylenes succinate) (PBS). Several PLA-degrading microorganisms, their enzymes, and substrate specificities are reported in Table 11. Upon disposal in the environment, PLA is hydrolyzed into low molecular weight oligomers and then mineralized into CO_2 and H_2O by the microorganisms present in the environment.

Microbial degradation of PLA should be studied for packaging of foods containing microorganisms including lactic acid bacteria, and fungi for their probable abilities of PLA degradation. Torres and others (1996) reported the ability of assimilation of lactic acid and racemic oligomer products of PLA for 2 strains of *Fusarium moniliforme* (widely distributed in soil) and on strain of *Penicillium roqueforti* (the main fungus in blue cheese, and can be isolated from soil).

Recycling diverts material from alternative waste streams such as land filling or incineration, as well as conserves natural resources and energy. PET and HDPE make up a large percentage of the plastic bottles that get recycled. Sorting PLA in recycling facilities is difficult due to low volumes and in many cases, the PLA container looks like PET. Because of this, the possibility of mixing the different materials together exists. As a result, there is concern in the recycling community that PLA bottles, at high enough levels, would contaminate the PET recycle stream due to chemical and thermal property differences. The National Association for PET Container Resources (NAPCOR) recently announced its concern for potential contamination of the PET recycling stream associated with PLA bottles. This trade association for the PET plastic industry in the U.S. and Canada cited its concerns involving cost of separation, increased contamination, yield loss, and impact on recycled PET (RPET) quality and processing (www.napcor.com).

Consequently, NatureWorks® and Primo Water Corp. conducted a commercial scale bottle recycling evaluation to demonstrate that automated systems being used today in the recycling industry are capable of separating PLA bottles from PET bottles with good accuracy and efficiency (93%). In this evaluation, near-infrared equipment was used since it is a common sorting technology in large recycling operations and can accurately identify many different types of polymers (NatureWorks® 2009).

Recycled bottles crushed, chopped into flakes, and pressed into bales. They enter to final recycling step and are changed to PLA monomers; L-lactic acid or L-lactide. There are 2 methods for PLA recycling, primarily hydrolysis or solvolysis to L-lactic acid or L-lactic acid-based compounds and, 2nd, depolymerization to the cyclic dimer, L-lactide. Both methods have problems with low yield of monomers in a short period and require the removal of catalysts and additives used for hydrolysis, solvolysis, or depolymerization (Tsuji and others 2003).

High-temperature hydrolysis, normally above the melting point, is an effective way to hydrolyze PLA rapidly to L-lactic acid without the aid of catalysts. The highest maximum yield of L-lactic acid (about 90%) in a high temperature and high pressure water was attained at 250 °C for 10 to 20 min (Tsuji and others 2001).

Conclusion

In previous years, the most negative point of PLA was its price in comparison with petrochemical-based polymers. Today, by using other sources of dextrose, optimizing lactic acid production processes and its costs, substituting electricity energy by wind and solar energy for PLA production, optimizing PLA production processes, and increasing PLA demands, reduction of its price can be attained. The present PLA price is much lower than in previous years, but it is not fixed and it even will be considerably lower in the future because, according to expert forecasts, beyond 2010 the global demand for biodegradable plastics will continue to increase by 30% each year and PLA will take a large part of this market because of its valuable properties (Bastioli 2005).

The linkage of a 100% bio-originated material and nanomaterials opens new windows for becoming independent from petrochemical-based polymers and also free of environmental and health concerns.

Substituting PET with PLA in food packages, which require high-barrier properties, is not feasible unless some modifications are applied to develop its permeability. Also, the brittleness of PLA may also limit its applications where toughness and impact resistance are critical. However, with the help of nanotechnology

Table 11–PLA-degrading microorganism, their enzymes substrate specificities, and detection methods used in degradation tests.

Microorganism	Enzyme	Substrate specificity	Detection method for PLA degradation
Amycolatopsis sp. strain HT 32	Protease	L-PLA	Film-weight loss; monomer production (lactic acid)
Amycolatopsis sp. strain 3118	Protease	L-PLA	Film-weight loss; monomer production
Amycolatopsis sp. strain KT-s-9	Protease	Silk fibroin, L-PLA	Clear-zone method
Amycolatopsis sp. strain 41	Protease	L-PLA, silk powder, casein, Suc-(Ala)3-pNA	Film-weight loss; monomer production
Amycolatopsis sp. strain K104–1	Protease	L-PLA, casein, fibrin	Turbidity method
Lentzea waywayandensis (formerly Saccharothrix waywayandensis)	Protease	L-PLA	Film-weight loss; monomer production
Kibdelosporangium aridum	Protease	L-PLA	Film-weight loss; monomer production
Tritirachium album ATCC 22563	Protease	L-PLA, silk fibroin, elastin	Film-weight loss; monomer production
*Brevibacillus (formerly Bacillus brevis)**	Protease	L-PLA	Change in molecular weight and viscosity
*Bacillus stearothermophilus**	Protease	D-PLA	Change in molecular weight and viscosity
*Geobacillus thermocatenulatus**	Protease	L-PLA	Change in molecular weight and viscosity
*Bacillus sinithii strain PL 21**	Lipase (Esterase)	L-PLA, pNP-fatty acid esters	Change in molecular weight
Paenibacillus amylolyticus strain TB-13	Lipase	DL-PLA, PBS, PBSA, PES, PCL, triolein, tributyrin	Turbidity method
Cryptococcus sp. strain S-2	Lipase (Curtinase)	L-PLA, PBS, PCL, PHB	Turbidity method

Adapted from Tokiwa and Calabia (2006).

and providing safe PLA nanocomposites, many of its weakness compared to petrochemical-based polymer will be resolved.

According to its safety, biodegradability, and ability for being improved in a tailor-made fashion, the authors predict the substituting of many petrochemical-based polymers by PLA for almost all pharmaceutical and direct food contact packaging materials in the near future.

Nomenclature

ASTM = American society for testing and materials;
EVOH = Ethylene vinyl alcohol;
GPPS = General purpose poly(styrene);
HDPE = High-density poly(ethylene);
HIPS = High-impact poly(styrene);
LDPE = Low-density poly(ethylene);
LLDPE = Linear low-density poly(ethylene);
MMT = Montmorillonite;
MWCNT = Multiwalled-carbon nanotube;
OMMT = Organo-montmorillonite;
OPLA = Oriented poly(lactic acid);
OPP = Oriented poly(propylene);
OPS = Oriented poly(Styrene);
PBAT = Poly(butylene adipate terephthalate);
PBS = Poly(butylenes succinate);
PBST = Poly(butylene succinate terephthalate);
PC = Poly(carbonate);
PCL = Poly(ε-caprolactone);
PEA = Poly(ester amide);
PEG = Poly(ethylene glycol);
PEGMA = Poly(ethylene-glycidyl methacrylate);
PEO = Poly(ethylene oxide);
PET = Poly(ethylene terephthalate);
PGA = Poly(glutamic acid);
PHB = Poly(3-hydroxybutyrate);
PHV = Poly(hydroxyl valerate);
PLLA = Poly(L-lactic acid);
PP = Poly(propylene);
PS = Poly(styrene);
PTMAT = Poly(tetramethylene adipate terephthalate);
PVA = Poly(vinyl alcohol);
PVC = Poly(vinyl chloride);
TPS = Thermoplastic starch.

References

Acioli-Moura R, Sun XS. 2008. Thermal degradation and physical aging of poly(lactic acid) and its blends with starch. Polym Eng Sci 48:829–36.

Alexandre M, Dubois P. 2000. Polymer-layered silicate nanocomposites: preparation, properties and uses of a new class of materials. Mater Sci Eng R: Rep 28:1–63.

Amass W, Amass A, Tighe B. 1998. A review of biodegradable polymers: uses, current developments in the synthesis and characterization of biodegradable polyesters, blends of biodegradable polymers and recent advances in biodegradation studies. Polym Int 47:89–144.

Ariyapitipun T, Mustapha A, Clarke AD. 1999. Microbial shelf life determination of vacuum-packaged fresh beef treated with polylactic acid, lactic acid, and nisin solutions. J Food Prot 62:913–20.

Auras R, Harte B, Selke S, Hernandez R. 2003. Mechanical, physical, and barrier properties of poly(lactide) films. J Plastic Film Sheet 19:123–35.

Auras R, Harte B, Selke S. 2004. An overview of polylactides as packaging materials. Macromol Biosci 4:835–64.

Auras RA, Singh SP, Singh JJ. 2005. Evaluation of oriented poly(lactide) polymers vs. existing PET and oriented PS for fresh food service containers. Packag Technol Sci 18:207–16.

Azeredo HMCD. 2009. Nanocomposites for food packaging applications. Food Res Int 42:1240–53.

Bao L, Dorgan JR, Knauss D, Hait S, Oliveira NS, Maruccho IM. 2006. Gas permeation properties of poly(lactic acid) revisited. J Membr Sci 285:166–72.

Bastioli C. 2005. Handbook of biodegradable polymers. 1st ed. Shropshire, U.K.: Rapra Technology Limited. 5 p.

Bogaert JC, Coszach P. 2000. Poly(lactic acids): a potential solution to plastic waste dilemma. Macromol Symp 153:287–303.

Bohlaman GM. 2005. General characteristics, processability, industrial applications and market evolution of biodegradable polymers. In: Bastioli C, editor. Handbook of biodegradable polymers. 1st ed. Shropshire, U.K.: Rapra Technology Limited. p 183–218.

Brandrup J, Immergut EH, Grulke EA. 1999. Polymer handbook. 4th ed. New York: John Wiley and Sons. 163 p.

Budhavaram NK, Fan Z. 2007. Lactic acid production from paper sludge using thermophilic bacteria. AIChE Annual Meeting.

Cai H, Dave V, Gross RA, McCarthy SP. 1996. Effects of physical aging, crystallinity, and orientation on the enzymatic degradation of poly(lactic acid). J Polym Sci, Part B: Polym Phys 34:2701–8.

Chandra R, Rustgi R. 1998. Biodegradable polymers. Prog Polym Sci 23:1273–335.

Chen C, Lv G, Pan C, Song M, Wu C, Guo D, Wang X, Chen B, Gu Z. 2007. Poly(lactic acid) (PLA)-based nanocomposites—A novel way of drug-releasing. Biomed Mater 2:L1–4.

Chollet E, Swesi Y, Degraeve P, Sebti I. 2009. Monitoring nisin desorption from a multi-layer polyethylene-based film coated with nisin-loaded HPMC film and diffusion in agarose gel by an immunoassay (ELISA) method and a numerical modeling. Innov Food Sci Emerg Technol 10:208–14.

Chow WS, Lok SK. 2009. Thermal properties of poly(lactic acid)/organo-montmorillonite nanocomposites. J Therm Anal Calorim 95:627–32.

Clarinval AM. 2002. Classification and comparison of thermal and mechanical properties of commercialized polymers. International Congress & Trade Show, The Industrial Applications of Bioplastics, 2002 February 3–5; York, UK.

Clarinval AM, Halleux J. 2005. Classification of biodegradable polymers. In: Smith R, editor. Biodegradable polymers for industrial applications. 1st ed. Boca Raton, FL, USA: CRC Press. p 3–31.

Coma V. 2008. Bioactive packaging technologies for extended shelf life of meat-based products. Meat Sci 78:90–103.

Conn RE, Kolstad JJ, Borzelleca JF, Dixler DS, Filer LJ, LaDu BN, Pariza MW. 1995. Safety assessment of polylactide (PLA) for use as a food-contact polymer. Food Chem Toxicol 33:273–83.

Cun D, Cui F, Yang L, Yang M, Yu Y, Yang R. 2008. Characterization and release mechanism of melittin-entrapped poly (lactic acid-co-glycolic acid) microspheres. J Drug Deliv Sci Technol 18:267–72.

Datta R, Henry M. 2006. Lactic acid: recent advances in products, processes and technologies: a review. J Chem Technol Biotechnol 81:1119–129.

Del Nobile MA, Conte A, Buonocore GG, Incoronato AL, Massaro A, Panza O. 2009. Active packaging by extrusion processing of recyclable and biodegradable polymers. J Food Eng 93:1–6.

Di Lorenzo ML. 2005. Crystallization behavior of poly(l-lactic acid). Eur Polym J 41:569–75.

Dorgan JR, Lehermeier H, Mang M. 2000. Thermal and rheological properties of commercial-grade poly(lactic acids)s. J Polym Environ 8:1–9.

FDA. 2001. FDA/CFSAN/OPA: Agency response letter: GRAS Notice No. GRN 000065.

FDA. 2002. Inventory of Effective Food Contact Substance (FCS) Notifications No. 178. http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=fcsListing&id=178.

Fukuzaki H, Yoshida M, Asano M, Kumakura M. 1989. Synthesis of copoly(D,L-Lactic acid) with relatively low molecular weight and in vitro degradation. Eur Polym J 25:1019–26.

Furukawa T, Sato H, Murakami R, Zhang J, Duan YX, Noda I, Ochiai S, Ozaki Y. 2005. Structure, dispersibility, and crystallinity of poly(hydroxybutyrate)/poly(L-lactic acid) blends studied by FT-IR microspectroscopy and differential scanning calorimetry. Macromol 38:6445–54.

Gajria AM, Davé V, Gross RA, McCarthy SP. 1996. Miscibility and biodegradability of blends of poly(lactic acid) and poly(vinyl acetate). Polymer 37:437–44.

Garlotta D. 2001. A literature review of poly(lactic acid). J Polym Environ 9:63–84.

Ghosh S, Viana JC, Reis RL, Mano JF. 2008. Oriented morphology and enhanced mechanical properties of poly(l-lactic acid) from shear controlled orientation in injection molding. Mater Sci Eng A 490:81–9.

Giles FH, Wagner JR, Mount EM. 2005. Extrusion, the definitive processing guide and handbook. 1st ed. New York: William Andrew Publishing. 547 p.

Grijpma DW, Altpeter H, Bevis MJ, Feijen J. 2002. Improvement of the mechanical properties of poly(D,L-lactide) by orientation. Polym Int 51:845–51.

Grossman EM. 1995. Annual Technical Conference—ANTEC 95, Conference Proceedings. SCORIM- principles, capabilities and applications. Society of plastic engineers 1995 p 461–76.

Gu H, Song C, Long D, Mei L, Sun H. 2007. Controlled release of recombinant human nerve growth factor (rhNGF) from poly[(lactic acid)-co-(glycolic acid)] microspheres for the treatment of neurodegenerative disorders. Polym Int 56:1272–80.

Harada M, Ohya T, Iida K, Hayashi H, Hirano K, Fukuda H. 2007. Increased impact strength of biodegradable poly(lactic acid)/poly(butylene succinate) blend composites by using isocyanate as a reactive processing agent. J Appl Polym Sci 106:1813–20.

Hartmann MH. 1998. High-molecular-weight polylactic acid polymers. In: Kaplan DL, editor. Biopolymers from renewable resources. Berlin: Springer. p 367–411.

Huang L, Sheng J, Chen J, Li N. 2008. 2nd International Conference on Bioinformatics and Biomedical Engineering, iCBBE 2008. Direct fermentation of fishmeal wastewater and starch wastewater to lactic acid by Rhizopus oryzae. 2008.

Hutchinson MH, Dorgan JR, Knauss DM, Hait SB. 2006. Optical properties of polylactides. J Polym Environ 14:119–24.

Jacobsen S, Fritz HG. 1996. Filling of poly(lactic acid) with native starch. Polym Eng Sci 36:2799–804.

Janorkar AV, Hirt DE. 2004. Annual Technical Conference—ANTEC, Conference Proceedings. Surface modification of poly(lactic acid) films via grafting hydrophilic polymers. 2004.

Jin T, Liu L, Zhang H, Hicks K. 2009. Antimicrobial activity of nisin incorporated in pectin and polylactic acid composite films against Listeria monocytogenes. Int J Food Sci Technol 44:322–9.

Jing F, Hillmyer MA. 2008. A bifunctional monomer derived from lactide for toughening polylactide. J Am Chem Soc 130:13826–7.

Jo YS, Kim MC, Kim DK, Kim CJ, Jeong YK, Kim KJ, Muhammed M. 2004. Mathematical modelling on the controlled-release of indomethacin-encapsulated poly(lactic acid-co-ethylene oxide) nanospheres. Nanotechnology 15:1186–94.

Joerger RD. 2007. Antimicrobial films for food applications: a quantitative analysis of their effectiveness. Packag Technol Sci 20:231–73.

Johnson RM, Mwaikambo LY, Tucker N. 2003. Biopolymers. Rapra Rev Rep 43:1–26.

Kale G, Auras R, Singh SP, Narayan R. 2007. Biodegradability of polylactide bottles in real and simulated composting conditions. Polym Test 26:1049–61.

Kawashima N, Ogawa S, Obuchi S, Matsuo M, Yagi T. 2002. Poly lactic acid "LACEA." In: Doi Y, Steinbuchel A, editors. Biopolymers polyesters III applications and commercial products. Weinheim: Wiley–VCH Verlag GmbH. p 251–74.

Ke T, Sun X. 2001. Thermal and mechanical properties of poly(lactic acid) and starch blends with various plasticizers. Trans Am Soc Agric Eng 44:945–53.

Ke T, Sun XS. 2003a. Starch, poly(lactic acid), and poly(vinyl alcohol) blends. J Polym Environ 11:7–14.

Ke T, Sun XS. 2003b. Thermal and mechanical properties of poly(lactic acid)/starch/methylenediphenyl diisocyanate blending with triethyl citrate. J Appl Polym Sci 88:2947–55.

Ke T, Sun SX, Seib P. 2003. Blending of poly(lactic acid) and starches containing varying amylose content. J Appl Polym Sci 89:3639–46.

Kerry JP, O'Grady MN, Hogan SA. 2006. Past, current and potential utilisation of active and intelligent packaging systems for meat and muscle-based products: a review. Meat Sci 74:113–30.

Kim YM, An DS, Park HJ, Park JM, Lee DS. 2002. Properties of nisin-incorporated polymer coatings as antimicrobial packaging materials. Packag Technol Sci 15:247–54.

Kim KI, Kim WK, Seo DK, Yoo IS, Kim EK, Yoon HH. 2003. Production of lactic acid from food wastes. Appl Biochem Biotechnol 107:637–48.

Kim JY, Park HS, Kim SH. 2007. Multiwall-carbon-nanotube-reinforced poly(ethylene terephthalate) nanocomposites by melt compounding. J Appl Polym Sci 103:1450–7.

Kim Y, Jung R, Kim HS, Jin HJ. 2009. Transparent nanocomposites prepared by incorporating microbial nanofibrils into poly(l-lactic acid). Curr Appl Phys 9:S69–71.

Koo GH, Jang J. 2008. Surface modification of poly(lactic acid) by UV/ozone irradiation. Fibers Polym 9:674–8.

Kylmä J, Härkönen M, Seppälä JV. 1997. The modification of lactic acid-based poly(ester-urethane) by copolymerization. J Appl Polym Sci 63:1865–72.

Labrecque LV, Kumar RA, Davé V, Gross RA, McCarthy SP. 1997. Citrate esters as plasticizers for poly(lactic acid). J Appl Polym Sci 66:1507–13.

Labuza TP, Breene W. 1989. Application of 'active packaging' technologies for the improvement of shelf-life and nutritional quality of fresh and extended shelf-life foods. Bibl Nutr Dieta 43:252–9.

Lee NC. 2006. The extrusion blow moulding system. In: Practical guide to blow moulding. Shawbury, U.K.: Rapra Technology Limited. p 81–98.

Lee CM, Kim ES, Yoon JS. 2005. Reactive blending of poly(L-lactic acid) with poly(ethylene-co-vinyl alcohol). J Appl Polym Sci 98:886–90.

Lee DS, Yam KL, Piergiovanni L. 2008. Food packaging science and technology. 1st ed. New York: Taylor and Francis 631 p.

Lehermeier HJ, Dorgan JR, Way JD. 2001. Gas permeation properties of poly(lactic acid). J Membr Sci 190:243–51.

Li H, Huneault MA. 2007. Effect of nucleation and plasticization on the crystallization of poly(lactic acid). Polymer 48:6855–66.

Li Y, Shimizu H. 2009. Improvement in toughness of poly(l-lactide) (PLLA) through reactive blending with acrylonitrile-butadiene-styrene copolymer (ABS): Morphology and properties. Eur Polym J 45:738–46.

Li BH, Yang MC. 2006. Improvement of thermal and mechanical properties of poly(L-lactic acid) with 4,4-methylene diphenyl diisocyanate. Polym Adv Technol 17:439–43.

Li L, Tang SC, Wang QH, Pan YK, Wang TL. 2006. Preparation of poly (lactic acid) by direct polycondensation in azeotropic solution. J East China Univ Sci Technol 32:672–5.

Li Y, Wang Y, Liu L, Han L, Xiang F, Zhou Z. 2009. Crystallization improvement of poly(L-lactide) induced by functionalized multiwalled carbon nanotubes. J Polym Sci Part A: Polym Chem 47:326–39.

Lim JY, Kim SH, Lim S, Kim YH. 2001. Improvement of flexural strengths of poly(L-lactic acid) by solid-state extrusion. Macromol Chem Phys 202:2447–53.

Lim JY, Kim SH, Lim S, Kim YH. 2003. Improvement of flexural strengths of poly(L-lactic acid) by solid-state extrusion, 2: extrusion through rectangular die. Macromol Mater Eng 288:50–7.

Lim HA, Raku T, Tokiwa Y. 2005. Hydrolysis of polyesters by serine proteases. Biotechnol Lett 27:459–64.

Lim LT, Auras R, Rubino M. 2008. Processing technologies for poly(lactic acid). Prog Polym Sci 33:820–52.

Liu FT, He R, Zhao YD, Gao F, Zhang YX, Cui DX. 2008. Modified biodegradable poly(D, L-lactic-co-glycolic acid) film implants for sustained release of 5-fluorouracil. Shanghai Jiaotong Daxue Xuebao/J Shanghai Jiaotong Univ 42:822–6,30.

Ljungberg N, Wesslén B. 2002. The effects of plasticizers on the dynamic mechanical and thermal properties of poly(lactic acid). J Appl Polym Sci 86:1227–34.

Ljungberg N, Colombini D, Wessleén B. 2005. Plasticization of poly(lactic acid) with oligomeric malonate esteramides: dynamic mechanical and thermal film properties. J Appl Polym Sci 96:992–1002.

Lopez-Rubio A, Almenar E, Hernandez-Munoz P, Lagaron JM, Catala R, Gavara R. 2004. Overview of active polymer-based packaging technologies for food applications. Food Rev Int 20:357–87.

Lopez-Rubio A, Gavara R, Lagaron JM. 2006. Bioactive packaging: turning foods into healthier foods through biomaterials. Trends Food Sci Technol 17:567–75.

Lu D, Zhang X, Zhou T, Ren Z, Wang S, Lei Z. 2008. Biodegradable poly (lactic acid) copolymers. Prog Chem 20:339–50.

Masaki K, Kamini NR, Ikeda H, Iefuji H. 2005. Cutinase-like enzyme from the yeast *Cryptococcus* sp. strain S-2 hydrolyses polylactic acid and other biodegradable plastics. Appl Environ Microbiol 7:7548–50.

Mehta R, Kumar V, Bhunia H, Upadhyay SN. 2005. Synthesis of poly(lactic acid): a review. J Macromol Sci Polym Rev 45:325–49.

Mehta R, Kumar V, Upadhyay SN. 2007. Mathematical modeling of the poly(lactic acid) ring-opening polymerization using stannous octoate as a catalyst. Polym Plast Technol Eng 46:933–7.

Miao LF, Yang J, Huang CL, Song CX, Zeng YJ, Chen LF, Zhu WL. 2008. Rapamycin-loaded poly (lactic-co-glycolic) acid nanoparticles for intraarterial local drug delivery: preparation, characterization, and in vitro/in vivo release. Acta Acad Med Sinicae 30:491–7.

Mills CA, Navarro M, Engel E, Martinez E, Ginebra MP, Planell J, Errachid A, Samitier J. 2006. Transparent micro- and nanopatterned poly(lactic acid) for biomedical applications. J Biomed Mater Res 76:781–7.

Mohanty AK, Misra M, Hinrichsen G. 2000. Biofibres, biodegradable polymers and biocomposites: an overview. Macromol Mater Eng 276–277:1–24.

Mutsuga M, Kawamura Y, Tanamoto K. 2008. Migration of lactic acid, lactide and oligomers from polylactide food-contact materials. Food Addit Contam Part A, Chem, Anal, Control, Expo Risk Assess 25:1283–90.

Nakayama N, Hayashi T. 2007. Preparation and characterization of poly(l-lactic acid)/TiO_2 nanoparticle nanocomposite films with high transparency and efficient photodegradability. Polym Degrad Stab 92:1255–64.

NatureWorks. 2005a. PLA ISBM bottle guide. Minnetonka, Minn.: NatureWorks LLC.

NatureWorks. 2005b. PLA processing guide for biaxially oriented film. Minnetonka, Minn.: NatureWorks LLC.

NatureWorks. 2005c. Processing guide for thermoforming articles. Minnetonka, Minn.: NatureWorks LLC.

NatureWorks. 2006a. PLA 2002D, 3001D, 3051D, 3251D, 4032D, 4042D, 4060D, 7000D, 7032D data sheets. Minnetonka, Minn.: NatureWorks LLC.

NatureWorks. 2006b. PLA injection molding guide for 3051D. Minnetonka, Minn.: NatureWorks LLC.

Natureworks. 2009. Using near-infrared sorting to recycle PLA bottles. Minnetonka, Minn.: Natureworks LLC.

Nijenhuis AJ, Colstee E, Grijpma DW, Pennings AJ. 1996. High-molecular-weight poly(L-lactide) and poly(ethylene oxide) blends: thermal characterization and physical properties. Polymer 37:5849–57.

Oda Y, Yonetsu A, Urakami T, Tonomura K. 2000. Degradation of polylactide by commercial proteases. J Polym Environ 8:29–32.

Ohkita T, Lee SH. 2006. Thermal degradation and biodegradability of poly(lacticacid)/cornstarch biocomposites. J Appl Polym Sci 100:3009–17.

Oliveira NS, Oliveira J, Gomes T, Ferreira A, Dorgan J, Marrucho IM. 2004. Gas sorption in poly(lactic acid) and packaging materials. Fluid Phase Equilib 222–223:317–24.

Oyama HT. 2009. Super-tough poly(lactic acid) materials: reactive blending with ethylene copolymer. Polymer 50:747–51.

Park S, Chang Y, Cho JH, Noh I, Kim C, Kim SH, Kim YH. 1998. Synthesis and thermal properties of copolymers of L-lactic acid and ε-caprolactone. Polymer 22:1–5.

Park SD, Todo M, Arakawa K. 2004. Effect of annealing on fracture mechanism of biodegradable poly(lactic acid). Key Eng Mater 261–263:105–10.

Patey W. 2010. Thermoforming PLA: how to do it right. Plastics Technol 56:30–1.

Platt K. 2006. The global biodegradable polymers market. In: Biodegradable polymers. Shawbury, UK: Smithers Rapra Technology Limited. p 31–48.

Poças MF, Oliveira JC, Oliveira FAR, Hogg T. 2008. A critical survey of predictive mathematical models for migration from packaging. Crit Rev Food Sci Nutr 48:913–28.

Pranamuda H, Tsuchii A, Tokiwa Y. 2001. Poly(L-lactide)-degrading enzyme produced by *Amycolatopsis* sp. Macromol Biosci 1:25–9.

Puaux JP, Banu I, Nagy I, Bozga G. 2007. A study of L-lactide ring-opening polymerization kinetics. Macromol Symp 259:318–26.

Quan D, Liao K, Zhao J. 2004. Effects of physical aging on glass transition behavior of poly(lactic acid)s. Acta Polym Sinica 5:726–30.

Ray SS, Bousmina M. 2005. Biodegradable polymers and their layered silicate nanocomposites: in greening the 21st century materials world. Prog Mater Sci 50:962–1079.

Reddy G, Altaf M, Naveena BJ, Venkateshwar M, Kumar EV. 2008. Amylolytic bacterial lactic acid fermentation—A review. Biotechnol Adv 26:22–34.

Ren Z, Dong L, Yang Y. 2006. Dynamic mechanical and thermal properties of plasticized poly(lactic acid). J Appl Polym Sci 101:1583–90.

Rhim JW. 2007. Potential use of biopolymer-based nanocomposite films in food packaging applications. Food Sci Biotechnol 16:691–709.

Rosato DV, Rosato DV, Rosato MG. 2000. Injection molding handbook. 3rd ed. Boston: Kluwer Academic Publishers 1488 p.

Sakata S, Kei T, Uchida K, Kaetsu I. 2006. Nano-particle of hydrophobic poly lactic acid for DDS. Polym Preprints Japan 55:2074.

Salmaso S, Elvassore N, Bertucco A, Lante A, Caliceti P. 2004. Nisin-loaded poly-L-lactide nano-particles produced by CO_2 anti-solvent precipitation for sustained antimicrobial activity. Int J Pharm 287:163–73.

Sanchez-Garcia MD, Gimenez E, Lagaron JM. 2007. Novel PET nanocomposites of interest in food packaging applications and comparative barrier performance with biopolyester nanocomposites. J Plastic Film Sheet 23:133–48.

Schnieders J, Gbureck U, Thull R, Kissel T. 2006. Controlled release of gentamicin from calcium phosphate-poly(lactic acid-co-glycolic acid) composite bone cement. Biomaterials 27:4239–49.

Scott G. 2000. 'Green' polymers. Polym Degrad Stab 68:1–7.

Sheth M, Kumar RA, Davé V, Gross RA, McCarthy SP. 1997. Biodegradable polymer blends of poly(lactic acid) and poly(ethylene glycol). J Appl Polym Sci 66:1495–505.

Shibata M, Someya Y, Orihara M, Miyoshi M. 2006. Thermal and mechanical properties of plasticized poly(L-lactide) nanocomposites with organo-modified montmorillonites. J Appl Polym Sci 99:2594–602.

Shogren R. 1997. Water vapor permeability of biodegradable polymers. J Environ Polym Degrad 5:91–5.

Sinha Ray S, Okamoto M. 2003. Polymer/layered silicate nanocomposites: a review from preparation to processing. Prog Polym Sci 28:1539–641.

Siparsky GL, Voorhees KJ, Dorgan JR, Schilling K. 1997. Water transport in polylactic acid (PLA), PLA/polycaprolactone copolymers, and PLA/polyethylene glycol blends. J Environ Polym Degrad 5:125–36.

Siracusa V, Rocculi P, Romani S, Rosa MD. 2008. Biodegradable polymers for food packaging: a review. Trends Food Sci Technol 19:634–43.

Södergård A, Stolt M. 2002. Properties of lactic acid based polymers and their correlation with composition. Prog Polym Sci (Oxford) 27:1123–63.

Sokolsky-Papkov M, Golovanevski L, Domb AJ, Weiniger CF. 2009. Prolonged local anesthetic action through slow release from poly(lactic acid co castor oil). Pharm Res 26:32–9.

Sozer N, Kokini JL. 2009. Nanotechnology and its applications in the food sector. Trends Biotechnol 27:82–9.

Suyama T, Tokiwa Y, Ouichanpagdee P, Kanagawa T, Kamagata Y. 1998. Phylogenetic affiliation of soil bacteria that degrade aliphatic polyesters available commercially as biodegradable plastics. Appl Environ Microbiol 64:5008–11.

Taubner V, Shishoo R. 2001. Influence of processing parameters on the degradation of poly(L-lactide) during extrusion. J Appl Polym Sci 79:2128–35.

Throne JL. 1996. Technology of thermoforming. 1st ed. New York: Hanser Publishers. 922 p.

Todo M. 2007. Effect of unidirectional drawing process on fracture behavior of poly(l-lactide). J Mater Sci 42:1393–6.

Tokiwa Y, Calabia BP. 2006. Biodegradability and biodegradation of poly(lactide). Appl Microbiol Biotechnol 72:244–51.

Torres A, Li SM, Roussos S, Vert M. 1996. Screening of microorganisms for biodegradation of poly(lactic acid) and lactic acid-containing polymers. Appl Environ Microbiol 62:2393–7.

Torres-Giner S, Ocio MJ, Lagaron JM. 2008. Development of active antimicrobial fiber-based chitosan polysaccharide nanostructures using electrospinning. Eng Life Sci 8:303–14.

Tsuji H, Ikada Y. 1996. Blends of aliphatic polyesters. I. Physical properties and morphologies of solution-cast blends from poly(DL-lactide) and poly(ε-caprolactone). J Appl Polym Sci 60:2367–75.

Tsuji H, Nakahara K, Ikarashi K. 2001. Poly(L-lactide), high-temperature hydrolysis of poly(L-lactide) films with different crystallinities and crystalline thicknesses in phosphate-buffered solution. Macromol Mater Eng 286:398–406.

Tsuji H, Daimon H, Fujie K. 2003. A new strategy for recycling and preparation of poly(L-lactic acid): hydrolysis in the melt. Biomacromol 4:835–40.

Tsuji H, Okino R, Daimon H, Fujie K. 2006. Water vapor permeability of poly(lactide)s: effects of molecular characteristics and crystallinity. J Appl Polym Sci 99:2245–52.

Uradnisheck J. 2009. Annual Technical Conference—ANTEC 2009, Conference Proceedings. Improved dimensional stability of thermoformed polylactic acid articles. 2009 June 22–26; Chicago IL, USA, p 1612–5.

Urayama H, Kanamori T, Kimura Y. 2002. Properties and biodegradability of polymer blends of poly(l-lactide)s with different optical purity of the lactate units. Macromol Mater Eng 287:116–21.

Uyama H, Ueda H, Doi M, Takase Y, Okubo T. 2006. Plasticization of poly (lactic acid) by bio-based resin modifiers. Polym Preprints Japan 55:5595.

Vainionpaa S, Rokkanen P, Tormall P.1989. Surgical application of biodegradable polymers in human tissues. Prog Polym Sci 14:679–716.

Van Aardt M, Duncan SE, Marcy JE, Long TE, O'Keefe SF, Sims SR. 2007. Release of antioxidants from poly(lactide-co-glycolide) films into dry milk products and food simulating liquids. Int J Food Sci Technol 42:1327–37.

Vermeiren L, Devlieghere F, Van Beest M, De Kruijf N, Debevere J. 1999. Developments in the active packaging of foods. Trends Food Sci Technol 10:77–86.

Vink ETH, Rabago KR, Glassner DA, Gruber PR. 2003. Applications of life cycle assessment to NatureWorks® polylactide (PLA) production. Polym Degrad Stab 80:403–19.

Vink ETH, Rajbago KR, Glassner DA, Springs B, O'Connor RP, Kolstad J, Gruber PR. 2004. The sustainability of NatureWorks polylactide polymers and ingeo polylactide fibers: an update of the future. Initiated by the 1st International Conference on Bio-based Polymers (ICBP 2003), November 2003, Saitama, Japan. Macromol Biosci 4:551–64.

Vink ETH, Glassner DA, Kolstad JJ, Wooley RJ, O'Connor RP. 2007. The eco-profiles for current and near-future NatureWorks® polylactide (PLA) production. Ind Biotechnol 3:58–81.

Wang S, Tao J, Guo T, Fu T, Yuan X, Zheng J, Song C. 2007. Thermal characteristics, mechanical properties and biodegradability of polycarbonates/poly(lactic acid) (PPC/PLA) blends. Lizi Jiaohuan Yu Xifu/Ion Exch Adsorp 23:1–9.

Wang N, Zhang X, Yu J, Fang J. 2008. Study of the properties of plasticised poly(lactic acid) with poly(1,3-butylene adipate). Polym Polym Compos 16:597–604.

White JR. 2006. Polymer ageing: physics, chemistry or engineering? Time to reflect. C.R Chimie 9:1396–408.

Williams DF. 1981. Enzymatic hydrolysis of polylactic acid. Eng Med 10:5–7.

Wolf O. 2005. Techno-economic feasibility of large-scale production of bio-based polymers in Europe. Institute for Prospective Technological Studies, Spain: European Communities. p 50–64.

Xu Q, Czernuszka JT. 2008. Controlled release of amoxicillin from hydroxyapatite-coated poly(lactic-co-glycolic acid) microspheres. J Control Release 127:146–53.

Yang L, Chen X, Jing X. 2008. Stabilization of poly(lactic acid) by polycarbodiimide. Polym Degrad Stab 93:1923–9.

Yu H, Huang N, Wang C, Tang Z. 2003. Modeling of poly(L-lactide) thermal degradation: theoretical prediction of molecular weight and polydispersity index. J Appl Polym Sci 88:2557–62.

Zee MV. 2005. Biodegradability of polymers–Mechanisms and evaluation methods. In: Bastioli C, editor. Handbook of biodegradable polymer. 1st ed. Shropshire, U.K.: Rapra Technology Limited. p 1–22.

Zhang B, He PJ, Ye NF, Shao LM. 2008. Enhanced isomer purity of lactic acid from the nonsterile fermentation of kitchen wastes. Bioresour Technol 99:855–62.

Chapitre 3

Les différentes approches de quantification et le mécanisme de libération contrôlée

Muhammad Imran, Anne-Marie Revol-Junelles and Stéphane Desobry

Laboratoire d'Ingénierie des Biomolécules, ENSAIA–INPL, Nancy Université, 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France

Predicting the future: Adaptations of food packaging trends via nanotechnology
In: Modified Atmosphere and Active Packaging. Taylor & Francis Publishers (2011)

1. Mother Nature's experiments: Nanotechnology as an inspiration from nature

„*Nature is a motherly protector'*. Above and beyond the copyright issues of ideas, when it comes to the work of *Mother Nature*, a large portion of nanoscience innovations is an attempt to imitate what has evolved in Nature. Living organisms are not just a compilation of nano-scale objects: Atoms and molecules are organized in hierarchical structures and dynamic systems that are the outcomes of millions of years of Mother Nature‟s experiments [1]. Of course, all physiological processes on some level occur at the *sub-micron*, *nanometer*, and even smaller *picometer* scale. The essential biomolecules such as sugars, amino acids, hormones, and DNA are in the nanometer range. Tenth-nanometer diameter ions such as potassium and sodium generate nerve impulses. Most protein and polysaccharide molecules have nanoscale dimensions (Figure 1). Every living organism on earth exists owing to the presence, absence, concentration, location, and interaction of these nanostructures [1, 2].

Nanoscience is currently enabling evolutionary changes in several technology areas but new paradigms will eventually have a much wider and revolutionary impact [3]. Nanoscience is "the study of phenomena and manipulation of materials at atomic, molecular, and macromolecular scales (0.2–100 nm), where properties differ significantly from those at a larger scale," whereas nanotechnology is "the design, characterisation, production, and application of structures, devices, and systems by controlling the shape and size at the nanometre scale" [4]. The US definition is that "nanotechnology is the understanding and control of matter at dimensions of roughly 1 to 100 nanometers, where unique phenomena enable novel applications." Encompassing nanoscale science, engineering, and technology, nanotechnology involves imaging, measuring, modelling, and manipulating matter at this length scale [5].

With the increased funding opportunities and interest in this field, the term "nano" is more frequently and often liberally used [6]. The Royal Commission on Environmental Pollution had published report on „*Novel Materials in the Environment: The case of nanotechnology',* which examined issues related to innovation in the materials sector and made important recommendations on how to deal with ignorance and uncertainty in this area. The small size of nanomaterials gives them specific or enhanced properties, compared with the same materials at macroscale, which generate great interest in their potential for development of different uses. The vital nanotechnological benefits in the food regime have been presented in Figure 2.

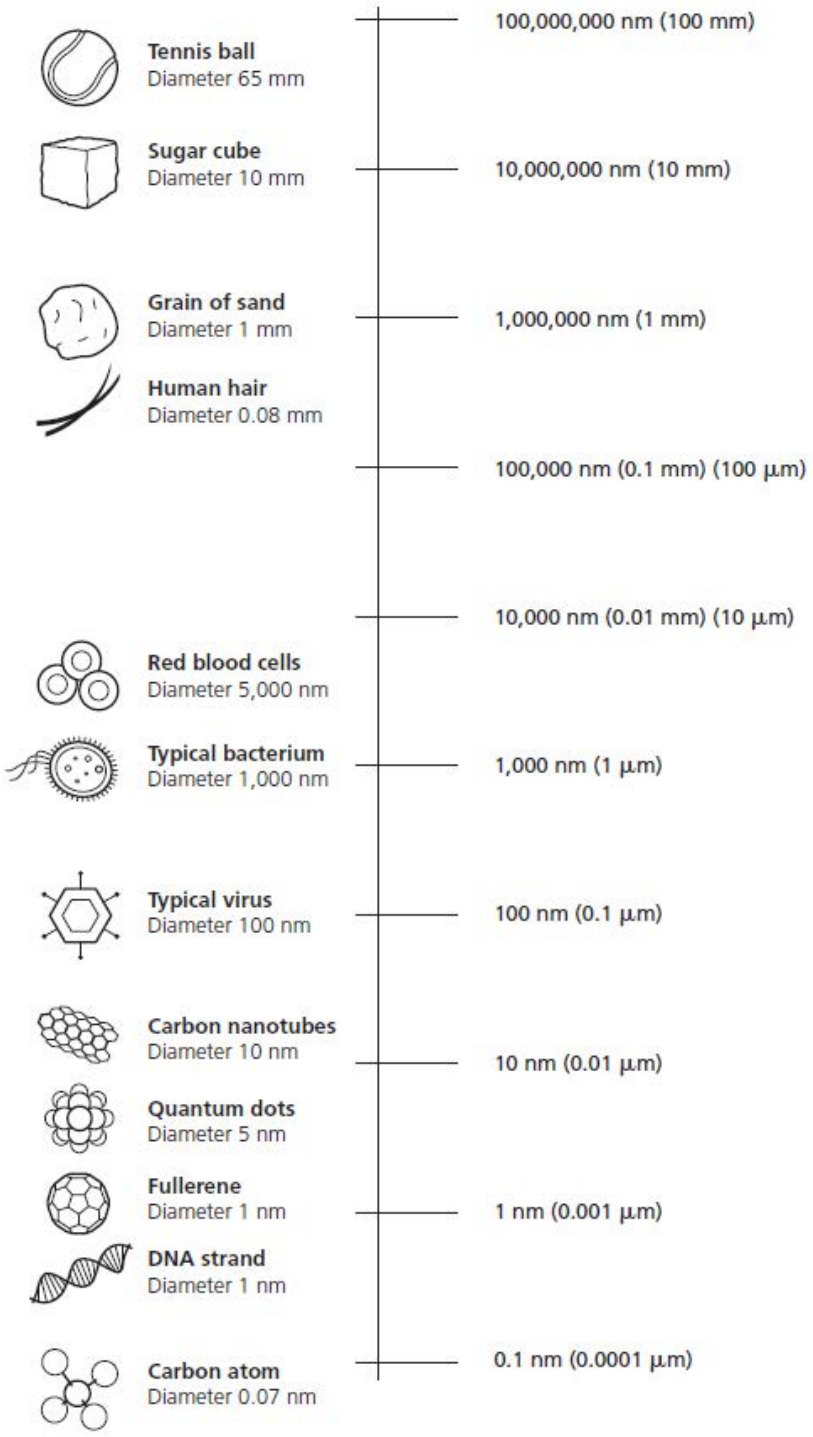


Figure 1: Length scale showing the nanometer in context. One nanometre (nm) is equal to one-billionth of a metre, 10^{-9} m. Most structures of nanomaterials which are of interest are below 100 nm. (Source: Royal Commission on Environmental Pollution Report 27, 2008 [7])

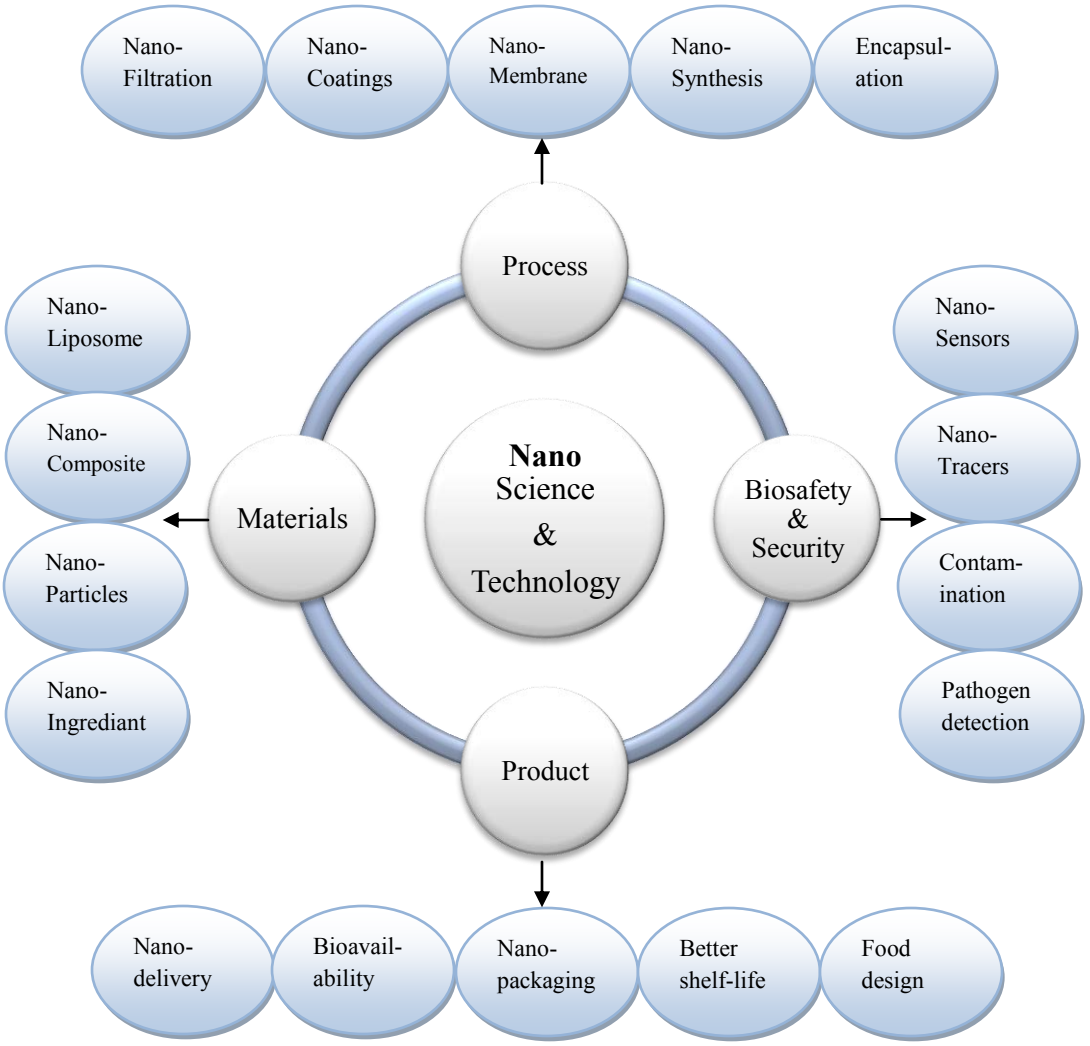


Figure 2: The impacts and needs of nanotechnology applications in foods

2. Nano-active packaging

Nanotechnology approaches are being broadened in food science, especially in packaging with high performances and low concentrations and prices, so this category of nano-research is estimated to be revolutionary in food packaging. The linkage of a 100% bio-originated material and nanomaterials opens new windows for answering environmental and health concerns [8]. It is believed the first area that nanotechnology will impact upon in the food industry is food

packaging. Nanotechnology is likely to be engaged for effective and efficient amendments of food products by bioactive and smart nano-packaging technology.

Nanocomposites packaging to create molecular barrier: In food packaging, a key issue is the development of high barrier properties against the migration of oxygen, carbon dioxide, flavor compounds, and water vapor. The nanoscale plate morphology of clays and other fillers helped the development of gas barrier properties. Thus, the development of nanocomposites may offer a new approach to improve mechanical strength, thermal stability, and gas barrier properties [9]. Numerous biopolymers have been utilized to develop materials for eco-friendly food packaging. However, the use of biopolymers has been restricted due to their usually poor mechanical and barrier properties, which may be improved by adding reinforcing compounds (fillers), forming composites. Nanoparticles have proportionally larger surface area than their microscale counterparts, which favors the filler-matrix interactions and the performance of the resulting material [10]. Incorporation of chitosan nanoparticles in the hydroxypropyl methylcellulose (HPMC) films improved their mechanical properties significantly, while also improving film barrier properties significantly. The chitosan poly(methacrylic acid) (CS-PMAA) nanoparticles tend to occupy the empty spaces in the pores of the HPMC matrix, inducing the collapse of the pores and thereby improving film tensile and barrier properties [11].

Nano-active packaging for controlled release of additives: Hydrophilic or hydrophobic active agents can be encapsulated into liposomes and then dispersed in the temperature-reversible chitosan-glycerophosphate hydrogel. The liposomes provided excellent sustained drug release from chitosan matrix when compared with that of free drugs, especially for hydrophilic drugs. The negative charge of the liposomes was complexed with the positive charge of the chitosan's protonized amine group that results in controlling the drugs release [12].

Nanotechnology to improve micro-nutrition: The ferrous glycinate liposomes possibly will be a category of capable iron fortifier. Ferrous glycinate liposomes might be obtained with high encapsulation efficiency of 84.80%. The stability of micro/nano-encapsulated ferrous glycinate in strong acid environment was greatly improved by protecting it from the extracapsular environment by lipid bilayer. The bioavailability of ferrous glycinate, as the iron source for biological activity including hemoglobin formation, may be increased [13].

To enhance the curcumin absorption by oral administration, liposome-encapsulated curcumin (LEC) was prepared from commercially available lecithins. High bioavailability of curcumin was evident in the case of oral LEC; a faster rate and better absorption of curcumin were observed as compared to the other forms. Thus liposome encapsulation of ingredients such as curcumin may be used as a novel nutrient delivery system [14].

Nanoactive packaging for improved shelf-life: The nano-packaging might provide an attractive alternative to improve preservation quality of the strawberry fruits during extended storage. A novel nano-packing material was synthesized by blending polyethylene with nano-powder (nano-Ag, kaolin, TiO,). This nano-packaging was able to maintain the sensory, physicochemical, and physiological quality of strawberry fruits at a higher level compared with the normal packing (polyethylene bags) at 4 °C. Additionally, nano-packing has the advantages of simple processing and feasibility to be industrialized in contrast with other storages [15].

Nano-sensors and bioswitches: In effect, the necessity to generate fast, reliable and precise information on the quality and security of foodstuffs and food industry has resulted in an intensive search for more selective and sensitive analytical methods. Chemical sensor and biosensor technology for use in this area has rather early taken advantage of the unique merits of nanomaterials. Various nano-based sensing approaches for exogenous compounds (e.g. pesticides, toxic anions, ripening gases or vitamin supplements) and endogenous compounds (from microorganisms to vitamins) in food are under development [16]. Researchers are working on nano-particle films and other packaging with embedded sensors that will detect food pathogens. With the electronic tongue technology, the sensors can detect substances in parts per trillion (PPT) and would trigger a colour change in the packaging to alert the consumer about food contamination or spoilage.

Nanotechnology to Develop Antimicrobial Packaging: The silver-based nanoclay showed strong antimicrobial activity against Gram-negative *Salmonella spp*. The active agent dispersed well throughout the PLA matrix to a nanoscale, yielding nanobiocomposites. The films were transparent with improved water barrier and strong antimicrobial properties. The migration levels of silver were within the specific migration levels referenced by the European Food Safety Agency (EFSA), and antimicrobial activity displayed supported the potential application of this

biocidal additive in nano-active food-packaging applications [17]. Nanocomposite low-density polyethylene films containing Ag and ZnO nanoparticles were prepared to preserve the orange juice. The microbial growth rate significantly reduced as a result of using this nanocomposite packaging material. Packaging made from nanocomposite film containing nanosilver showed a more pronounced antimicrobial effects, as compared with nano-ZnO during 112 days storage of inoculated orange juice [18].

3. Bioactives delivery mechanisms at micro- or nanoscale:

To date, literature study reveals that relatively low attention has been given to release mechanism of diverse active agents from liposome at micro- or nanoscale. The research studies related to the application of encapsulation concept in the food are mainly focused on methodology, liposomal composition, encapsulation efficiency and prolonged stability. However, little emphasis has been made on release phenomenon occurring at molecular or membrane level.

The question arises, what type of mechanisms are involved with the binding of active agents to the liposomal membrane. Is this binding really irreversible and just caused by their lipo- or hydro-philicity? Do other factors participate in leakage kinetics from the liposomal bilayer membrane? The appreciation of the observed reversible character of the active agent binding is of paramount importance, since the term "encapsulated" is often over interpreted as being "irreversibly encapsulated" [19].

In case of the active agents capable of forming pore, like antimicrobial bacteriocins, different postulates of mechanism of factors of release include: liposomes are "kinetically stable" for defined period of time; "fusion" between liposome and pathogen outer membrane; core (antimicrobial) material induced leakage; liposome membrane permeability which for instance had been reduced by cholesterol; diffusion due to low molecular weight of the antimicrobials; certain antimicrobial like nisin induce leakage by changing membrane structure; interaction between liposome and fat globule membrane may result in destabilization of liposomal membrane and subsequent release of active compounds; bivalent ions Ca^{++}/ Mg^{++} induce de-stability of liposomes especially in the food products containing higher bivalent ions [20-23].

Table 1 summarizes the critical features of food active agents (antioxidant, antimicrobial, enzyme, amino acids, vitamin, enzyme, fortifier, Essential fatty acids) by enca--sulation in the micro- or nanopaticles (liposome), with release mechanisms/factors under *in vitro* or *in vivo* conditions. Few research studies had proved the actual phenomenon underlying the release from liposome. In case of antimicrobial release for minimizing food-borne illness, transmission electron microscope reveals the fusion of liposome with bacterial cell envelope to discharge the encapsulated nisin in liposome [24]. Other interesting facts had revealed the effects of pH and bile salts for increasing the permeation of iron (ferrous glycinate) through liposomal membrane due to bilayer instability and surfactant action respectively [13]. Various logical hypotheses had been formulated for different active agent release from liposomal encapsulation but they are yet to be proved (Table 1).

4. Release efficiency of bioactives at micro/nano-scale:

Encapsulation and targeting the bioactive agents – including nutrients, drugs, vaccines, and cosmetics – and their protection from degradation and inactivation have been investigated extensively using microencapsulation systems [25, 26]. The complex term „release" includes two major phenomenons, diffusion of active agent from liposome core through uni/multi lamellar bilayers and desorption from liposome into the medium where its bioavailability is required [27].

A recent study has investigated the first phase of release process of lipophilic agents in aqueous liposomal dispersions. The sequential steps included: active agent dissolved in the lipid domain of the membrane, (2) departure of the drug from the membrane into the aqueous phase, (3) association of the drug component in the aqueous phase with the acceptor membrane, followed by (4) dissolving of the drug in the acceptor membrane. These steps may differ at high phospholipid concentrations where it is believed that collision between the lipid vesicles is the main transferring mechanism [19]. Different mechanisms of active agent/drug delivery from nanoparticles to the target cell are illustrated in Figure 3.

Table 1: Micro- or nano-encapsulation of various bioactive agents in food systems, their key features and mechanisms of release (*in vitro/in vivo*)

Bioactive agent	Particle/Vesicle Composition	Encapsulation Objective	Encapsulation Method	Size (nm)	Entrapment Efficiency %	Mechanisms of release H : Hypothetical ; P: Proved	Ref.
Antioxidant							
- Curcumin	Commercial Soya lecithin liposome (SLP-PC70/white)	To improve gastrointestinal absorption	Micro-fluidization	220	68	H: The mechanisms in uptake include steps of the diffusion of particles through mucus and accessibility to an enterocyte surface, epithelial interaction, cellular trafficking, exocytosis and systemic dissemination	[14]
- Quercetin	Eudragit® E nano-particles	Solubility	Precipitation method	82 - 473	95 - 99.9	H: The dissolution increased due to particle size reduction and high energy amorphous state formation	[31]
Bacteriocin	Phosphatidyl-choline (PC) liposomes	Slow release, lesser amount	Reverse phase evaporation	570	26	H: Weak hydrophobic interaction between BLS and PC result in slow release	[32]
- Bacteriocine like substance (BLS)							
- Nisin	Phospholipon mixture liposomes	Long term preservation, nisin protection	Mozafari method	190-284	12 - 54	H/P: Fusion of liposome with bacterial membrane proved with electron microscope and lipid mixing assay, but intracellular nisin delivery is not yet proved	[24]
- Nisin	Proliposome	Longer stability	Heating method	725-800	9.5 - 47	H: Extra liposomal environmental conditions, bivalent ions like Ca^{+2} and Mg^{+2}, fat concentration in medium	[20]
- Nisin	PC:Phosphatidyl-glycerol (PG) liposome	Stability against pH	Reverse phase evaporation	100-240	72 – 89 (Calcein dye)	H: Nisin rapidly creates pores in membranes that contain high levels of anionic lipids H: Brownian motion inducing rearrangement of liposome and thus reducing its stability	[33]
-Nisin and Lysozyme (LZ)	PC:PG:Cholesterol Liposome	Microbiological safety of food products	Reverse phase evaporation	85-145 161-174	54-71 (Nisin) 60-61 (LZ) (Calcein dye)	H: Unstable pore formation due to binding of nisin to negatively charged head groups of phospholipids H: Insertion of antimicrobial into liposome membrane due to its low molecular weight	[29]
Enzyme							
- Casein hydrolysates	PC:PG:Cholesterol Liposome	Masking the bitterness	Reverse phase evaporation	500-1000	56-62	H: Chemical stability affected by oxidation	[34]

Bioactive agent	Particle/Vesicle Composition	Encapsulation Objective	Encapsulation Method	Size (nm)	Entrapment Efficiency %	Mechanism of release H : Hypothetical ; P: Proved	Ref.
Enzyme							
- Flavourzyme (FZ)	Chitosan:alginate Capsules	Controlled proteo -lysis of cheese	Extrusion	Micro scale	7-84	H: Enzyme release by the pressure applied during pressing the cheese curd	[35]
-Chymotrypsin	Proliposome	Cheese acceler-ated ripening	Agitation/hydrat ion	--	63	H: The Lipid exchange between milk fat globule membranes and liposomes might explain the early release of enzymes	[36]
- Glucose oxidase	Egg PC: Cholesterol Liposome	Sustained release of hydrogen peroxide	Dehydration rehydration (DRV) method	--	24	H: The liposomal membrane was considered as semi-permeable thus enzyme release by permeation	[37, 38]
- Lipase/ protease/FZ	Proliposome VPF	Cheese texture and flavour improvement	Agitation/hydrat ion	Micro scale	20-36	H: Rupturing of liposome during cheese making H: Release of encapsulated agents due to liposome membrane collapse by lipase action	[39]
Fortifier							
- Iron (Ferrous glycinate)	Egg lecithin liposome	To improve bio-availability	Reverse phase Evaporation	207-559	66-84.8	P: The leakage is attributed to low pH resulting in instability of liposome related to permeation of protons P: Bile salts are surfactants that seem to break down the bilayer membranes	[13]
Nutrient							
- PUFA + Vitamin E + Flavonoid	Marine lecithin emulsion	PUFA Stability against oxidation	High pressure homogenisation	160-207	Emulsion based	H: Release due to thermodynamic instability of emulsions affected by temperature, fat and lecithin ration	[40]
Miscellaneous							
- Free amino acids (FAA)	Soy lecithin liposome	To enhance nutritional value	Reverse phase Evaporation and Rehydration of Freeze-dried	2000-8000	42.6	H: Release tested under in vitro simulating condition but no mechanism of release investigated	[41]
-Cucuminoids	Polybutylcyano-acrylate	To improve bio-availability and stability against photo degradation	Solvent evaporation method	173-281	66-82	H/P: Release due to environment changes in the cell like acidic value, however what phenomenon occur on membrane level due to low pH is not proved	[42]

PUFA: Poly unsaturated fatty acid

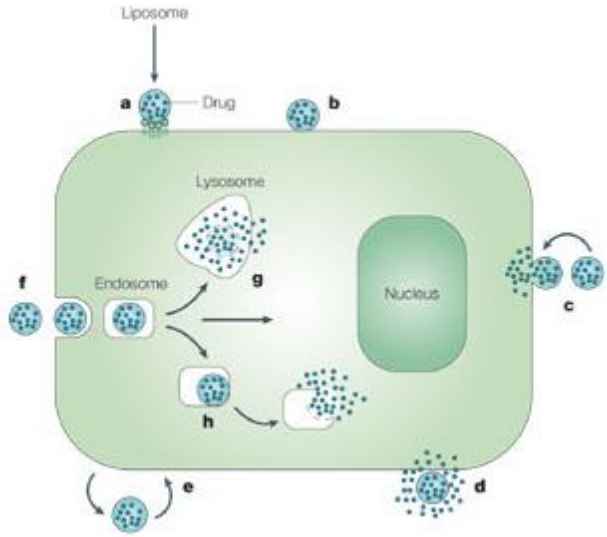


Figure 3: Drug-loaded liposomes can specifically (a) or nonspecifically (b) adsorb onto the cell surface. Liposomes can also fuse with the cell membrane (c), and release their contents into the cell cytoplasm, or can be destabilized by certain cell membrane components when adsorbed on the surface (d) so that the released drug can enter cell via micropinocytosis. Liposome can undergo the direct or transfer-protein-mediated exchange of lipid components with the cell membrane (e) or be subjected to a specific or nonspecific endocytosis (f). In the case of endocytosis, a liposome can be delivered by the endosome into the lysosome (g) or, en route to the lysosome, the liposome can provoke endosome destabilization (h), resulting in drug liberation in the cytoplasm (Torchilin, V.P. 2006, Nature Reviews Drug Discovery[28])

Either due to the lack of advanced tools to study the nano-structured materials or novelty of this field, literature study shows no significant work done to elaborate the release process of food active agents from liposomes. An exciting work related to antimicrobials release from liposome had analysed this phenomenon by utilizing calcein (fluorescent dye) [29]. However, the major complication to imitate the nisin release in this manner is its capability to form pore in membrane models (liposome), and thus results of its release will be quite different from fluorescent dye as high concentration of this antimicrobial bioactive would disrupt the membrane instead of slow inside out migration.

Table 2 summarizes the different approaches utilized till date for the quantification of release efficiency of encapsulated active agents being employed in food sector. More than half of

Table 2: Different approaches of quantification the release efficiency of encapsulated active/fortification agents

Bioactive	Release efficiency	Quantification D : Direct ; ID : Indirect	Ref.
Antioxidant			
Quercetin	Higher solubility of Nano-system, 74 fold higher release	D: Partition coefficient analysis based on UV-spectrometer ID : Antioxidant activities assays	[31]
Bacteriocin			
BLS	The encapsulated BLS remained with approximately 90% its initial activity for 30 days, as compared to 14 days for free BLS	ID: Residual antimicrobial activity against *Listeria monocytogenes*	[32]
Nisin	Indirectly correlated to stability of liposome	ID: Size stability and image analysis	[24]
Nisin	After 18 days storage 39.2 to 78.2 % (of initially encapsulated) nisin was released in different food medium	D: Competitive enzyme immunoassay (c-EIA) based quantification	[20]
Nisin and Lysozyme	During 35 minutes, 5-20% release was observed for a mixture of nisin and lysozyme for different PC:PG:Cholesterol liposome	ID: Fluorescent assay based on calcein dye release	[29]
Enzyme			
Flavourzyme	During 4 to 16 hours under simulated cheese block pressing, only 30 percent of enzyme was release from microcapsules and thus beneficial for initial stages of cheese maturation	ID: Enzyme quantification analysed indirectly by its activity against substrate L-leucine-p-nitroanilide expressed as leucine amino peptidase units (LAPU) using spectrophotometer	[35]
Chymotrypsin	During first 3 weeks, the liposome enzyme treated cheese had little decreased in residual activity as compared to >20% decrease for free enzyme treated cheese model	ID: Residual enzymatic activity in cheese was characterized against fluorescein isothyocyanate labelled casein (FITC)	[36]
Fortifier			
- Iron	Bile salt micelles and vesicles can solubilize phospholipid bilayer membranes. A small amount of ferrous glycinate was released from liposomes in the first 4 h in the medium of pH 1.3. However, after 20 hours more than 50 % ferrous glycinate was released.	D: The in vitro (simulated gastrointestinal conditions) release of ferrous glycinate from liposomes was measured by bathophenanthrolin colorimetry and dialysis method.	[13]
Miscellaneous			
Curcuminoids	Initial burst release obtained for first 4–5 hours attributed to the drug adsorbed on the surface of the nano-particles. After 24 hours, 77 and 85 % cumulative release was observed for neutral and acidic medium respectively	D: HPLC method using UV–vis detector and compared with standard curcuminoids solution	[42]

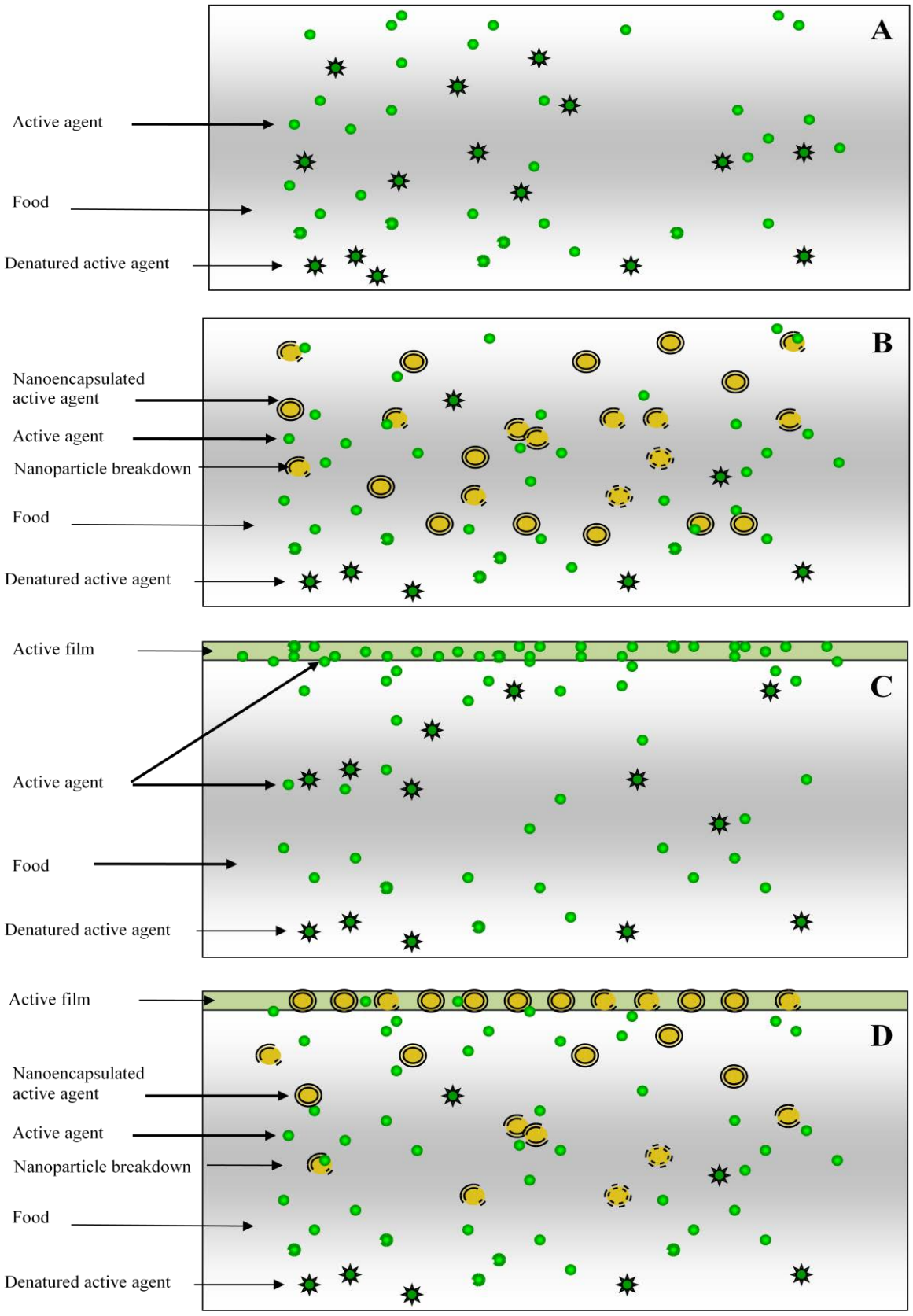

A
Active agent
Food
Denatured active agent
B
Nanoencapsulated active agent
Active agent
Nanoparticle breakdown
Food
Denatured active agent
Active film
C
Active agent
Food
Denatured active agent
Active film
D
Nanoencapsulated active agent
Active agent
Nanoparticle breakdown
Food
Denatured active agent

Figure 4: Illustration of different approaches opted for food security. (A) Direct incorporation of active agent i.e. free form into food; major disadvantages - degradation/denaturation of active agent by enzymes/ions/food matrice interaction/fat, no protection at food surface where microbial/contamination load is maximum, costly as excessive quantity of active agent required, and low shelf-life of food. (B) Nanoencapsulation of active and subsequent incorporation in food; major disadvantage – inadquate quality due to contamination and microbial spoilage at food surface. (C) Active packaging, incorporation of active agent in food coatings for controlled release; major disadvantage - inactivation of additive by complex food system, however sustained desorption/diffusion of active agent from package towards food (D) Nanoactive packaging, fusion of all above approaches to eliminate the respective disadvantages for longer food shelf-life

the research data comprised on indirect evaluation of bioactive agent through residual enzymatic or antimicrobial activity measurement. It can be foreseen that with improvements in manufacturing technologies, new strategies for stabilization of fragile nutraceuticals and development of novel approaches to site-specific carrier targeting, encapsulation carriers will play an important role in increasing the efficacy of functional foods [30]. At the present time, greater fundamental understanding of polymer-polymer and polymer–active agent interactions at the molecular level is still required to ensure design of ideal nutraceutical carriers for use in the food industry. The labelling of the active agents with coloured dyes or radioactive probes may result as an exciting approach towards the direct quantification of bioactive‟s bioavailability and its mechanism of release.

5. Nano-active packaging: concluding notes

Thus far at its infancy stage, nano-active packaging is an innovative cutting edge approach to enhance food safety and security. The nano-active packaging strengthens the pre-existing principal objectives of packaging, which includes: physical protection (mechanical parameters), molecular barrier (oxygen, water vapor, dust or chemicals), sensory attributes (transparency, homogenous topographical attributes), high quality food (biosensors, smart nano-systems) and extended shelf life or food safety (nano-active particles, controlled release of antimicrobials and target delivery)… The need-based evolution towards nano-active packaging

for superior packaged food quality is illustrated in Figure 4. Furthermore the nano-active concept complements very well with eco-friendly biodegradable polymers for environmental conservation. The adaptations towards the bioactive, biodegradable, and bio-nanocomposite concepts in nano-active packaging is most likely to be the smartest development yet to be seen in modern packaging innovations to provide healthier food.

References:

[1] J. Weiss, P. Takhistov, D.J. McClements, Journal of Food Science, 71 (2006).

[2] R. Ravichandran, International Journal of Green Nanotechnology: Physics and Chemistry, 1 (2010) 72 - 96.

[3] M. Imran, A.M. Revol-Junelles, A. Martyn, E.A. Tehrany, M. Jacquot, M. Linder, S. Desobry, Critical Reviews in Food Science and Nutrition, 50 (2010) 799-821.

[4] RSRAE, Available at (http://www.nanotec.org.uk/finalreport. pdf)., Accessed december 14, 2010., (2004).

[5] NNI, Available at (http://www.nano.gov/)., Accessed December 14, 2010, (2001).

[6] A. Nel, T. Xia, L. Madler, N. Li, Science, 311 (2006) 622-627.

[7] RCEP, Available at (www.rcep.org.uk/reports/27), Accessed December 10, 2010, (2008).

[8] M. Jamshidian, E.A. Tehrany, M. Imran, M. Jacquot, S. Desobry, Comprehensive Reviews in Food Science and Food Safety, 9 (2010) 552-571.

[9] A. Arora, G.W. Padua, Journal of Food Science, 75 (2010).

[10] H.M.C.d. Azeredo, Food Research International, 42 (2009) 1240-1253.

[11] M.R. De Moura, R.J. Avena-Bustillos, T.H. McHugh, J.M. Krochta, L.H.C. Mattoso, Journal of Food Science, 73 (2008).

[12] H. Chiang, Y.C. Huang, H.Y. Yeh, S.Y. Yeh, Y.Y. Huang, Biomedical Engineering - Applications, Basis and Communications, 21 (2009) 107-114.

[13] B. Ding, S. Xia, K. Hayat, X. Zhang, Journal of Agricultural and Food Chemistry, 57 (2009) 2938-2944.

[14] M. Takahashi, S. Uechi, K. Takara, Y. Asikin, K. Wada, Journal of Agricultural and Food Chemistry, 57 (2009) 9141-9146.

[15] F.M. Yang, H.M. Li, F. Li, Z.H. Xin, L.Y. Zhao, Y.H. Zheng, Q.H. Hu, Journal of Food Science, 75 (2010).

[16] M.G. Valdes, A.C.V. Gonzalez, J.A.G. Calzon, M.E. Diaz-Garcia, Microchimica Acta, 166 (2009) 1-19.

[17] M.A. Busolo, P. Fernandez, M.J. Ocio, J.M. Lagaron, Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 27 (2010) 1617-1626.

[18] A. Emamifar, M. Kadivar, M. Shahedi, S. Soleimanian-Zad, Food Control, 22 (2010) 408-413.

[19] A. Fahr, P.V. Hoogevest, S. May, N. Bergstrand, M.L.S. Leigh, European Journal of Pharmaceutical Sciences, 26 (2005) 251-265.

[20] R. Laridi, E.E. Kheadr, R.O. Benech, J.C. Vuillemard, C. Lacroix, I. Fliss, International Dairy Journal, 13 (2003) 325-336.

[21] T.M. Taylor, P.M. Davidson, B.D. Bruce, J. Weiss, Critical Reviews in Food Science and Nutrition, 45 (2005) 587-605.

[22] L.M. Were, B. Bruce, P.M. Davidson, J. Weiss, Journal of Food Protection, 67 (2004) 922-927.

[23] A.K. Anal, H. Singh, Trends in Food Science and Technology, 18 (2007) 240-251.

[24] J.C. Colas, W. Shi, V.S.N.M. Rao, A. Omri, M.R. Mozafari, H. Singh, Micron, 38 (2007) 841-847.

[25] M.R. Mozafari, J. Flanagan, L. Matia-Merino, A. Awati, A. Omri, Z.E. Suntres, H. Singh, Journal of the Science of Food and Agriculture, 86 (2006) 2038-2045.

[26] M.R. Mozafari, C. Johnson, S. Hatziantoniou, C. Demetzos, Journal of Liposome Research, 18 (2008) 309-327.

[27] C.P. Champagne, P. Fustier, Current Opinion in Biotechnology, 18 (2007) 184-190.

[28] V.P. Torchilin, Nature Reviews Drug Discovery, 4 (2005) 145-160.

[29] L.M. Were, B.D. Bruce, P.M. Davidson, J. Weiss, Journal of Agricultural and Food Chemistry, 51 (2003) 8073-8079.

[30] L. Chen, G.E. Remondetto, M. Subirade, Trends in Food Science and Technology, 17 (2006) 272-283.

[31] T.H. Wu, F.L. Yen, L.T. Lin, T.R. Tsai, C.C. Lin, T.M. Cham, International Journal of Pharmaceutics, 346 (2008) 160-168.

[32] M.L. Teixeira, J. dos Santos, N.P. Silveira, A. Brandelli, Innovative Food Science and Emerging Technologies, 9 (2008) 49-53.

[33] T.M. Taylor, S. Gaysinsky, P.M. Davidson, B.D. Bruce, J. Weiss, Food Biophysics, 2 (2007) 1-9.

[34] H.A. Morais, C.M. Da Silva Barbosa, F.M. Delvivo, H.S. Mansur, M.C. De Oliveira, M.P.C. Silvestre, Journal of Food Biochemistry, 28 (2004) 21-41.

[35] K. Anjani, K. Kailasapathy, M. Phillips, International Dairy Journal, 17 (2007) 79-86.

[36] E. Laloy, J.C. Vuillemard, P. Dufour, R. Simard, Journal of Controlled Release, 54 (1998) 213-222.

[37] J.M. Rodriguez-Nogales, Journal of Chemical Technology and Biotechnology, 79 (2004) 72-78.

[38] J.M. Rodriguez-Nogales, M. PÃ©rez-Mateos, M.D. Busto, Journal of Chemical Technology and Biotechnology, 79 (2004) 700-705.

[39] E.E. Kheadr, J.C. Vuillemard, S.A. El-Deeb, Food Research International, 36 (2003) 241-252.

[40] N. Belhaj, E. Arab-Tehrany, M. Linder, Process Biochemistry, Article in press (2009).

[41] Y. Barr, S. Helland, Journal of Liposome Research, 17 (2007) 79-88.

[42] R. Mulik, K. Mahadik, A. Paradkar, European Journal of Pharmaceutical Sciences, 37 (2009) 395-404.

III. Résultats et Discussion

Chapitre 4: Enrobages actifs à base du dérivé cellulose: Effets de la nisine et de plastifiant sur les propriétés physico-chimiques et antimicrobiennes des films

Pour répondre aux considérations environnementales, mais également afin de prolonger la durée de vie et la qualité des produits alimentaires, la réduction des déchets d'emballage a fait croître très fortement l'exploration de nouveaux emballages alimentaires biologiques tels que les films comestibles et biodégradables (Burke, 2006 ; Tharanathan, 2003). Les matériaux à base de cellulose ont été largement utilisés pour l'enrobage car ils offrent les avantages d'être comestibles, biocompatibles, d'avoir des propriétés barrière, un faible coût, et une apparence esthétique, et d'être non toxiques ainsi que non polluants (Vancomez *et al*., 2009). L'hydroxy propyle méthyle cellulose (HPMC) est autorisée en utilisation alimentaire par la FDA (21 CFR 172.184) et l'UE (UE, 1995).

La nisine est de plus en plus utilisée en tant que « molécule biopréservatrice » pour une incorporation directe dans l'aliment ou *via* des films comestibles/actifs. La nisine exerce une action inhibitrice vis-à-vis des bactéries Gram-positive et de la germination des spores de *Bacillus* et *Clostridium* (de Arauz *et al*., 2009). La Nisaplin® est la forme commerciale disponible de la nisine et contient 2,5% de nisine, 74,5% de NaCl, 23,8% de protéines de lait et 1,7 % d'humidité (Dawson *et al.,* 2003). La nisine pure n'étant pas disponible, l'utilisation de la Nisaplin® est intéressante au niveau industriel en raison de sa disponibilité commerciale, de son large spectre d'action vis-à-vis des germes pathogènes, de son statut GRAS (EU, 2004 ; FDA, 2008) et de son étiquette d'additif «bio» pour les emballages comestibles. Cependant, la Nisaplin® conduit à une forte hétérogénéité des films d'emballage, surtout en raison de sa forte teneur en sel. Pour remédier à ce problème, des agents plastifiants peuvent être utilisés. Ils peuvent entrainer une diminution de la fragilité des films en réduisant les forces inter-moléculaires, en augmentant la mobilité des chaines de polymère, en diminuant la température de transition vitreuse et en augmentant leur fléxibilité (Galdeano *et al*., 2009 ; Zhang & Han, 2008).

L'objectif de cette étude est d'étudier les effets de la Nisaplin® et de différentes concentrations d'agents plastifiants sur les propriétés physico-chimiques et antibactériennes.de film comestible à base d'HPMC.

Chapter 4: Cellulose derivative based active coatings: Effects of nisin and plasticizer on physico-chemical and antimicrobial properties of hydroxypropyl methylcellulose films

To address the environmental issues, and concurrently extend the shelf life and food quality, reducing packaging waste has catalysed the exploration of new bio-based packaging materials such as edible and biodegradable films (Burke, 2006; Tharanathan, 2003). Cellulose-based materials are being widely used as edible coatings they offer the advantages like edibility, biocompatibility, barrier properties, aesthetic appearance, being non-toxic, non-polluting and having low cost (Vasconez et al., 2009). Hydroxypropyl methylcellulose (HPMC) is approved for food uses by the FDA (21 CFR 172.874) and the EU (EC, 1995).

Nisin has been increasingly used as „bio-preservative" for direct incorporation in food as well as in active/edible films. Nisin effectively inhibits Gram-positive bacteria and outgrowth spores of *Bacillus* and *Clostridium* (de Arauz et al., 2009). Nisaplin® is the commercially available form containing 2.5% nisin, 74.5% NaCl and 23.8% denatured milk solids and 1.7% moisture (Dawson et al., 2003). The pure nisin is not available for commercial / industrial use, thus the Nisaplin® (heterogeneous blend of nisin) applications are interesting at industrial scale due to its commercial availability, broad spectrum against food borne pathogen, only approved bacteriocin by FDA i.e. status as GRAS (EU, 2004; FDA, 2001) and its „bio-additive "notion for edible packaging. However, due to its heterogeneity, Nisaplin renders the packaging films with undesirable imperfections.

To address this problem, plasticizers may help to decrease inherent brittleness of films by reducing intermolecular forces, increasing the mobility of polymer chains, decreasing the glass transition temperature and improving their flexibility (Galdeano et al., 2009; Zhang & Han, 2008). So in the present study our objective was to study the effect of Nisaplin® or various concentrations of plasticizer on HPMC-based edible film individually, and to further analyse their simultaneous utilization for improving the physicochemical attributes and antimicrobial efficacy.

Chapter 4

Cellulose Derivative Based Active Coatings: Effects of Nisin and Plasticizer on Physico-chemical and Antimicrobial Properties of Hydroxypropyl methylcellulose Films

Muhammad Imran, Soumaya El-Fahmy, Anne-Marie Revol-Junelles, Stéphane Desobry

Laboratoire d'Ingénierie des Biomolécules, ENSAIA–INPL, Nancy Université, 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France

(Carbohydrate Polymers (81), 219–225. 2010)

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Cellulose derivative based active coatings: Effects of nisin and plasticizer on physico-chemical and antimicrobial properties of hydroxypropyl methylcellulose films

Muhammad Imran, Soumaya El-Fahmy, Anne-Marie Revol-Junelles, Stéphane Desobry*

Nancy Université, ENSAIA–INPL, Laboratoire d'Ingénirie des Biomolécules (LIBio), 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France

ARTICLE INFO

Article history:
Received 4 December 2009
Received in revised form 3 February 2010
Accepted 9 February 2010
Available online 4 March 2010

Keywords:
Carbohydrate edible coating
HPMC
Polypeptide nisin
Biodegradable packaging
Listeria

ABSTRACT

Bioactive composite coatings based on hydroxypropyl methylcellulose (HPMC), broad-spectrum food preservative nisin (Nisaplin®), and hydrophilic plasticizer glycerol were evaluated for mechanical, barrier (O_2, H_2O), transparency and microbiological effectiveness. Incorporation of Nisaplin® into cellulose derivative, i.e. HPMC-based films strongly increased the film thickness due to salt crystallization while glycerol had normalized it by homogenous dispersibility. The tensile strength of composite films decreased, however ultimate elongation was increased significantly. The dynamic vapour sorption experimental data fitted by different models had shown lesser values of respective energy constants for composite films. The transparency and water permeability of HPMC films were negatively affected by the additives as an effect individual but conversely as combined effect for film transparency. Film bioactivity demonstrated efficacy against *Listeria* > *Enterococcus* > *Staphylococcus* > *Bacillus* spp. These cellulose derivative based active films may thus be a key approach towards eradicating post-process contamination of healthy foods.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Post-process contamination caused by product mishandling and faulty packaging is responsible for about two-thirds of all microbiologically related class I recalls in the USA with most of these recalls originating from contamination of ready-to-eat food products (Cagri, Ustunol, & Ryser, 2004). Over the last few years, consumer demand for food stuff of natural origin (termed as "Bio"), high quality, elevated safety, minimally processed, longer shelf-life, ease-to-eat with a fresh taste and appearance have turned out to be the cardinal importance (Sobrino-Lopez & Martin-Belloso, 2008). Currently, there is an escalating tendency to employ environmental friendly materials with the intention of substituting non-degradable materials, thus reducing the environmental pollution resulting from waste accumulation. To address the environmental issues, and concurrently extend the shelf-life and food quality, reducing packaging waste has catalysed the exploration of new bio-based packaging materials such as edible and biodegradable films (Burke, 2006; Tharanathan, 2003). One of the approaches is to use renewable biopolymers such as polysaccharides, proteins, gums, lipids and their complexes, derived from animal and plant origin (Ray & Bousmina, 2005). Such biodegradable/edible packaging not only ensures food safety but at the same instant are good source of nutrition (Reppas, Swidan, Tobey, Turowski, & Dressman, 2009).

Cellulose-based materials are being widely used as they offer the advantages like edibility, biocompatibility, barrier properties, aesthetic appearance, being non-toxic, non-polluting and having low cost (Vasconez, Flores, Campos, Alvarado, & Gerschenson, 2009). Hydroxypropyl methylcellulose edible films are attractive for food applications because it is a readily available non-ionic edible plant derivative shown to form transparent, odourless, tasteless, oil-resistant, water-soluble films with very efficient oxygen, carbon dioxide, aroma and lipid barriers, but with moderate resistance to water vapour transport (Villalobos, Chanona, Hernandez, Gutiaerrez, & Chiralt, 2005). HPMC is used in the food industry as an emulsifier, film former, protective colloid, stabilizer, suspending agent, or thickener. HPMC is approved for food uses by the FDA (21 CFR 172.874) and the EU (EC, 1995); its safety in food use has been affirmed by the JECFA (Burdock, 2007). The tensile strength of HPMC films is high and flexibility neither too high nor too fragile, which make them suitable for edible coating purposes (Brindle & Krochta, 2008).

Within the scope of natural food preservation, the application of antimicrobial peptides from lactic acid bacteria (LAB) in bioactive packaging films has received great attention (Cleveland,

* Corresponding author. Tel.: +33 3 83 59 58 80; fax: +33 3 83 59 57 72.
E-mail address: Stephane.Desobry@ensaia.inpl-nancy.fr (S. Desobry).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.02.021

Montville, Nes, & Chikindas, 2001). Nisin has been increasingly used as 'bio-preservative' for direct incorporation in food as well as in active/edible films. Nisin effectively inhibits Gram-positive bacteria and outgrowth spores of *Bacillus* and *Clostridium*. Structurally, it is a 34 amino acid polypeptide with a molar mass of 3500 Da. This lantibiotic contains unusual amino acids responsible for the important functional properties, i.e. acid tolerance, thermo stability at low pH and a specific bactericidal mode of action (de Arauz, Jozala, Mazzola, & Vessoni Penna, 2009). Nisaplin® is the commercially available form containing 2.5% nisin, 74.5% NaCl and 23.8% denatured milk solids and 1.7% moisture (Dawson, Hirt, Rieck, Acton, & Sotthibandhu, 2003). In a recent study for edible films (Sebti, Chollet, Degraeve, Noel, & Peyrol, 2007) using HPMC/chitosan and incorporating the pure nisin (Aplin and Barrett Ltd. now merged in as Danisco), the author had evaluated the effect of nisin on physical characteristics of films. However, the pure nisin is not available for commercial/industrial use and is provided in very low amount for research purposes (under conditions). For this reason the use of Nisaplin® (heterogeneous blend of nisin) in food industry becomes essential due to its commercial availability, broad spectrum against food borne pathogen, only approved bacteriocin by FDA, i.e. status as GRAS (EU, 2004; FDA, 2001) and its 'bio-additive' notion for edible packaging. Thus it is imperative to verify the contribution, either positively or negatively, played by commercially available Nisaplin® on the various physico-chemical characteristics of biodegradable/edible films of HPMC.

Plasticizers impressively affect the physical properties of biopolymer films (Zhang & Han, 2008). The plasticizers help to decrease inherent brittleness of films by reducing intermolecular forces, increasing the mobility of polymer chains, decreasing the glass transition temperature of these materials and improving their flexibility (Galdeano, Mali, Grossmann, Yamashita, & Garcia, 2009; Zhang & Han, 2008). Thus it is important to study the effect of commonly used polyol 'glycerol' on the homogenous dispersion of Nisaplin® (nisin, salt and milk solid) for the formation of composite active films of improved quality. However, plasticizer generally causes the increased water permeability so it must be added at a certain amount to obtain the films with improved flexibility, thickness and transparency without significant decrease of mechanical strength and barrier property to mass transfer (Brindle & Krochta, 2008; Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006; Möller, Grelier, Pardon, & Coma, 2004). No information regarding the sorption behaviour, light transmission, tensile characteristics and bioactivity spectrum against potential pathogens of HPMC-Nisaplin®-plasticizer composite film has been reported. Therefore, the objective of this investigation was to study the effect of Nisaplin® or various concentrations of plasticizer on HPMC-based edible film individually, and to further analyse their simultaneous utilization for improving the above-mentioned physico-chemical attributes and antimicrobial efficacy against a spectrum of bacteria.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

HPMC powder with hydroxypropoxyl content ~9% and viscosity ~15 mPa s (2% in H_2O, 25 °C) was obtained from Fluka-Biochemika, Japan. Ethanol 96.2% (Pharmaceutics CARLO Erba) was used to improve hydration of HPMC, it also helped in reduction of air bubbles in film forming solution (FFS) and eventually the drying of film. Glycerol (>97% purity) was used as a plasticizer and was purchased from Merck (Darmstadt, Germany). Nisin solution was prepared by using Nisaplin® (Sigma Chemical Co.) that contains 2.5% nisin and the rest are mainly salt and milk proteins. According to the supplier, the activity of 1 g Nisaplin® is ~10^6 IU.

2.1.2. Microorganism and culture media

Listeria, *Staphylococcus*, *Enterococcus* and *Bacillus* strains were purchased from different institute collections (*Staphylococcus aureus* CIP 677, *S. aureus* CIP 76.25, *S. aureus* CIP 4.83, *S. aureus* CIP 57.10, *Listeria seeligeri* SLCC 3954^T, *L. grayi* CIP 6818^T, *L. monocytogenes* CIP 7831, *L. monocytogenes* CIP 82110^T, *L. innocua* CIP 12511, *L. ivanoii* CIP 12510, *Bacillus cereus* CIP 6624, *B. licheniformis* CIP 5271, *B. subtilis* CIP 5265, *Enterococcus facium* DSM 20477^T, *E. durans* CIP 55125^T). The strains from these genera were cultivated in trypticase soy broth (Biokar Diagnostics, Beauvais, France) supplemented with 6 g L^{-1} of bacto-yeast extract (Biokar) (TSB-YE) except *Enterococcus* strains for which Ellikar (Biokar) medium was utilized. Incubation was performed at 37 °C except for *Bacillus* (30 °C). All strains were stored in appropriate culture medium supplemented with glycerol (10%) at −30 °C and propagated twice before use. Agar medium was prepared by addition of 12 g L^{-1} of bacteriological agar.

2.2. Film preparation

FFS were prepared by dissolving 6 g of HPMC in solution of ethanol (35 mL) and distilled water (65 mL). For better dissolving the Nisaplin®, the pH of solvent was adjusted to ~3 with HCl 0.1 N. The solutions were mixed for 40 min at 65 °C using a heating magnetic stirrer (Fisher Bio-block Scientific). Composition of the HPMC biodegradable active film formulations and variables of concern, such as concentration of plasticizer and Nisaplin® amount are shown in Table 1. Selected amounts of glycerol and Nisaplin® were added during heating and stirring. Nisaplin® concentration

Table 1
Mechanical properties and thickness of HPMC-Nisaplin®-plasticizer composite films (mean of triplicate analysis).

Film composition	Thickness, *X* (μm)	Tensile strength, TS (MPa)	Ultimate elongation, UE (%)	Young's modulus, *Y* (MPa)
HPMC	47 ± 2	63 ± 8	13 ± 1	2334 ± 99
HPMC + 10% G	49 ± 3^{NS}	47 ± 11*	33 ± 8*	1462 ± 63***
HPMC + 20% G	53 ± 1^{NS}	27 ± 10***	33 ± 3*	1098 ± 165***
HPMC + 30% G	57 ± 5^{NS}	21 ± 2***	41 ± 13**	961 ± 92***
HPMC + 50% G	56 ± 3^{NS}	16 ± 3***	50 ± 6***	421 ± 16***
HPMC + N	70 ± 12**	43 ± 9*	26 ± 14^{NS}	856 ± 229***
HPMC + N + 10% G	71 ± 12**	28 ± 4***	30 ± 13^{NS}	783 ± 269***
HPMC + N + 20% G	72 ± 11**	23 ± 6***	41 ± 4**	656 ± 335***
HPMC + N + 30% G	64 ± 7^{NS}	20 ± 4***	30 ± 1^{NS}	591 ± 209***
HPMC + N + 50% G	58 ± 2^{NS}	20 ± 3***	31 ± 3^{NS}	722 ± 97***

HPMC = hydroxypropyl methylcellulose; G = glycerol; N = Nisaplin® 1%, i.e. 10^4 IU.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.
^{NS} $p > 0.05$.

was adjusted to give a final activity of 10^4 IU. Its solution was prepared by taking 1/10th part of solvent for a formulation (H_2O and ethanol—pH 3) before adding HPMC or glycerol, the solution was centrifuged at 4000 rpm for 15 min at 4 °C and the supernatant recovered. As a homogenous solution was achieved after mixing, it was degassed at 50–60 °C under vacuum (Yamato®). Films were made by pouring approximately 5 g FFS in the lids of Petri-dishes (Optilux - Nunclon™ Fisher, DK-4000 Roskilde, Denmark) and left to dry them at room temperature (20 °C) and relative humidity (~50%) for 24–48 h. Films were either stored under similar conditions of drying or at approximately zero relative humidity using phosphorus pentaoxide (P_2O_5) depending upon film characterization experiment.

2.3. Film characterization

2.3.1. Film thickness measurement

The thickness of films was measured using the standard NF Q 03-016 with a manual micrometer (Messmer, London, England) equipped with a head measuring 1 cm in diameter and a sensitivity of 2 μm. The thickness was measured in 10 randomly selected points on each film prepared by using identical amount (5 g) of FFS and then an average value was determined.

2.3.2. Tensile properties

The mechanical characteristics of films were evaluated at 20 ± 1 °C and 50 ± 2% RH. It consists of tensile strength (TS, MPa), ultimate elongation (UE, percent at break point), and Young's modulus (*Y*, MPa). Maximum tensile strength is the largest stress that a film is able to sustain. Ultimate elongation is the maximum percentage change in the length of a film before breaking. Young's modulus, calculated from the slope of the initial linear region of the stress–strain curves, reflects the film stiffness. The tests were performed using the tension testing machine Lloyd instrument (Hants, United Kingdom) according to standard NF T 54-102 (1971) on 6 specimens previously stored for 7 days at 20 ± 1 °C and 50 ± 2% RH. Sample films of approximately 5 cm × 2 cm (analysed area = 3 cm × 2 cm) uniaxially stretched (sensor of force of 5 kN and constant speed of 20 mm/min). The stress–strain curves were computer-recorded and exploited with Nexygen software.

2.3.3. Water sorption isotherms

Sorption isotherms of films were obtained using a dynamic vapour sorption system (DVS, SMS Ltd., UK). The sample is equilibrated at a constant temperature for different relative humidity values. Film pieces were cut into small pieces (5 mm × 5 mm) and dried in a vacuum dessicator at 20 °C over phosphorus pentoxide (P_2O_5) for 2 weeks. The programmed relative humidities were from 0 to 95%, divided in 10% increments (10 points). The temperature was set at 25 °C. The samples were considered to be at equilibrium when the value d*m*/d*t* (slope of the changing in mass with time) was set to be <0.002 mass%/min.

The modelisation of the sorption isotherms was done using BET (Brunauer–Emmett–Teller) (Eq. (1)), GAB (Guggenheim–Anderson–de Boer) (Eq. (2)), and TSS (three sorption stage) (Eq. (3)) models. The procedure used for estimating the parameters for different models was non-linear regression (curve fitting), using Origin 6.1 software (Origin Lab Corporation, USA).

$$X = \frac{X_m \cdot C_{BET} \cdot a_w}{(1 - a_w)(1 - a_w + C \cdot a_w)} \quad (1)$$

$$X = \frac{X_m \cdot C_{GAB} \cdot K \cdot a_w}{(1 - K \cdot a_w)(1 - K \cdot a_w + C \cdot K \cdot a_w)} \quad (2)$$

$$X = \frac{X_m \cdot C_{TSS} \cdot K \cdot a_w \cdot h_{TSS}}{(1 - K \cdot a_w)(1 + (C \cdot h_{TSS} - 1) \cdot K \cdot a_w)} \quad (3)$$

where *X* is the mass of water adsorbed at p/p_0 (a_w), X_m is the monolayer value (% db). C_{BET} is a temperature-dependent constant, and p/p_0 is the water partial pressure (p = water partial pressure; p_0 = water vapour pressure at saturation). Constant C_{GAB} and C_{TSS} are related to the energy associated with the binding between the water molecules and the matrix primary interactions sites or monolayer. There is also a temperature-dependent value (*K*) related to the heat of sorption of the multilayer. The parameter h_{TSS} is a correction factor concerning the information related to sorption at high relative humidity (≥90%) and the energies (heat of sorption) involved during the sorption process.

2.3.4. Measurement of transparency/light transmission

Film transparency against ultraviolet (UV) and visible light was measured for a wavelength spectrum between 200 and 900 nm, using a UV–visible recording spectrophotometer (Ultrospec 4000 UV/visible, Pharmacia Biotech, UK) according to the procedure given by Fang, Tung, Britt, Yada, and Dalgleish (2002). The transparency of the films was calculated by the equation: transparency = $-\log T_{600}/X$ (Han & Floros, 1997), where T_{600} is the transmittance at 600 nm and *X* is the film thickness. Three replicates of each treatment were tested.

2.3.5. Water vapour permeability (WVP)

Water vapour permeability of the composite films was determined with the gravimetric method described in the AFNOR NFH00-030 standard (1974). The film was sealed in a permeation cell containing a desiccant (silica gel). The glass permeation cells were 5.8 cm (i.d.) × 7.8 cm (o.d.) × 3.6 cm deep with an exposed area of 26.42 cm^2. The permeation cells were placed in a controlled temperature (38 ± 1 °C) and RH (~100%) chamber via ventilation. The water vapour transport was determined from the weight gain of the cell. Three replicates were made from each film composition. Water vapour transmission rate (WVTR) and WVP of the films were calculated as follows (Khwaldia, Banon, Perez, & Desobry, 2004). CWVT was determined from the slope obtained from the regression analysis of weight gain data as a function of time, once the steady state was reached.

$$\mathrm{WVTR} = \frac{dm}{dt} A \quad (\mathrm{g\ h^{-1}\ m^{-2}}) \quad (4)$$

$$P = \frac{\mathrm{WVTR}}{\Delta p\, 3600} \quad (\mathrm{g\,s^{-1}\,m^{-2}\,Pa^{-1}}) \quad (5)$$

$$\mathrm{WVP} = P \times X \quad (\mathrm{g\ m^{-1}\ s^{-1}\ Pa^{-1}}) \quad (6)$$

where d*m* is the weight gain of the cup over time (d*t*), *A* is the area of exposed film, Δp is the vapour pressure differential across the film, and *X* is the film thickness.

2.3.6. Microbiological analysis

To find out the antibacterial activity of films, 1 cm diameter disks were cut from different composite bioactive films and placed on inoculated nutrient medium. The method was previously standardized by adjusting the microbial inoculation rate (0.1%, v/v) and the volume of the agar medium layer (12 mL). Dishes were refrigerated at 4 °C for 4 h to allow the process of bacteriocin diffusion without microbial growth and then incubated at 37 °C (*Listeria*, *Staphylococcus*, *Enterococcus strains*) or 30 °C (*Bacillus* strain). Data was expressed as growth inhibitory zone diameter (cm) and measured at the nearest 1 mm for three replicates.

2.3.7. Statistical analysis

Statistical analyses were carried out by using the software KyPlot version 2.0 (Koichi Yoshioka, Department of Biochemistry

and Biophysics, Graduate School of Allied Health Sciences, Tokyo, Japan). For comparison between HPMC film and films containing either glycerol or Nisaplin®, a parametric multiple test (Dunnett test with HPMC film as control) was performed. Furthermore composite films containing both Nisaplin® and glycerol were compared with their respective formulations without Nisaplin® using Tukey parametric multiple test.

3. Results and discussion

3.1. Film thickness measurement

Film thickness depended greatly on film nature and composition (Table 1). In the first step the addition of plasticizer alone (10–50%, w/w, d.m.) produced the HPMC films with thickness statistically indifferent (Dunnett test, $p > 0.05$). The narrow range variation in thickness might be there because elevated glycerol content maintained higher moisture content at the end of film drying (Chen & Lai, 2008). However, the incorporation of Nisaplin® radically increased the film thickness (70 ± 12 μm) due to the formation of salt crystals (salt present in Nisaplin® formulation) in course of drying and the film was non-homogenous. Previous studies had demonstrated that the film thickness depended primarily on the biopolymer (nature, concentration) and/or the additives incorporated (e.g. nisin, glycerol, …) in the biodegradable films (Mali, Grossmann, Garcia, Martino, & Zaritzky, 2004; Sebti et al., 2007). In the present study, the composite films of Nisaplin® with 30 and 50% glycerol normalized the crystals effect by homogenous dispersibility because plasticizer could reduce the intermolecular forces and increase the mobility of polymer chains.

3.2. Tensile properties

One of the primary tasks of bioactive packaging is either provision of physical shield to food or slow release of active agent. The capacity of these composite bioactive films for preserving the integrity of food stuff was evaluated by measuring the tensile strength (TS), Young's modulus (Y) and ultimate elongation at break (%UE).

As expected the concentration of plasticizer had profound influence on the tensile strength (Table 1). While the concentration of glycerol increased 20% or more, the TS decreased very significantly (Dunnett test, $p \leq 0.001$) to 16 ± 3 MPa for glycerol content 50% (w/w, d.m.). The drop in TS with increasing concentration of plasticizer is in accordance with the results presented by previous studies for plastic and oat starch films (Galdeano et al., 2009; Guiga et al., 2009). However, when Nisaplin® was added in HPMC formulation the TS was decreased (Dunnett test, $p \leq 0.05$) due to the role of milk solids and salt as hydrophilic compounds. On the other hand the composite film containing both antimicrobial and plasticizer had shown significant decrease in tensile strength as compared to HPMC film, but pair-wise comparison of TS for films containing both Nisaplin® and glycerol with respective formulations without Nisaplin® presented non-significant variations (Tukey test, $p > 0.05$). The observed behaviour could be related to the structural modification of HPMC network. The plasticizer and hydrophilic compounds resulted in less dense film matrix, facilitating movements of polymer chains under stress, hence declining the film resistance.

An inverse correlation was observed between the TS and % elongation at break (%UE) characteristics for active composite films. With increasing concentration of the glycerol, the %UE increased very significantly up to 50 ± 6% for glycerol content 50% (Dunnett test, $p \leq 0.001$). Thus the films prepared with glycerol were more flexible and more stretchable than non-plasticized formulation.

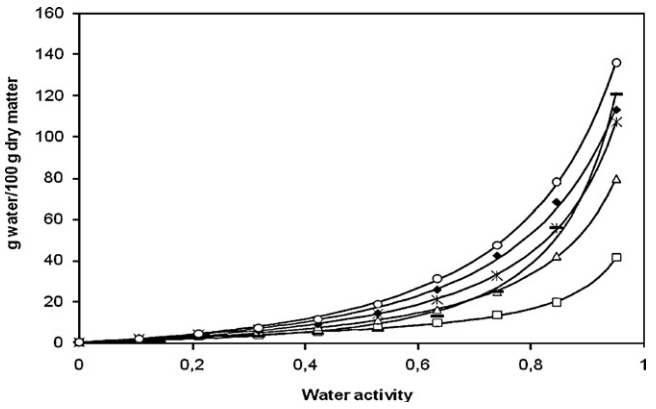


Fig. 1. Moisture sorption isotherms of HPMC, Nisaplin® and glycerol composite films at 25 °C. Experimental DVS values were averaged and fitted by isotherm equation; solid lines represent the GAB model fitted to the data. (□) HPMC film, (△) HPMC + 30% glycerol, (✳) HPMC + 50% glycerol, (—) HPMC + 10^4 IU Nisaplin®, (♦) HPMC + 30% glycerol + 10^4 IU Nisaplin®, (○) HPMC + 50% glycerol + 10^4 IU Nisaplin®.

Nisaplin® had a non-significant increase in the %UE but variation was quite high caused by random break due to presence of salt crystals of Nisaplin® once the film were dried. Concerning the Nisaplin® films with 30% or 50% glycerol, the homogenous dispersion of Nisaplin® by plasticizer resulted in formation of a film network normalized towards moderate elasticity (Dunnett test, $p > 0.05$).

Nisaplin® incorporation in non-plasticized HPMC film reduced the Young's modulus (Y) significantly indicating a decrease in film rigidity. NaCl, milk proteins and carbohydrates present in the Nisaplin® preparation could have interacted with HPMC and modified its mechanical conduct. In addition, these compounds have high affinity for water, known to be very effective plasticizer for most biopolymers, which induces lowering of elastic modulus. With glycerol incorporation, Y decreased significantly (Dunnett test, $p \leq 0.001$) which is in accordance to the results obtained earlier (Pushpadass, Marx, & Hanna, 2008) with starch coatings. Hence the key function in decreasing the film rigidity for composite film was carried out by plasticizer.

3.3. Water sorption isotherm

The isotherms acquired presented a slow initial increase in moisture content with water activity (a_w) increase up to 0.6 and a quick augmentation in film water adsorption with further rise of a_w implying a swelling phenomenon as water activity is increased (Fig. 1). Such a negligible convexity obtained at low a_w was related with type III sorption isotherm (Villalobos, Hernandez-Munoz, & Chiralt, 2006), a characteristic of components rich in hydrophilic components and are frequently reported in literature (Guiga et al., 2009; Kristo, Biliaderis, & Zampraka, 2007; Kristo, Koutsoumanis, & Biliaderis, 2008; Müller, Laurindo, & Yamashita, 2009; Sebti, Delves-Broughton, & Coma, 2003).

For plasticized films, glycerol contributed to an increase in moisture uptake for RH > 60%. At low vapour pressures, hydrogen bonding is the main force involved in the adsorption mechanism (Enrione, Hill, & Mitchell, 2007), thus the first step of DVS corresponds to the fixation of water molecules on the specific hydrophilic groups of polymer; afterwards, the amount of sorbed water depends on the swelling capacity of the polymer (Fringant et al., 1996). That is why incorporation of glycerol modified the water sorption only at high a_w. Similar behaviour was detected for active films with Nisaplin® due to its heterogeneity. Na salt is well acknowledged as hygroscopic reagent (Rougier, Bonazzi, & Daudin, 2007) for the biopolymers, and as a matter of fact it accounts for 74.5% in Nisaplin® formulation.

Table 2
Parameter values obtained from the curves fitted to various composite films with BET, GAB and TSS models.

Model	Formulation	X_m	C	K	h_{TSS}
BET	HPMC	4.30	2.86	–	–
	HPMC + 30% G	7.88	1.61	–	–
	HPMC + 50% G	10.88	1.35	–	–
	HPMC + N	3.67	2.68	–	–
	HPMC + N + 30% G	13.69	0.80	–	–
	HPMC + N + 50% G	19.61	0.70	–	–
GAB	HPMC	4.14	6.23	0.94	–
	HPMC + 30% G	14.51	0.70	0.89	–
	HPMC + 50% G	19.45	0.65	0.90	–
	HPMC + N	27.1	0.23	0.91	–
	HPMC + N + 30% G	41.17	0.38	0.82	–
	HPMC + N + 50% G	51.38	0.35	0.82	–
TSS	HPMC	4.33	4.45	0.94	0.71
	HPMC + 30% G	14.58	0.71	0.89	0.96
	HPMC + 50% G	19.39	0.63	0.90	0.99
	HPMC + N	26.84	0.33	0.91	0.68
	HPMC + N + 30% G	44.36	0.41	0.82	0.88
	HPMC + N + 50% G	50.97	0.38	0.83	0.90

HPMC = hydroxypropyl methylcellulose; G = glycerol; N = Nisaplin® 1%, i.e. 10^4 IU.

3.3.1. Modelling of the sorption isotherms

The experimental sorption data of biodegradable films was first fitted with the two parameter BET model (Eq. (1)), between 0 and 50% RH. GAB and TSS models (Eqs. (2) and (3)) were fitted to the whole range data (0–95% RH). For all the formulations, the coefficient of determination R^2 was 0.99 (except for GAB fitted HPMC containing Nisaplin® film with 0.98 R^2), verifying the suitability of equations in explaining the data.

The X_m value is of high concern, as it refers to strongly adsorbed water to specific sites and is considered as optimum value at which the film is most stable. For all models, X_m value of 4 ± 0.3 g/100 g for HPMC film was observed (Table 2) which is close to the previous findings (Villalobos et al., 2006). Glycerol increased the X_m values obtained from BET, GAB and TSS models for plasticized films. For active films containing nisin, the values of X_m were varied for BET and GAB or TSS models. The X_m values of BET and TSS were generally lower than those from the GAB fitted value. Even for the BET the monolayer value was slightly lower than HPMC which is possibly due to the fact that denatured milk solids in Nisaplin® adsorb water significantly when a_w is >0.5. As the BET model is applied to 0–50% RH, the presence of denatured milk solids with nisin effect the sorption behaviour in contrary manner by engaging the active sites of HPMC. Nonetheless, TSS model normalizes the higher elevation for X_m value by GAB for nisin incorporated films through its extra correction factor adjusting the rapid increase at higher a_w region.

The thermodynamic parameter C is represented in Table 2 after model fitting. A marked decrease in C_{BET}, C_{GAB} and C_{TSS} values was observed for different plasticizer concentrations. Its decrease suggested that nisin or polyol may perhaps occupy sorption sites of polymer and in consequence reduced the bonding energy.

On the other hand, the larger difference of K value from 1 would point towards a decrease in sorption energy of the multilayer. As a result, lower value of K suggested the higher water content in multilayers (Quirijns, Van Boxtel, Van Loon, & Van Straten, 2005). Thus Nisaplin® and glycerol molecules had reduced the interaction energies between the water molecules, on the second and higher water layers, and the polymer. When the h_{TSS} parameter value appears to be 1, it means that TSS model is reduced to GAB expression (Timmermann, 1989). The relative lower values (<1) for this constant observed were conceivably due to taking into account the solubility or quick sorption behaviour in third sorption stage.

Table 3
Light transparency of composite active films as affected by nisin, plasticizer or both (mean of triplicate analysis).

Formulation	Transparency = −log(T600/X)
HPMC	3.19 ± 0.01
HPMC + 10% G	2.90 ± 0.50^{NS}
HPMC + 20% G	2.85 ± 0.50^{NS}
HPMC + 30% G	2.73 ± 0.43^{NS}
HPMC + 50% G	2.81 ± 0.48^{NS}
HPMC + N	2.41 ± 0.35**
HPMC + N + 10% G	2.93 ± 0.01^{NS}
HPMC + N + 20% G	2.84 ± 0.08^{NS}
HPMC + N + 30% G	2.80 ± 0.13^{NS}
HPMC + N + 50% G	2.63 ± 0.36**

G = glycerol; N = Nisaplin® 1%, i.e. 10^4 IU.
** $p < 0.01$.
^{NS} $p > 0.05$.

3.4. Measurement of transparency/light transmission

Transparency of the film is relevant property of film since it has a direct impact on the appearance of packaged product. Compared to the effect of each mixture component on transparency, Nisaplin® was the primary factor reducing the film transparency (Table 3). Plasticizers are compounds used to increase film transmission (Jongjareonrak et al., 2006) but the control HPMC film showed such an excellent transparency characteristic that even addition of glycerol at higher content resulted in slight decrease in the light transmission. This attribute of the HPMC edible film justified its use as edible film biopolymer primarily to fulfil the consumer eagerness to see food through packaging. Transmission percentage had depended on the concentration of plasticizer (glycerol) and addition of active agent Nisaplin® (Fig. 2). The transparency of HPMC film was inversely proportional to the Nisaplin® addition and glycerol concentration as an effect individual.

Glycerol played a positive role to improve the transparency of films containing Nisaplin® due to improved dispersibility of Nisaplin® in network of HPMC film and thus provided homogenous film which had higher transparency values. HPMC film containing Nisaplin® only had given transmission value close to bottom which was improved with the addition of glycerol. But higher addition of plasticizer decreased the transparency, as HPMC films containing both Nisaplin® (10^4 IU) and glycerol (50%, db) showed minimum transmission value second to Nisaplin® addition alone. This result was in accordance with previous findings (Villalobos et al., 2005), which observed that lower ratios of surfactants infer lesser anisotropy degree in the physical properties throughout the matrix. In this logic, the observed effect of glycerol on transparency could be related with the smaller number of discontinuities in the refractive index through the internal homogenous film structure.

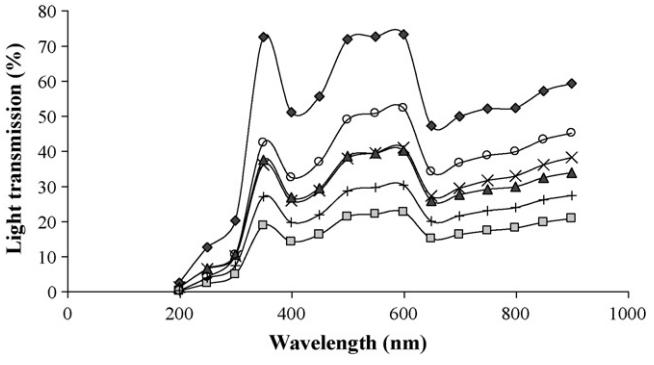


Fig. 2. Light transmission (%T) of UV, visible and NIR for HPMC composite/active films (♦) HPMC film, (×) HPMC + 30% glycerol, (▲) HPMC + 50% glycerol, (■) HPMC + 10^4 IU Nisaplin®, (○) HPMC + 30% glycerol + 10^4 IU Nisaplin®, (+) HPMC + 50% glycerol + 10^4 IU Nisaplin®.

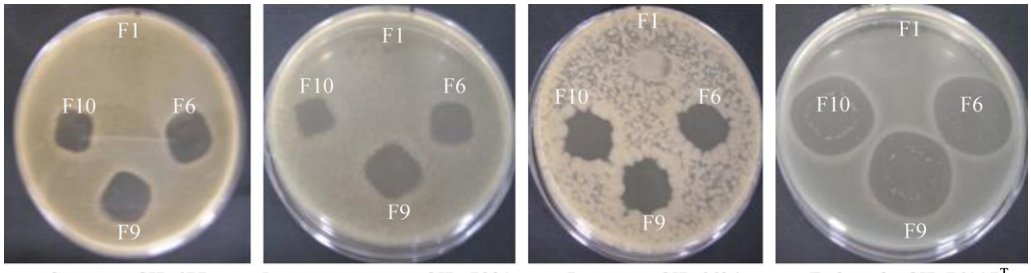


Fig. 3. Inhibition zone of composite active films against bacteria of food origin F1 = HPMC film, F6 = HPMC + 10^4 IU Nisaplin®, F9 = HPMC + 30% glycerol + 10^4 IU Nisaplin®, F10 = HPMC + 50% glycerol + 10^4 IU Nisaplin®.

Table 4
Water vapour permeability of HPMC films as a function of nisin and glycerol concentration at ~ 100% RH gradient (mean of triplicate analysis).

Films	C.W.V.T ($g\,m^{-2}\,h^{-1}$)	Water vapour permeability ($g\,m^{-1}\,s^{-1}\,Pa^{-1}$) × 10^{-10}
HPMC	32 ± 1	4.2 ± 0.1
HPMC + 30% G	48 ± 2***	6.5 ± 0.3***
HPMC + 50% G	57 ± 2***	8.8 ± 0.3***
HPMC + N	25 ± 1*	4.9 ± 0.1NS
HPMC + N + 30% G	58 ± 4***	10.4 ± 0.7***
HPMC + N + 50% G	59 ± 4***	9.5 ± 0.6***

G = glycerol; N = Nisaplin® 1%, i.e. 10^4 IU.
* $p < 0.05$.
*** $p < 0.001$.
NS $p > 0.05$.

3.5. Water vapour permeability (WVP)

WVP estimate the ease with which moisture penetrates through a barrier. Among the plasticized films containing 30% or 50% glycerol (w/w, d.b.) showed higher WVP values (Table 4). As expected, glycerol is an effective plasticizer with a high capacity to interact with water, facilitating its solubilisation and permeation through film. These results are similar to the findings of WVP using plasticizer for skin gelatine film (Jongjareonrak et al., 2006), oat starch film (Galdeano et al., 2009), tapioca starch (Chen & Lai, 2008) and sodium caseinate edible films (Schou et al., 2005). While the concentration of glycerol increased 30% or more, the WVP increased very significantly (Dunnett test, $p \leq 0.001$).

Regardless of the ever-increasing interest in the incorporation of antimicrobial compounds in edible films the literature is rather scarce on findings about their effect on modifying film structure and properties. Water permeation through edible films is the sum of three phenomena: (i) sorption already investigated with isotherms, (ii) water diffusion inside the polymer, and (iii) water desorption. Measurements of WVTR for HPMC plus Nisaplin® and HPMC plus Nisaplin® and glycerol were in total accordance with water sorption isotherms. The WVP of films made from HPMC with Nisaplin® were slightly more but not statistically different ($p < 0.05$). According to a prior study (Khwaldia, Linder, Banon, & Desobry, 2005), the re-structuring of biopolymer inside film matrix due to active agent/plasticizer incorporation significantly affect WVP which may explain the relative increase of WVP in composite active films.

3.6. Microbiological analysis

To identify whether using plasticizer for improving physicochemical properties of film, would influence negatively or not by inhibiting nisin release from composite film network, microbial test were used. The common method for testing antimicrobial activity of active film is to measure the inhibition zone of pre-inoculated agar gel containing the indicator strain. As expected, HPMC film had not shown any antimicrobial activity against 16 bacterial strains from food origin (*Listeria*, *Staphylococcus*, *Bacillus* and *Enterococcus*). Glycerol had not affected the release of nisin from films and thus the inhibition zone from active film was similar to the plasticized active films (Table 5). The only difference observed for antimicrobial effectiveness was due to the respective sensibility of specific strain against nisin (Galvez, Abriouel, Lopez, & Omar, 2007). The interesting fact was to observe that not only the active films had

Table 5
Inhibition zone of composite active films against food borne pathogens.

Bacterial strains	HPMC + N (cm)	HPMC + N + 30% G (cm)	HPMC + N + 50% G (cm)
Staphylococcus aureus CIP 677	1.4 ± 0.10	1.6 ± 0.05	1.5 ± 0.16
S. aureus CIP 76.25	1.4 ± 0.08	1.4 ± 0.10	1.4 ± 0.05
S. aureus CIP 4.83	1.7 ± 0.12	1.9 ± 0.02	1.9 ± 0.07
S. aureus CIP 57.10	1.4 ± 0.22	1.6 ± 0.10	1.6 ± 0.17
Listeria seeligeri SLCC 3954^T	1.7 ± 0.12	1.9 ± 0.10	1.8 ± 0.08
L. grayi CIP 6818^T	2.2 ± 0.15	2.1 ± 0.22	2.3 ± 0.08
L. monocytogenes CIP 7831	1.4 ± 0.22	1.7 ± 0.07	1.6 ± 0.15
L. monocytogenes CIP 82110^T	1.5 ± 0.05	1.7 ± 0.02	1.7 ± 0.07
L. innocua CIP 12511	1.3 ± 0.22	1.3 ± 0.23	1.3 ± 0.25
L. ivanoii CIP 12510	3.5 ± 0.98	3.4 ± 0.17	3.5 ± 0.14
Bacillus cereus CIP 6624	1.5 ± 0.08	1.6 ± 0.16	1.5 ± 0.02
B. licheniformis CIP 5271	1.6 ± 0.18	1.6 ± 0.14	1.6 ± 0.23
B. subtilis CIP 5265	1.6 ± 0.17	1.4 ± 0.36	1.5 ± 0.14
Enterococcus facium DSM 20477^T	2.5 ± 0.02	2.6 ± 0.10	2.5 ± 0.02
E. durans CIP 55125^T	2.1 ± 0.18	2.4 ± 0.56	2.4 ± 0.20
E. faecalis CIP 76117^T	2.6 ± 0.05	2.6 ± 0.07	2.7 ± 0.13

G = glycerol; N = Nisaplin® 1%, i.e. 10^4 IU.
CIP: Collection of Institute Pasteur, Paris, France.
DSM: Deutsche Sammlung von Mikio-Orgonismer and Zellkuturen, Göttingen, Germany.
SLCC: Special *Listeria* Culture Collection, University of Wurzburg, Germany.

inhibited bacteria below its surface but nisin had also diffused in the inoculated media to hamper bacterial survival (Fig. 3).

4. Conclusion

The imperative aim of this study was to provide information that may escort to the development of antimicrobial containing HPMC bio-packaging by means of commercially available form of nisin (Nisaplin®). It was demonstrated in the study that along-with nisin the presence of salt and denatured protein in Nisaplin® greatly affected the transparency, thickness and water sorption behaviour of active films. In addition, the presence of plasticizer substantially improved the stretch-ability and transparency but adversely altered the permeability and tensile strength. Furthermore, the results clearly demonstrated that formulation containing HPMC, nisin and 30% glycerol is a promising bioactive film due to its transparent and homogenous matrix, stable structure with good stretch-ability, moderate water sorption and good antimicrobial efficiency.

Acknowledgements

The authors would like to thank C. Jeandel and C. Perroud for their technical assistance.

References

Brindle, L. P., & Krochta, J. M. (2008). Physical properties of whey protein-hydroxypropylmethylcellulose blend edible films. *Journal of Food Science*, *73*(9).
Burdock, G. A. (2007). Safety assessment of hydroxypropyl methylcellulose as a food ingredient. *Food and Chemical Toxicology*, *45*(12), 2341–2351.
Burke, J. R. (2006). Biodegradeable-compostable packaging: The promise and the problems. *Paper and Packaging*, *47*(5).
Cagri, A., Ustunol, Z., & Ryser, E. T. (2004). Antimicrobial edible films and coatings. *Journal of Food Protection*, *67*(4), 833–848.
Chen, C. H., & Lai, L. S. (2008). Mechanical and water vapor barrier properties of tapioca starch/decolorized hsian-tsao leaf gum films in the presence of plasticizer. *Food Hydrocolloids*, *22*(8), 1584–1595.
Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: Safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, *71*(1), 1–20.
Dawson, P. L., Hirt, D. E., Rieck, J. R., Acton, J. C., & Sotthibandhu, A. (2003). Nisin release from films is affected by both protein type and film-forming method. *Food Research International*, *36*(9–10), 959–968.
de Arauz, L. J., Jozala, A. F., Mazzola, P. G., & Vessoni Penna, T. C. (2009). Nisin biotechnological production and application: A review. *Trends in Food Science and Technology*, *20*(3–4), 146–154.
Enrione, J. I., Hill, S. E., & Mitchell, J. R. (2007). Sorption behavior of mixtures of glycerol and starch. *Journal of Agricultural and Food Chemistry*, *55*(8), 2956–2963.
EU. (2004). Regulation (EC) No. 1935/2004 European Parliament and the Council of 27 October 2004 on materials and articles intended to come into contact with food repealing.
Fang, Y., Tung, M. A., Britt, I. J., Yada, S., & Dalgleish, D. G. (2002). Tensile and barrier properties of edible films made from whey proteins. *Journal of Food Science*, *67*(1), 188–193.
FDA. (2001). FDA/CFSAN/OPA: Agency response letter: GRAS notice no. GRN 000065.
Fringant, C., Desbrieres, J., Milas, M., Rinaudo, M., Joly, C., & Escoubes, M. (1996). Characterisation of sorbed water molecules on neutral and ionic polysaccharides. *International Journal of Biological Macromolecules*, *18*(4), 281–286.
Galdeano, M. C., Mali, S., Grossmann, M. V. E., Yamashita, F., & Garcia, M. A. (2009). Effects of plastic izers on the properties of oat starch films. *Materials Science and Engineering C*, *29*(2), 532–538.
Galvez, A., Abriouel, H., Lopez, R. L., & Omar, N. B. (2007). Bacteriocin-based strategies for food biopreservation. *International Journal of Food Microbiology*, *120*(1–2), 51–70.
Guiga, W., Galland, S., Peyrol, E., Degraeve, P., Carnet-Pantiez, A., & Sebti, I. (2009). Antimicrobial plastic film: Physico-chemical characterization and nisin desorption modeling. *Innovative Food Science and Emerging Technologies*, *10*(2), 203–207.
Han, J. H., & Floros, J. D. (1997). Casting antimicrobial packaging films and measuring their physical properties and antimicrobial activity. *Journal of Plastic Film and Sheeting*, *13*(4), 287–298.
Jongjareonrak, A., Benjakul, S., Visessanguan, W., & Tanaka, M. (2006). Effects of plasticizers on the properties of edible films from skin gelatin of bigeye snapper and brownstripe red snapper. *European Food Research and Technology*, *222*(3–4), 229–235.
Khwaldia, K., Banon, S., Perez, C., & Desobry, S. (2004). Properties of sodium caseinate film-forming dispersions and films. *Journal of Dairy Science*, *87*(7), 2011–2016.
Khwaldia, K., Linder, M., Banon, S., & Desobry, S. (2005). Effects of mica, carnauba wax, glycerol, and sodium caseinate concentrations on water vapor barrier and mechanical properties of coated paper. *Journal of Food Science*, *70*(3).
Kristo, E., Biliaderis, C. G., & Zampraka, A. (2007). Water vapour barrier and tensile properties of composite caseinate-pullulan films: Biopolymer composition effects and impact of beeswax lamination. *Food Chemistry*, *101*(2), 753–764.
Kristo, E., Koutsoumanis, K. P., & Biliaderis, C. G. (2008). Thermal, mechanical and water vapor barrier properties of sodium caseinate films containing antimicrobials and their inhibitory action on *Listeria monocytogenes*. *Food Hydrocolloids*, *22*(3), 373–386.
Mali, S., Grossmann, M. V. E., Garcia, M. A., Martino, M. N., & Zaritzky, N. E. (2004). Barrier, mechanical and optical properties of plasticized yam starch films. *Carbohydrate Polymers*, *56*(2), 129–135.
Möller, H., Grelier, S., Pardon, P., & Coma, V. (2004). Antimicrobial and physicochemical properties of chitosan–HPMC-based films. *Journal of Agricultural and Food Chemistry*, *52*(21), 6585–6591.
Müller, C. M. O., Laurindo, J. B., & Yamashita, F. (2009). Effect of cellulose fibers addition on the mechanical properties and water vapor barrier of starch-based films. *Food Hydrocolloids*, *23*(5), 1328–1333.
Pushpadass, H. A., Marx, D. B., & Hanna, M. A. (2008). Effects of extrusion temperature and plasticizers on the physical and functional properties of starch films. *Starch/Staerke*, *60*(10), 527–538.
Quirijns, E. J., Van Boxtel, A. J. B., Van Loon, W. K. P., & Van Straten, G. (2005). An improved experimental and regression methodology for sorption isotherms. *Journal of the Science of Food and Agriculture*, *85*(2), 175–185.
Ray, S. S., & Bousmina, M. (2005). Biodegradable polymers and their layered silicate nanocomposites: In greening the 21st century materials world. *Progress in Materials Science*, *50*(8), 962–1079.
Reppas, C., Swidan, S. Z., Tobey, S. W., Turowski, M., & Dressman, J. B. (2009). Hydroxypropylmethylcellulose significantly lowers blood cholesterol in mildly hypercholesterolemic human subjects. *European Journal of Clinical Nutrition*, *63*(1), 71–77.
Rougier, T., Bonazzi, C., & Daudin, J. D. (2007). Modeling incidence of lipid and sodium chloride contents on sorption curves of gelatin in the high humidity range. *LWT - Food Science and Technology*, *40*(10), 1798–1807.
Schou, M., Longares, A., Montesinos-Herrero, C., Monahan, F. J., O'Riordan, D., & O'Sullivan, M. (2005). Properties of edible sodium caseinate films and their application as food wrapping. *LWT - Food Science and Technology*, *38*(6), 605–610.
Sebti, I., Chollet, E., Degraeve, P., Noel, C., & Peyrol, E. (2007). Water sensitivity, antimicrobial, and physicochemical analyses of edible films based on HPMC and/or chitosan. *Journal of Agricultural and Food Chemistry*, *55*(3), 693–699.
Sebti, I., Delves-Broughton, J., & Coma, V. (2003). Physicochemical properties and bioactivity of nisin-containing cross-linked hydroxypropylmethylcellulose films. *Journal of Agricultural and Food Chemistry*, *51*(22), 6468–6474.
Sobrino-Lopez, A., & Martin-Belloso, O. (2008). Use of nisin and other bacteriocins for preservation of dairy products. *International Dairy Journal*, *18*(4), 329–343.
Tharanathan, R. N. (2003). Biodegradable films and composite coatings: Past, present and future. *Trends in Food Science and Technology*, *14*(3), 71–78.
Timmermann, E. O. (1989). A B.E.T.-like three sorption stage isotherm. *Journal of the Chemical Society Faraday Transactions 1: Physical Chemistry in Condensed Phases*, *85*(7), 1631–1645.
Vasconez, M. B., Flores, S. K., Campos, C. A., Alvarado, J., & Gerschenson, L. N. (2009). Antimicrobial activity and physical properties of chitosan-tapioca starch based edible films and coatings. *Food Research International*, *42*(7), 762–769.
Villalobos, R., Chanona, J., Hernandez, P., Gutiaerrez, G., & Chiralt, A. (2005). Gloss and transparency of hydroxypropyl methylcellulose films containing surfactants as affected by their microstructure. *Food Hydrocolloids*, *19*(1), 53–61.
Villalobos, R., Hernandez-Munoz, P., & Chiralt, A. (2006). Effect of surfactants on water sorption and barrier properties of hydroxypropyl methylcellulose films. *Food Hydrocolloids*, *20*(4), 502–509.
Zhang, Y., & Han, J. H. (2008). Sorption isotherm and plasticization effect of moisture and plasticizers in pea starch film. *Journal of Food Science*, *73*(7).

Chapitre 5: Systèmes liposomiques de nano-libération : l'encapsulation de la nisine par la nouvelle stratégie de microfluidisation

De façon similaire à l'inclusion simple de la nisine dans des enrobages actifs, un moyen d'augmenter la sécurité alimentaire est l'encapsulation du composé actif qui sera par la suite introduit dans le système alimentaire. L'utilisation directe de la nisine sous sa forme libre étant couteuse et possédant des inconvénients tels qu'une baisse d'activité et de stabilité (Roberts and Zottola, 1993), il est nécessaire d'avoir recourt à l'une ou l'autre de ces méthodologies. L'encapsulation réduit la réactivité du composé actif avec l'environnement, protège le composé actif d'éventuelle dégradation durant le process et le stockage, augmente la répartition et permet une libération graduelle du composé actif (Nongoniema *et al*., 2009). Les liposomes sont particulièrement appropriés pour une utilisation en industrie alimentaire comme système de libération de composés car ils sont bien caractérisés, réalisable facilement, et modulable dans leurs propriétés de transporteur de composés et ont un statut GRAS (Xiu and Xiu, 2005).

Peu d'informations sont disponibles sur la méthodologie à suivre pour l'encapsulation de composés antimicrobiens. Les méthodes traditionnelles existantes soit impliquent l'utilisation de solvants organiques, soit ont de faibles niveaux de production et d'efficacité d'encapsulation ou conduisent à une hétérogénéité et une dispersion en taille des capsules.

Le principal objectif de cette étude est de développer une nouvelle méthode de micro/nano-encapsulation de la nisine utilisant une approche de microfluidisation, par l'utilisation d'un système de désintégration de cellules en continu. Un tel système possède l'avantage majeur de ne pas utiliser de solvants organiques, largement utilisés dans les autres méthodes. De plus cette technique est efficace, rapide, écologiquement non agressive et utilisable pour des productions à grande échelle. Dans cette étude des lécithines de soja et d'origine marine ont été employées pour l'encapsulation de la nisine par cette nouvelle méthode. Les caractéristiques des systèmes obtenus ont été comparées à celles de proliposomes. Les paramètres ayant une importance critique majeure sur les performances du procédé tels que la pression et le nombre de passages à travers l'homogénéisateur et la concentration en lécithine ont été analysés. La morphologie, la taille, l'indice de polydispersité, la mobilité électrophorétique/le potentiel zéta, la composition lipidique, l'efficacité d'encapsulation et la stabilité physique des structures formés ont été déterminés.

Chapter 5: Liposomal nanodelivery systems to encapsulate food biopreservative nisin by novel strategy of microfluidic format

Analogous to immobilization of nisin in active coatings, the current appealing technique to improve the food safety is active agents‟ encapsulation and subsequent incorporation inside the food systems. Either one or other above mentioned concepts are mandatory to improve food safety as the use of nisin in its free form (direct incorporation in foods) is costly and has drawbacks including lower activity, stability, and bio availability (Roberts and Zottola, 1993). Encapsulation reduces reactivity of active agent with the environment, protect the active ingredient against possible denaturation during processing and storage, improve the distribution of the active ingredient, and provide gradual release for the active ingredients (Nongonierma et al. 2009). Liposomes are particularly well suited for use in the food industry as delivery systems because they are well characterized, easily made, highly versatile in their carrier properties, highly biocompatible, and GRAS materials (Xia and Xu, 2005).

Little information is available on the methodology of encapsulation for antimicrobial agents. The existing traditional methods involve either organic solvent, have less production and encapsulation efficiency or result in heterogeneous and uncontrolled polydispersity in size and lamellarity. The main objective of the present study was to develop a new method for nisin micro/nano-encapsulation using a microfluidization approach i.e. continuous cell disruption system. Such a system entitles a major advantage that organic solvents often used in other liposome preparation methods are not required and therefore this technique is effective, rapid, environment friendly and suitable for large-scale production. In the present study soy lecithin and marine lecithin were employed for nisin encapsulation by novel method and compared for critical characteristics against commercially available Proliposomes. Parameters having critical importance on their performance such as the effect of pressure and the number of passes through the homogeniser, lecithin concentration, morphology, size, polydispersity index, electrophoretic mobility/zeta potential, lipid composition, encapsulation efficiency, and physical stability are studied. Owing to this study, it would be possible to add the active ingredients with improved bioavailability to pharmaceutical/food systems at industrial scale.

Chapter 5

Liposomal Nanodelivery Systems to Encapsulate Food Biopreservative Nisin by Novel Strategy of Microfluidic Format

Muhammad Imran [1], Anne-Marie Revol-Junelles [1], Cedric Paris[1], Emmanuel Guedon [2], Michel Linder [1], Stéphane Desobry [1]

[1] Laboratoire d'Ingénierie des Biomolécules, ENSAIA–INPL, Nancy Université, 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France

[2] Laboratoire de Réactions et Génie des Procédés, UPR CNRS 3349, ENSAIA–INPL, Nancy Université, 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France

(Colloids and Surfaces, Soumise 2011)

Abstract:

Commercial purified nisin was encapsulated in different nanoliposomes (Marine-, Soy-, Pro-liposomes) using an innovative, rapid, large scale and nontoxic continuous cell disruption system (CCDS) method of microfluidic format. Soy-lecithin had higher omega-6 and polar lipids as compared to marine-lecithin while proliposomes contained only saturated phospholipids. Lantibiotic nisin was entrapped in soy-liposomes with highest encapsulation efficiency of 47% at 5% soy-lecithin concentration. Average size of these liposomes ranged from 151±4 to 181±5 nm, without or with nisin respectively. The electrophoretic mobility was influenced by type and concentration of lecithin; however, incorporation of nisin reduced the negative charge of liposomes significantly. Physical stability of nanoliposome-encapsulated nisin was demonstrated for 6 weeks at 4°C, though transmission electron microscopic studies revealed pore-formation by nisin and fusion phenomenon. Antimicrobial assay had revealed that blend of unencapsulated/free and encapsulated nisin (1:1) exhibited better control of *Listeria monocytogenes* as compared to free or 100% encapsulated nisin alone.

Keywords: polypeptide bacteriocin, nano encapsulation, liposome, microfluidization, PUFA, zeta-potential, *Listeria monocytogenes*

1. Introduction:

Preservatives are additives incorporated in food, for which controlled release can be valuable during either processing, storage, consumption, or in the human body (*1*). Currently the customized mode of application of antimicrobial agents is direct introduction to the food system in free form (*2*). However, undesirable interactions of these active agents with food components reduce their efficacy against pathogens and thus require the addition of larger antimicrobial quantities to reduce the microbial number within limit (*3*).

Natural compounds, such as nisin, chitosan or lysozyme, were investigated to replace chemical preservatives and to obtain „green label" products (*2*). Among all the antimicrobial peptides known, only a very few of them are actually allowed to be used either as a preservative in the food industry or as an antibiotic in the health care. The 34-residue-long peptide nisin is one of these few, and has been used as a food preservative for a long time (*4*). Nisin effectively inhibits Gram-positive bacteria and outgrowth spores of *Bacillus* and *Clostridium* (*5*). Among bacteriocins, use of nisin has become vital in food industry due to its broad spectrum against food borne pathogens, generally recognized as safe (GRAS) status, and its „bio-additive" notion (*6, 7*).

Use of nisin in its free form (unpackaged or unencapsulated) is expensive and is associated with loss of activity due to degradation or deactivation and emergence of nisin-resistant bacterial strains (*8, 9*). Jung *et al.* (*10*) found significant loss of nisin activity in milk because of its interactions with milk components. Divalent cations associated with bacterial cell wall surfaces were shown to induce electrostatic repulsion preventing the cationic polypeptide nisin from interacting with the bacterial pathogens, thus reducing its activity (*11, 12*). In the cheese industry, the use of nisin in its free form is costly and has drawbacks, including lower activity, stability, and bio availability (*13*).

Currently, some novel encapsulation methods have been introduced in order to overcome these limitations (*14*). Encapsulation reduces reactivity of active agent with the environment, protect the active ingredient against possible denaturation during processing and storage, improve the distribution of the active ingredient, provide gradual release for the active ingredients (*15*), act as a long-term preservative in food and drug systems, protect nisin from inhibitors or unfavorable conditions e.g. those that naturally occur in food matrix (*9*), (iv) decrease the risk of emergence of resistant strains and provide a mean for targeting the bacteria (*16*).

Nanoliposomes are under intensive research and development by the pharmaceutical, cosmetic, and food industries as nanocarrier systems for the protection and delivery of bioactive agents (*17, 18*). In consequence of enhanced stability and targeting at nano-scale, the quantity of bioactive required for a specific effect when encapsulated in a liposome is much less than the amount required when unencapsulated (*19*). Liposomes are particularly well suited for use in the food industry as delivery systems because they are well characterized, easily made, highly versatile in their carrier properties, highly biocompatible, and GRAS materials (*20*). As liposomes structure encloses both aqueous (core) and lipid (bilayer) phases, they can be utilized in the entrapment, delivery, and release of water-soluble, lipid-soluble, and amphiphilic materials. Because nisin is amphiphilic in nature (*21*), it is entrapped simultaneously in the core and bilayers of liposome. It generates the possibility for superior controlled release of nisin by phospholipids based nano-delivery systems, such as onion-shaped liposomes in the form of multilamellar vesicles (MLV) (*22*).

Little information is available on the methodology of encapsulation for antimicrobial agents. The traditional liposome preparation methods includes freeze-thaw cycling (*12*), film hydration (*23*), reversed phase evaporation (*24*), dual asymmetric centrifugation (*25*), heating method (*16*) etc. However, these traditional methods involve either organic solvent, have less production and encapsulation efficiency (EE) or result in heterogeneous and uncontrolled polydispersity in size and lamellarity. Thus additional post processing steps are required, such as solvent removal and membrane extrusion to yield homogeneous liposome populations (*26*).

A simpler procedure for preparing liposomes is by using preformed bilayers, called „„Pro-liposomes"" (*9, 27*). In contrast, marinosomes® are liposomes based on a natural marine lipid extract containing a high polyunsaturated fatty acids (PUFAs) ratio (*28*). ω-3 fatty acids, which include decosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), appeared to have positive effects on the treatment of different cardiovascular disorders, cancer and rheumatoid arthritis (*29*). Similarly previous studies had indicated that soy lecithin (good source of the essential fatty acid linoleic acid) displays health benefits due to hypocholesterolemic properties thus helpful in reducing significant risk for cardiovascular diseases (*30, 31*). Thus developing marine liposomes and soy liposomes may not only provide a nanodelivery system for nisin but a nutritional element for health benefits as well.

The main objective of the present study was to develop a new method for nisin micro/nano-encapsulation using a microfluidization approach i.e. continuous cell disruption system (CCDS). Such a system entitles a major advantage that organic solvents often used in other liposome preparation methods are not required and therefore this technique is effective, rapid, environment friendly and suitable for large-scale production. The previous investigations on nisin loaded liposome were performed with purified phospholipids using traditional techniques (*8, 9, 32*). However in the present study soy lecithin (SL) and marine lecithin (ML) were employed for nisin encapsulation by our novel method and compared for critical characteristics against commercially available Proliposomes. Certain parameters pertained to each of the newly developed micro/nano-carrier systems must be thoroughly assessed. In these different types of liposomes, parameters having critical importance on their performance such as the effect of pressure and the number of passes through the homogenizer, lecithin concentration, morphology, size, polydispersity index, electrophoretic mobility/zeta potential, lipid composition, encapsulation efficiency, and physical stability are studied. Owing to this study, it would be possible to add the active ingredients in lower concentrations to pharmaceutical/food systems at industrial scale while improving their bioavailability through controlled release from nanodelivery system.

2. Materials and methods:

2.1. Materials

Commercial preparation of Proliposomes H was obtained from Lucas Meyer (Chelles, France). The Pro-lipos H was made up of hydrogenated phosphatidylcholine. Commercial soybean lecithin (SL) was used, extracted according to method describe previously (*33*). Marine lecithin (ML) is a mixture of different phospholipids, extracted from raw salmon (*Salmo salar*) head lipidic material by Folch method (*34, 35*). The liposomal ingredients were kept under nitrogen atmosphere at the recommended storage temperatures (0-4 °C). Ammonium molybdate and all solvents e.g. chloroform (purity = 99%); methanol (purity = 99%); diethyl ether (purity > 99%) and hexane (purity = 97%) used for Iatroscan, gas chromatography and lipid extraction were purchased from Fisher Scientific (France).

Nisin was purchased from Honghao Chemical Co. (Shanghai, China). Nisin used in this study contained >90 % pure nisin (according to the manufacturer, the formulation contains 3.84 x 10^6 I.U. per gram and 6.88 % moisture content). Millipore nylon filters (0.2 μm) were obtained from Millipore (Cork, Ireland). Bicinchoninic acid (BCA) reagents were obtained from Sigma Chemical Co. (Lyon, France).

2.2. LC/MS characterization of nisin

Liquid chromatography mass spectrometry (LCMS) was performed using Thermo electron HPLC system (Thermo electron corporation, Waltham, MA, USA) equipped with Finnigan LTQ MS (Thermo electron corporation, USA). Chromatographic separation was performed on RP-18 (150 mm x 2.1 mm x 5 μm) Alltima C-18 reverse phase column (Alltech) equipped with pre-column RP-18 (7.5 mm x 2.1 mm x 5μm) at 25°C. Mobile phase A and B were prepared containing water:acetonitrile:TFA as 90:10:0.1 and 10:90:0.06 respectively. The column was equilibrated with mobile phase A, and 10 μL of the samples (dissolved in mobile phase A) were injected. Elution was performed by 40-min programme at a flow rate of 0.2 ml/min as follows: a linear gradient ranging from 10% to 35% (v/v) mobile phase B for 10 min, 35% to 42% (v/v) mobile phase B in 20 min, maintained up to 100% B during 5 min, and equilibration in last 5 min. MS detection was performed during the elution programme by PDA-MS coupled detectors. The conditions of ESI-MS were as follows: ionization mode, ESI (electro spray ionization); polarity, positive; Source gas (A.U.), shealth (40), auxiliary (5), sweep (5); spray voltage, 5 KV; capillary temperature, 275 °C; capillary voltage, 34 V; tube lens voltage, 220 V. The ion optics parameters were optimized by automatic tuning using nisin Z solution directly infused into the MS source. The full scan chromatograms were taken in a range from m/z 50 to 2000. Furthermore, zoom scans for multiple charge ions (2^+, 3^+) were carried out to obtain the charge state of nisin. The data acquisitions were performed using Xcalibur 1.4 software.

2.3. Fatty acid profile and lipid composition

Gas chromatography (GC) was used for analyzing fatty acid composition and percentage. Fatty acid methyl esters (FAMEs) were prepared (*36*). Lecithin (100 mg) was dissolved in 5 mL

hexane, which was added in 200 µL (2 M) KOH methanol solution (1.29 g of potassium hydroxide/10 mL methanol). The tubes were then vortexed for 1 min under nitrogen. After mixing, the solutions were kept at rest for 30 min till both phases were clearly separated; upper phase was separated and evaporation was done under a stream of nitrogen in order to obtain 100 mg/mL FAME hexane. The separation of the FAMEs was carried out on a Perichrom^{TM} 2000 gas chromatograph (Perichrom, Saulx-lès-Chartreux, France), equipped with a flame-ionization detector. A fused silica capillary column was used (50m, 0.25 mm i.d. × 0.25 µm film thickness, CP 7419 Varian, Middelburg, Netherlands). Injector and detector temperatures were set at 260 °C. A temperature programme of column initially set at 145 °C for 5 min, then rising to 210 °C at a rate of 2 °C/min and held at 210 °C for 10 min was used. Standard mixtures (marine and vegetable source; Supelco, Sigma-Aldrich, Bellfonte, PA, USA) were used to identify fatty acids (internal standard C21:0). The results were presented as triplicate analyses.

The neutral and polar lipid classes were determined by Iatroscan MK-5 TLC-FID (Iatron Laboratories, Inc., Tokyo, Japan). Each sample was spotted on ten Chromarod S-III silica coated quartz rods held in a frame. The migration was done for 20 min in solution comprising hexane/diethyl ether/formic acid (80: 20: 0.2 v:v:v), then oven-dried for 1 min at 100 °C and finally scanned in the Iatroscan analyzer. The Iatroscan was operated under the following conditions: flow rate of hydrogen, 160 mL/min; flow rate of air, 2 L/min. The recording and integration of the peaks were carried out by ChromStar internal software.

2.4. Optimization of nanovesicles production

Liposomes were prepared from proliposome mixture following the procedure of Dufour, Vuillemard, and Laloy (*37*). The deionized water was boiled and degassed in order to remove all oxygen traces. One, 5 and 10 % (w/v) of proliposome mixture was mixed with an aqueous solution containing nisin [3.8 x 10^4 I.U.]. The mixture was stirred (50–60 rpm) for 15 min, diluted with deionized water to obtain 1 mg/mL of nisin Z concentration and re-stirred for 15 min. The entrapment process was carried out above transition temperature i.e. at 65 °C for Pro-lipos H.

In case of CCDS technique, the method was optimized using SL with different concentrations (1, 5, 10 % w/v), number of cycles (3 to 5) and pressure (1400, 1700, 2000, 2500

bars). From this data optimum pressure and number of passes were used for preparing liposome-encapsulated-nisin. Soy/marine lecithins and water were mixed for 15 min, by magnetic stirring. Further it was thoroughly mixed by Ultraturax T-25 (Avantek, Strasbourg, France) at 13500 rpm for 3 min. The phospholipids dispersion was then passed through CCDS (Constant cell disruptor system, Northants, UK) with a vertical interaction chamber for specific number of cycles at given pressure (Figure 1). Cell disruptors and homogenizers are both positive displacement pumps each differs in the way that they create pressure on the sample and transfer it from pressurized chamber to another chamber which is at lower pressure. Homogenizers pressurize the sample in a chamber, which is then released, into a chamber of lower pressure through a homogenizing valve. Cell disruptors use a hydraulic force to accelerate the sample to high pressure and forcing them through a miniature orifice to hit on a disruption head, which is at a lower pressure. The homogenization temperature was kept below 10°C by using a flow of cold water flowing (4°C) as cooling jacket.

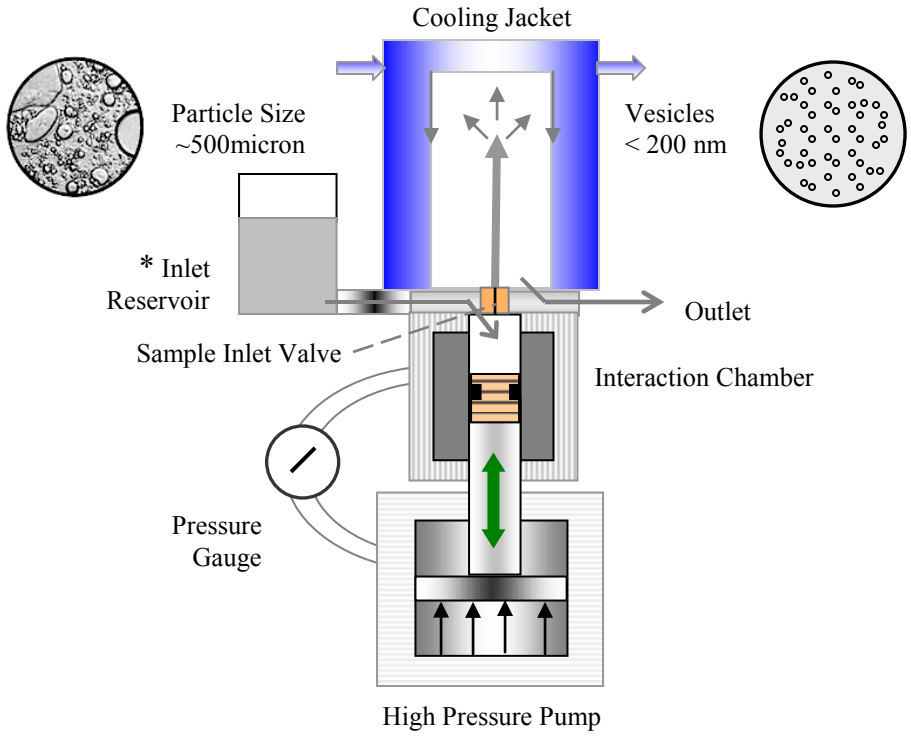


Figure 1: Schematic diagram of continuous cell disruption system (CCDS)

To recover the liposomes containing nisin, emulsions were passed through Sephadex G-50 (fractionation range for protein 1500-30000 M.W.) column and nisin-encapsulating liposomes were eluted by size exclusion chromatography using a centrifuge (Eppendorf, Hambourg, Germany).

2.5. Particle size characterization

The mean diameter and particle size distribution of liposomes were determined using dynamic light scattering (DLS) technique employing a Zetasizer Nano-ZS (Malvern instruments, UK). The apparatus is equipped with a 4 mW He/Ne laser emitting 633 nm, measurement cell, photo multiplier and correlator. Prior to size measurement, the samples were diluted (1:400) with ultra pure water. The samples were taken in vertical cylindrical cuvettes (10 mm diameter). The scattering intensity was measured at a scattering angle of 173° relative to the source using an avalanche of photodiode detector, at 25°C. Results are presented as an average diameter of the liposome suspension (z-average mean) with the polydispersity index (PDI). This index ranges from 0.0 for an entirely monodisperse system up to 1.0 for a polydisperse particle dispersion (*16*). The particle size distribution was characterized using PDI, which is a measure of the width of the size distribution. In dispersion, particles are in a constant random Brownian motion, so that this causes the intensity of scattered light to fluctuate as a function of time. Therefore, droplets sizes were obtained from the correlation function calculated by the dispersion technology software (DTS) using various algorithms. All measurements were carried out at 25°C, with a medium refractive index 1.335. The measurements were performed in five replicates.

2.6. Electrophoretic mobility / ζ – potential

The effect of lecithin type and nisin on the electrophoretic mobility of liposomes was studied by means of a Zetasizer Nano-ZS apparatus (Malvern Co. Ltd., UK). Samples were prepared as explained above in size measurement. This equipment allows measuring zeta potential by a combination of electrophoresis and laser Doppler velocimetry, also called laser Doppler electrophoresis. By means of this method, it can be determined how fast a droplet moves in a liquid when an electric field is applied, i.e., its electrophoretic mobility. From the

electrophoretic mobilities, the Zetasizer software calculates the zeta potential using the Hückel and Smoluchowski limits. A few studies had used ζ-potential as surface charge (*12*) but several studies have shown that zeta potential determinations using these limits are only valid for small rigid spheres (*38, 39*).

For liquid–liquid emulsions this approximation may lead to erroneous zeta potentials, since the electric field applied causes a momentum transfer across the interface at the droplet surface. As a result, there is a coupling of continuous phase fluid flow and internal circulation, through a zero shear stress boundary condition at the interface, modifying the droplet hydrodynamic mobility coefficient, α. Therefore, in the present study, to avoid deviations of the zeta potential, both the electrophoretic mobility and zeta potential were used to measure the droplet surface charge.

2.7. Encapsulation efficiency (EE)

The concentration of entrapped nisin in the liposomes was determined using the bicinchoninic acid (BCA) assay. In this assay, the BCA reagent (Sigma) was added to liposome sample containing protein i.e. nisin. After incubation at 37 °C for 30 min, the absorbance at 562 nm was measured using a UV–Vis spectrophotometer (Ultrospec 4000 UV/visible, Pharmacia Biotech, UK). Bovine serum albumin containing 1 mg/ml protein was diluted and used as the protein standard. The EE was calculated using the following equation:

EE% = Encapsulated nisin / encapsulated nisin + unencapsulated nisin *Eq. 1*

2.8. Negative stain electron microscopy (TEM)

Liposome samples encapsulating nisin were diluted 10-folds with ultra pure water to dilute the concentration of the vesicles. Equal volumes of the diluted sample and a 2 % ammonium molybdate solution were combined and left for 3 min at room temperature. A drop of this solution was placed on a copper mesh for 5 min before the excess liquid was drawn off using filter papers. The mesh was examined using a Transmission Electron Microscope (Philips CM-20) at an operating voltage of 200 KV.

2.9. Microbiological analysis

The minimal inhibitory concentration (MIC) of nisin was determined by the critical dilution method in 96-well plates (Nunc, Roskilde, Denmark). The target strain *Listeria monocytogenes* CIP 82110^T was prepared in TSB-YE medium to a final OD 660 nm of 0.2. Equal volume of inoculated medium and nisin dilutions were added to the 96 well-plate. The plates were shacked and the initial OD 660 nm was determined with a Titertek Multiscan MCC/340 (Huntsville, AL). The plates were incubated for 24 h at 37 °C. Afterwards, the corresponding MIC dilutions of nisin, liposome encapsulated (soy-lecithin 5%) nisin, and liposome encapsulated plus unencapsulated nisin were incubated with the target strain and its growth kinetics were recorded at 660 nm absorbance.

2.10. Statistical analyses

Statistical analyses were carried out by using the software KyPlot version 2.0 (Koichi Yoshioka, Department of Biochemistry and Biophysics, Graduate school of Allied health Sciences, Tokyo, Japan). For comparison a parametric multiple Tukey test ($p \leq 0.05$) was performed.

3. Results and discussion:

The choice of the encapsulating polymer has noteworthy influence not only on liposome physico-chemical characteristics but also its acceptability by consumers. SL and ML are non-toxic, enhance absorption and assist in solubility. Furthermore they are considered as bio-additives and nutritive which entitle them as ideal choice for nanoencapsulation of bioactive agents. One of the major concerns in health care today is the increasing resistance of microbial pathogens to current antimicrobial therapeutics. Consequently, much research is being conducted to formulate new classes of antimicrobials. One parallel approach is the encapsulation and targeting the antimicrobial agents using phospholipids membranes.

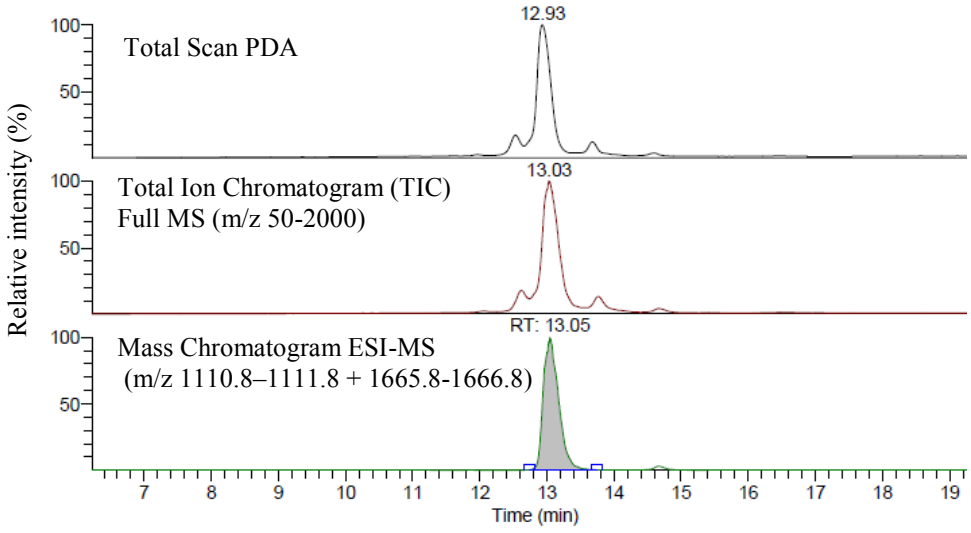
3.1. Characterization of active agent and encapsulating polymers:

3.1.1. LC/MS characterization of nisin

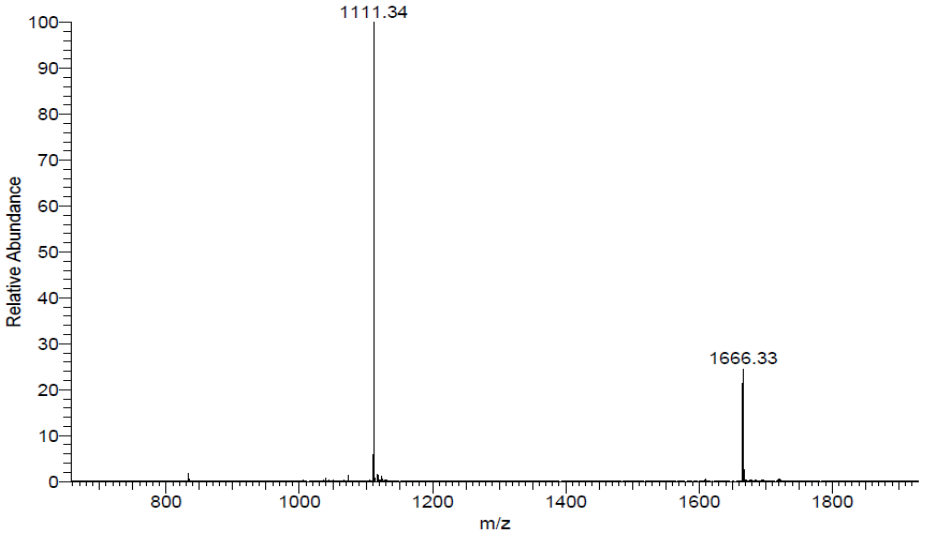
No previous study revealed the availability of pure nisin for commercial / industrial use except provision in very low amount for research purposes (under strict conditions). Nevertheless commercial pure nisin was purchased from Honghao chemicals Co. (Shanghai, China) and characterized by LCMS. The results of known bacteriocin variants showed that ions derived from bacteriocins could be detected in a range higher than m/z 1000 (*40*). The nisin solution (dissolved in mobile phase A) was first separated by LC and then detected simultaneously by PDA and MS detectors. LCMS permitted to obtain the total PDA chromatogram and total ion chromatogram (TIC). The relative area percentage on PDA and MS (TIC) chromatogram indicated that nisin purity was higher than 90 % (Figure 2a). Bacteriocin-derived ions were scanned by extracting m/z 50 – 2000. Peak detected at 13.03 min in the mass chromatogram had been identified as nisin. The mass spectrum at the retention time showed that this molecule was detected as m/z 1111.34 and 1666.33, which corresponded to $[M+3H]^{+3}$ and $[M+2H]^{+2}$, respectively (Figure 2b).

Therefore, the molecular mass of the bacteriocin was determined to be 3331. Consequently, the detected ions as well as the molecular mass agreed completely with those of nisin Z (*40*). The charge state of ions^{+3} (1111.34) was verified by zoom scan and it corresponded to isotopic massif with Δ = 0.34 (Figure 2c). Through the LC/MS analysis, the bacteriocin purchased was identified as nisin Z with > 90 % purity.

(a):



(b):



(c):

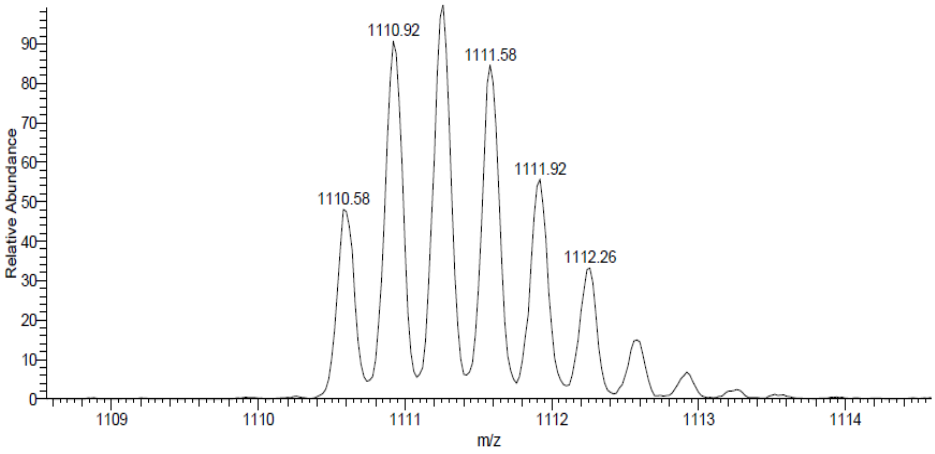


Figure 2: Detection of nisin Z by liquid chromatography/mass spectrometry. (a): HPLC chromatogram; total ion mass chromatogram; nisin Z identified peak (b) Mass spectrum at the retention time (12.85-13.39 min) showed that nisin Z was detected as $[M+3H]^{3+}$ (m/z 1111.34) and $[M+2H]^{2+}$ (m/z 1666.33) ions (c) zoom scan to confirm charge state $[M+3H]^{+3}$ of nisin

3.1.2. Fatty acid profile and lipid composition of encapsulating polymer

The fatty acid profile of different lecithins used in this study was analyzed to determine the effect of lipid class and fatty acid chain length on the EE % of nisin. The percentage of total polyunsaturated fatty acids was the highest (60 ± 0.4 %) in SL, as compared to 38 ± 0.1 % in salmon head lecithin (*41*) and zero percent in proliposomes respectively (Table 1). SL contains >50 % omega-6 (linoleic acid C18:2 n-6) and >5 % omega-3 (linolenic acid C18:3 n-3) which belong to the group of essential fatty acids (EFAs). EFAs are the "good fats" and help to support the cardiovascular, reproductive, immune, and nervous systems (*42, 43*). However, the most significant proportions of polyunsaturated fatty acids of C20:5 and C22:6 was found only in ML. Highest saturated fatty acids contents (C16 and C18) were found in Proliposomes, which contained 87.4 ± 0.3 % stearic acid.

Table 1: Fatty acid profile of different lecithins by gas chromatography (area %). Results are grouped as saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA) and poly-unsaturated fatty acids (PUFA).

Fatty acid	Soy Lecithin	Marine Lecithin	Proliposome H
C14:0		5.24 ± 0.46	
C16:0	16.6 ± 0.34	18.95 ± 0.48	12.56 ± 0.33
C18:0	3.82 ± 0.03	4.06 ± 0.05	87.44 ± 0.34
SFA	**20.42**	**28.25**	**100**
C16:1		4.88 ± 0.28	
C18:1(n-9)	19.20 ± 0.17	18.62 ± 1.25	
C18:1(n-7)		2.73 ± 1.03	
C20:1(n-7)		1.27 ± 0.56	
C20:1(n-9)		6.13 ± 0.62	
MUFA	**19.2**	**33.63**	**0**
C18:2(n-6)	54.63 ± 0.29	4.25 ± 0.06	
C18:3(n-3)	5.74 ± 0.11		
C18:4(n-3)		1.80 ± 0.18	
C20:4(n-6)		5.07 ± 0.78	
C20:4(n-3)		1.24 ± 0.03	
C20:5(n-3)		8.31 ± 0.02	
C22:5(n-3)		3.65 ± 0.03	
C22:6(n-3)		13.78 ± 0.20	
PUFA	**60.37**	**38.11**	**0**

The neutral and polar lipid classes of lecithins were separated by thin-layer chromatography (Iatroscan). SL contained 69.6 ± 2.2 % of phospholipids and 25.4 ± 1.4 % neutral lipids. Polar lipid fraction of the ML was found 41.5 ± 1.5 % which is significantly (Tukey test, $p \leq 0.001$) lower than SL. The commercial proliposomes contained no traces of neutral lipids. These results obtained from lipid profile of lecithin is particularly interesting for characterizing the liposome for encapsulation efficiency, surface properties and stability which has been discussed in the subsequent parts under liposome characterization. Soya and marine lecithin are less costly than purified commercial products and thus more appropriate for large-scale applications.

3.2. Choice of conditions for liposome fabrication:

In order to prepare the multilamellar vesicles for imposing multiple barriers in active agent release, the size diameter of the liposome must range >100 nm (*44*). To optimize the average size of liposomes near this limit, the influence of pressure (1400, 1700, 2000, 2500 bars) and number of passes (3-5) was measured on the mean diameter of liposomes at 5% SL concentration (Figure 3).

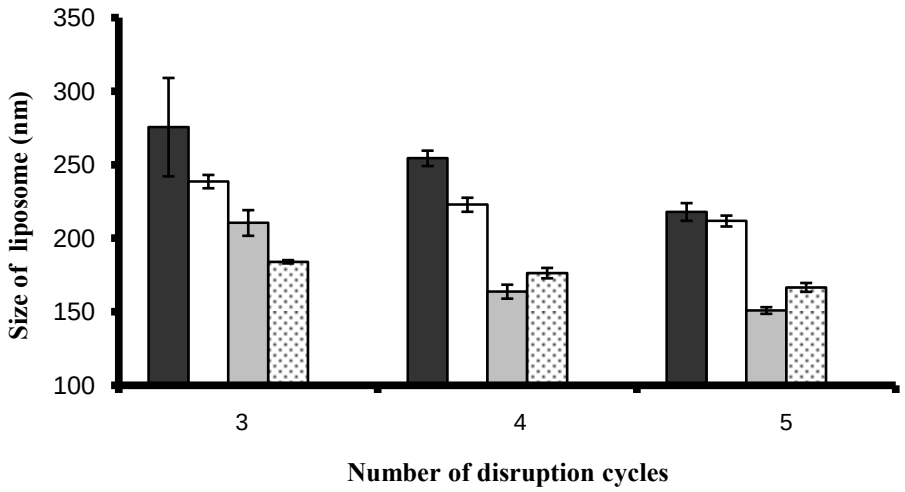


Figure 3: Mean size of soy liposomes using 5 percent concentration of SL; effect of pressure (■1400; □1700; ▒2000; ⊠2500 Bar) and number of disruption cycles (3, 4, 5) at 25 °C. Mean of averaged size from 5 replicates.

The resulted liposome size decreased along with the number of process cycles. At 2000 bar pressure, five passes resulted in appropriate average size (151 ± 4) with a polydispersity index (PDI) 0.23 ± 0.02. However increasing inlet pressure to maximum limit of apparatus 2500 bar results in unexpected higher average size either due to over disruption of liposome or probable formation of multivesicular vesicles (MVV). These results are in coherence with previous study of liposome preparation using milk fat globule membrane (MFGM) phospholipids (*45*). More significantly, one cycle of the treatment was attained within 5 min even for large volume of the liposome (100 mL). From these results, 2000 bar pressure and 5 numbers of passes were chosen as effective parameters for liposome fabrication.

3.3. Characterization of liposomes

3.3.1. *Encapsulation efficiency (EE)*

The high entrapment efficiency of active agents with respect to increasing concentration and lipid classes composition was reported previously (*12, 46*). The protein content entrapped in liposome preparations using different concentrations of lecithins was measured to determine the total amount of nisin entrapped (Figure 4).

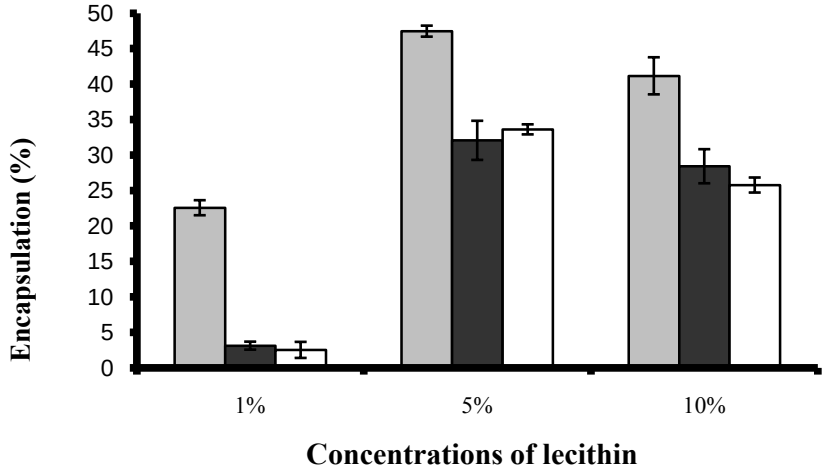


Figure 4: Percent encapsulation efficiencies of nisin Z (1mg/mL) at a 2000 bar pressure of CCDS with five disruption cycles for soy liposomes and marine liposomes while heating method for proliposomes; effect of lecithin types (□ SL; ■ ML; □Proliposomes) at one, five and ten percent lecithin concentrations.

With equivalent concentrations of nisin (1 mg/mL; 3.8 x 10^4 IU) added to liposome formulations, the highest concentration of antimicrobials was obtained with 5 % SL liposomes. These soy liposomes containing nisin had a protein content entrapped 47.4 ± 1 % which was significantly higher than SL 1 and 10 % and other composition liposomes at similar concentration (Tukey test, $p \leq 0.001$). The marine- and pro-liposomes had highest EE % for 5 % lecithin ranging 32 ± 3 % and 33.6 ± 1 % respectively.

Relatively higher EE of soy and marine liposomes may be related to the high pressure disruption method utilized as compared to traditional heating method for proliposomes. Once the size of the liposomes decreased, it resulted in better dispersibility and number of vesicles that could be effective for higher retention. The decreased antimicrobial concentration in higher neutral-lipids containing marine liposomes was in agreement with results reported in earlier studies in which increased concentration decreased the amount of encapsulated active agent (*9*). It has been suggested that introduction of other than phospholipids affects the bilayer curvature (*47, 48*). Lower phospholipids ratio in ML could reduce the polypeptide affinity, thus reducing the concentration of antimicrobials that can be incorporated. In conclusion, protein content measurement indicated that nisin encapsulated formed stable liposomes for 5 and 10% lecithin concentration with 5 % as highly effective; however, the stability depended on the lipid composition. There is no previous study that has tested the effect of fatty acid chain length on liposome stability or EE. The present study revealed that having other factors constant, SL with shorter fatty acid chain length than ML (Table 1) had an advantage for encapsulating nisin. The determination of the exact location and distribution of antimicrobials in the liposomes (e.g., inside the liposomal core or incorporated in the vesicle membrane) will require further investigations.

3.3.2. Surface charge properties

As discussed earlier, it is more reliable to assess the droplet surface electrical properties by electrophoretic mobility. However, if Smoluchowski limit is applied, the approximate zeta potential values are obtained (Table 2). Instability of liposomes was attributed to collisions and eventual merging of liposomal membranes of two or more liposomes (*44*). This process is

Table 2: Influence of nisin on electrophretic mobilities and zeta potential (0: liposomes without nisin, +: liposomes containing nisin) of soy liposomes, marine liposomes and proliposomes

Lecithin Percentage	Nisin content	Liposome Type Soy liposome	Marine liposome	Proliposome
Electrophoretic mobility (μm/s)/(V/cm)				
1	0	-4.31 ± 0.16	-3.46 ± 0.07	-3.63 ± 0.07
	+	-3.47 ± 0.13	-2.75 ± 0.10	-2.80 ± 0.21
5	0	-4.32 ± 0.05	-3.77 ± 0.06	-4.10 ± 0.16
	+	-3.41 ± 0.04	-3.32 ± 0.11	-3.20 ± 0.12
10	0	-4.85 ± 0.04	-3.93 ± 0.07	-4.15 ± 0.03
	+	-3.42 ± 0.06	-3.64 ± 0.10	-3.29 ± 0.05
Zeta potential (mV)				
1	0	-55.1 ± 2.1	-44.1 ± 0.9	-46.4 ± 0.7
	+	-44.3 ± 1.6	-35.1 ± 1.2	-35.8 ± 2.4
5	0	-55.3 ± 0.7	-48.1 ± 0.8	-52.4 ± 2.2
	+	-43.5 ± 0.5	-42.4 ± 1.4	-39.9 ± 2.7
10	0	-61.9 ± 0.6	-50.2 ± 0.7	-53.0 ± 0.8
	+	-43.7 ± 0.7	-46.4 ± 0.8	-42.0 ± 0.6

thermodynamically driven because of the tendency of the system to decrease the energetically unfavorable curvature of the bilayer membrane in spherical liposomes. Collisions may occur because of random (Brownian) movement of vesicles in solution or because of superimposed convection. Increasing the repulsive interactions may reduce the frequency of collision. Thus, liposomes composed of charged polar lipids carrying higher electrical charges with an electrophoretic mobility of −4.32 and −4.10 (μm/s)/(V/cm), for soy liposomes and proliposomes respectively, can be expected to be more stable than marine liposomes with significantly lower (Tukey test, $p \leq 0.001$) electrophoretic mobility of -3.77 (μm/s)/(V/cm) (Table 2). The change of surface charge with emulsifier concentration is also directly linked to the variation of salinity caused by the emulsifier. As it might be expected the cationic emulsifier provides a positive charge on the droplet surface, whereas the anionic and non-ionic surfactants lead to a negatively-charged surface. The measurement of zeta potential values provide useful information on the electrostatic repulsion between liposomes, closely related with coalescence and stability phenomena.

Electrophoretic mobility or ζ-Potential measurements pointed out that introduction of nisin in liposomes significantly affected net surface charge of liposomes compared with nisin-free liposomes (Table 2). The addition of nisin was reported to perturb membranes, increasing fluidity and altering the overall bilayer structure. Model membrane studies have shown that nisin has a high specificity for anionic phospholipids, which is in fact part of the basis for its antimicrobial activity (*49*). Nisin is a member of a class of small antimicrobial polypeptides known as lantibiotics that are thought to self-assemble in the bacterial membrane to form pores that prevent bacteria from maintaining homeostasis (*21*). Whereas nisin rapidly creates pores in membranes, these pores are transient in nature with nisin possibly translocating entirely across the membrane bilayer after pore formation. Accordingly, cationic peptide neutralized the charge of liposomes significantly (Tukey test, $p \leq 0.001$). As bacteria carry a negative electric charge, the decrease in negative charge of liposomes containing nisin may result in less repulsion between both, thus favoring nisin antimicrobial action.

3.3.3. Physical stability

3.3.3.1. Nano-sizer measurement

Zeta-sizer results indicated that physical stability of liposomes at 4°C over the 6-week period investigated depended greatly on lecithin composition, concentration and method employed. The presence of nisin generally increased liposome size e.g. soy liposomes 5 % had demonstrated 151 ± 4 and 181 ± 5 nm sizes without nisin and with nisin, respectively (Figure 3 & 5). The increase in liposome size when nisin was encapsulated in soy liposomes could be due to the creation of a more swollen membrane structure. Increase in liposome size upon nisin encapsulation is in agreement with results reported by Were et al. (*3*). For 5 and 10 % lecithin concentration, the PDI of soy liposomes and marine liposomes encapsulating nisin (prepared with constant disruptor method) was <0.3. Proliposomes prepared with heating method had given a PDI value >0.4. The higher PDI values found for 1% lecithin formulations may be due to the fact that liposomes become unstable at lower phospholipids concentration by the pore formation activity of nisin. The protein may alter the packing of phospholipids in the liposomal membrane and promote curvature changes. Liposome size thus was dependent on the lipid composition

(fatty acid chain length), nisin and fabrication method. During the 6-week storage of liposomes containing nisin at 4 °C, there were slight fluctuations in effective diameter (Figure 5) for 5 and 10 % lecithin formulations, which were, however, not statistically significant (Tukey test, $p > 0.05$).

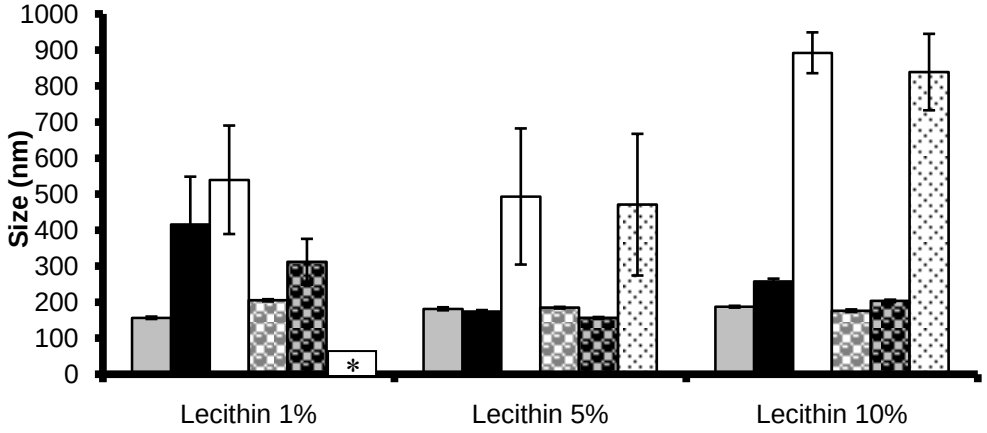


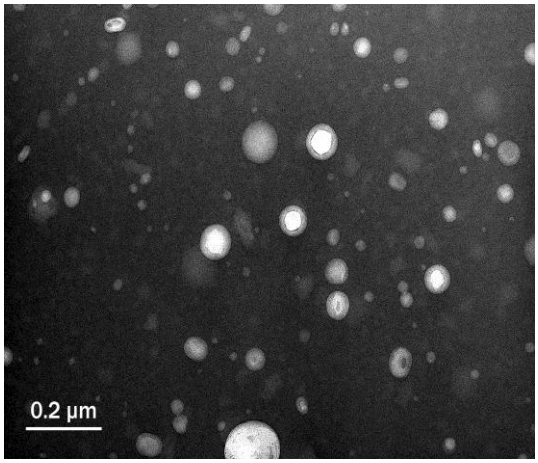
Figure 5: Mean diameter values of liposomes encapsulating nisin (1mg/mL) during storage at 4 °C. Mean size for different concentrations of lecithins (1, 5, 10%) for soy liposomes ▫, marine liposomes ■ and □ proliposomes at day 0. Size measurements of liposomes encapsulating nisin after 6 week storage period for soy liposomes ▫, marine liposomes ■ and proliposomes ▫ have shown stability of liposomes prepared with CCDS method. (* Proliposomes were unstable after 6 weeks i.e. separation of 2 phases)

3.3.3.2. Negative stain electron microscopy (TEM)

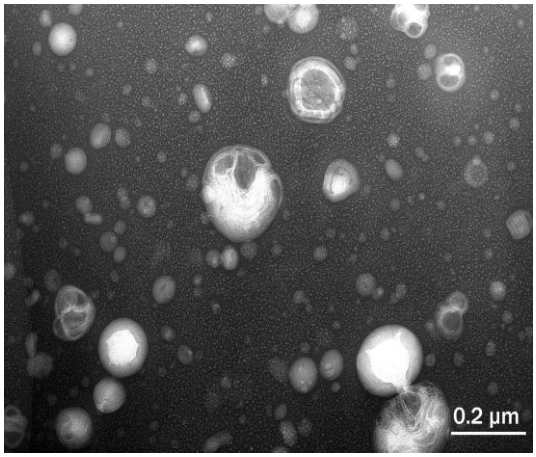
The microscopical approach is commonly used to characterize the structure/ morphology/ geometry of the nanocarriers. Electron microscopy techniques have been widely used to measure the size and interaction in the form of aggregation or fusion (*22*). In order to prove the stability during storage period and ability of the new method to efficiently produce liposomes and nanoliposomes (and not other lipidic structures such as micelles and emulsions) TEM studies

were performed. TEM experiments confirmed the presence of predominantly spherical and bilayered structures (Figure 6a, c).

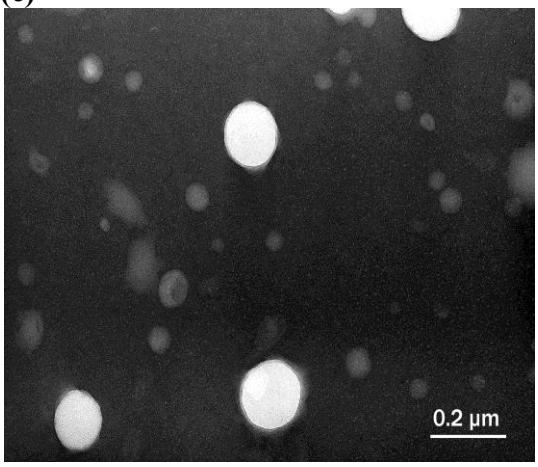
(a)



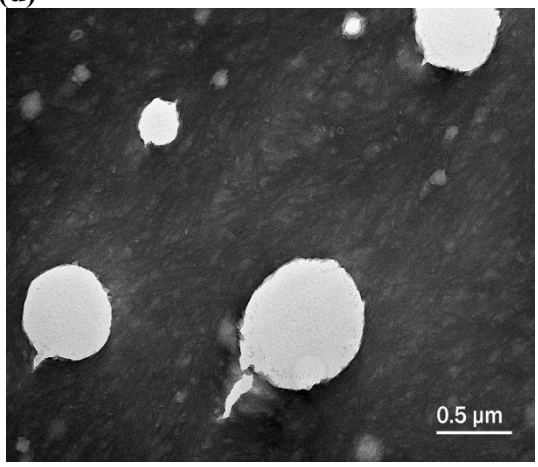
(b)



(c)



(d)



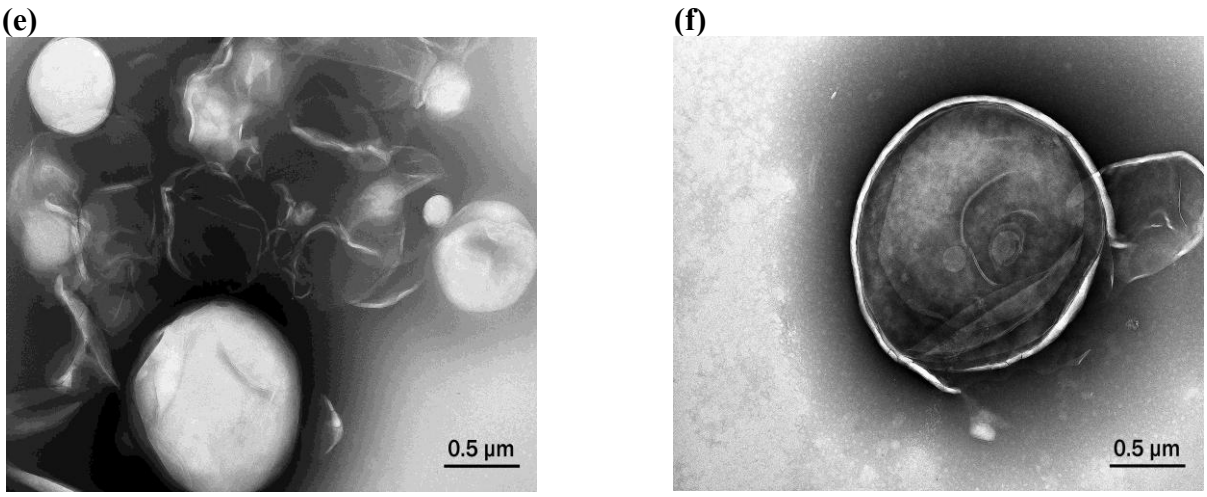


Figure 6: Structure and stability study of different liposomes at 4 °C for 20 weeks. Soy liposomes, marine liposomes and proliposomes at 0 day (a, c, e) and after 20 weeks (b, d, f) respectively

Apparently soy liposomes (5% lecithin and 1 mg/mL nisin) kept at 4 °C were still intact after 20 weeks according to physical stability study by zeta-sizer, however TEM experiments revealed changes in their morphology and occurrence of fusion phenomenon (Figure 6b). These modifications could be due to pore formation and release of nisin. On the other hand proliposomes were unstable during storage period while presence of glycerin and alcohol in their formulation exposed them as nano-gel structure (Figure 6e, f).The pore formation phenomenon was clearly observed in case of marine liposomes where pore structure is semi-attached with liposome (Figure 6d). These are very promising results referring to controlled release of nisin either through pore formation or modification in membrane structure but further research will be required to measure the diffusion kinetics of active agents from inside out. Therefore, it can be believed that the new method is fully capable of producing nanoliposomes, with different compositions, to the real definition of the liposome (*22*).

3.4. Antimicrobial activity

The minimal inhibitory concentration (MIC) value is the inverse of the highest dilution where no growth was detected. As a primary test for the antilisterial activity, free nisin (non-encapsulated) had revealed a MIC value of 7.8 mg L^{-1} against *Listeria monocytogenes* CIP 82110^{T} in inoculated tryptic soya broth (TSB-YE) medium. After wards, different solutions including control (without nisin addition), non-encapsulated free nisin solution (MIC and ½

MIC), encapsulated nisin (with soy-lecithin 5%), and blend of free plus encapsulated nisin (1:1) at corresponding MIC value were tested for antimicrobial activity against *Listeria monocytogenes*. As expected the control sample, without any nisin inoculation, had not shown antimicrobial action against *Listeria monocytogenes* (Figure 7).

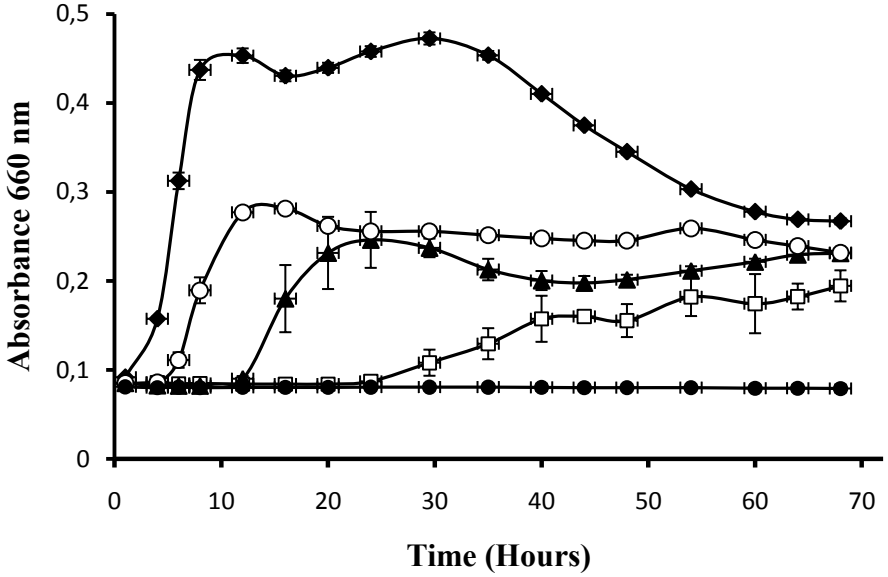


Figure 7: Growth kinetics of *Listeria monocytogenes* incubated at 37 °C with unencapsulated (free), encapsulated (nanoliposome prepared with soy-lecithin 5%, 2000 bar pressure, 5 cycles), and blend of free and encapsulated (1:1) nisin at corresponding minimal inhibitory concentration (7.8 mg L^{-1}). The treatments include; (◆) control, (□) unencapsulated/free nisin MIC, (▲) unencapsulated nisin ½ MIC, (○) encapsulated nisin, and (●) blend of nisin (free/unencapsulated and encapsulated nisin)

Active solution with nisin (MIC value) inhibited the listerial development up to 24 hours but afterwards there was gradual and steady growth of bacteria due to nisin deactivation and bacterial resistance. However, by reducing the concentration of nisin to 3.9 mg L^{-1} (1/2 MIC) could only control the increase in bacterial population up to 15 H and then steady growth of *Listeria* was observed. The nano-active liposomes (encapsulated nisin) had demonstrated lesser antimicrobial activity as bacterial growth started before 10 hours of incubation possibly due to insufficient nisin bioavailability. Nevertheless the bacterial growth was reduced by half (peak

values) as compared to control. This reduction in pathogen growth was due to slow release of nisin from liposome and probably as a result of active nano-liposomes interactions with bacteria. These results are in accordance with the previous study using phosphatidylcholine/phosphatidylglycerol (PC:PG) liposome-encapsulated and free nisin and EDTA against *Listeria monocytogenes* and *Escherichia coli* at 24 and 48 h, where inhibition by free and encapsulated active agents was essentially equivalent (*32*). In a recent study in milk system at refrigeration temperature, a decrease of 3-4 log cycles in *L. monocytogenes* counts was observed for free and encapsulated nisin at 0.5 mg/ml concentration. Thus liposome encapsulation of antimicrobial peptides may be important to overcome stability issues and interaction with food components (*50*). The interaction of liposome with target cells can occur by adsorption onto the cell surface, fusion with the cell membrane, release of drug by micropinocytosis, or due to a specific or nonspecific endocytosis (*51*). However in the present study, blend of free and encapsulated nisin (1:1) had indicated better control of pathogen as compared to 100% encapsulated nisin or free nisin (Figure 7). This effect is possibly due to the fact that free nisin controlled the initial bacterial growth burst and eventually fresh nisin release from liposome and nano-active-liposome interactions with *Listeria monocytogene*s could have improved the antimicrobial potential. Nonetheless, in future more extensive microbiological study against a spectrum of pathogens using model food system for longer storage time will be required to exhibit actual potential of these nano-active liposomal solutions encapsulating nisin for improving food shelf-life.

As conclusion, active-nano-liposome production with new method utilized in the present study is the first report for encapsulation of nisin with different biopolymers. This is innovative, without solvent, rapid, efficient and industrially applicable method. Soy liposomes prepared from SL 5 % provided best possible EE (47%) and physical stability. All of the ingredients used in the present study are non-toxic (even nutritive as PUFA), GRAS (nisin) with bio-additive notion. The microscopical study confirmed successful formation of nanoliposomes as well as the fusion and pore formation phenomenon in larger liposomes that indicated slow release of lantibiotic nisin. The initial microplate antimicrobial assay had revealed that blend of free and encapsulated nisin (1:1) had indicated better control of *Listeria monocytogenes* as compared to 100% encapsulated nisin or free nisin. In future, it‟‟ll be great to analyze the diffusion rates of active

agent from nanoparticles for predicting the bioavailability of antimicrobials intended for food bio-preservation.

Acknowledgement:

The authors acknowledge the technical assistance of Céline Caillet at Laboratoire Environnement et Minéralurgie (LEM) with ζ-sizer.

References:

1. Luykx, D. M. A. M.; Peters, R. J. B.; Van Ruth, S. M.; Bouwmeester, H., A review of analytical methods for the identification and characterization of nano delivery systems in food. *Journal of Agricultural and Food Chemistry* **2008,** 56, (18), 8231-8247.
2. Devlieghere, F.; Vermeiren, L.; Debevere, J., New preservation technologies: Possibilities and limitations. *International Dairy Journal* **2004,** 14, (4), 273-285.
3. Were, L. M.; Bruce, B. D.; Davidson, P. M.; Weiss, J., Size, Stability, and Entrapment Efficiency of Phospholipid Nanocapsules Containing Polypeptide Antimicrobials. *Journal of Agricultural and Food Chemistry* **2003,** 51, (27), 8073-8079.
4. Breukink, E.; De Kruijff, B., The lantibiotic nisin, a special case or not? *Biochimica et Biophysica Acta - Biomembranes* **1999,** 1462, (1-2), 223-234.
5. de Arauz, L. J.; Jozala, A. F.; Mazzola, P. G.; Vessoni Penna, T. C., Nisin biotechnological production and application: a review. *Trends in Food Science and Technology* **2009,** 20, (3-4), 146-154.
6. EU, *Regulation (EC) No. 1935/2004 European Parliament and the Council of 27 October 2004 on materials and articles intended to come into contact with food repealing* **2004**.
7. FDA, *FDA/CFSAN/OPA: Agency Response Letter: GRAS Notice No. GRN 000065* **2001**.
8. Benech, R. O.; Kheadr, E. E.; Laridi, R.; Lacroix, C.; Fliss, I., Inhibition of Listeria innocua in cheddar cheese by addition of nisin Z in liposomes or by in situ production in mixed culture. *Applied and Environmental Microbiology* **2002,** 68, (8), 3683-3690.
9. Laridi, R.; Kheadr, E. E.; Benech, R. O.; Vuillemard, J. C.; Lacroix, C.; Fliss, I., Liposome encapsulated nisin Z: Optimization, stability and release during milk fermentation. *International Dairy Journal* **2003,** 13, (4), 325-336.

10. Jung, D. S.; Bodyfelt, F. W.; Daeschel, M. A., Influence of fat and emulsifiers on the efficacy of nisin in inhibiting Listeria monocytogenes in fluid milk. *Journal of dairy science* **1992,** 75, (2), 387-393.

11. Davies, E. A.; Milne, C. F.; Bevis, H. E.; Potter, R. W.; Harris, J. M.; Williams, G. C.; Thomas, L. V.; Delves-Broughton, J., Effective use of nisin to control lactic acid bacterial spoilage in vacuum-packed bologna-type sausage. *Journal of Food Protection* **1999,** 62, (9), 1004-1010.

12. Taylor, T. M.; Gaysinsky, S.; Davidson, P. M.; Bruce, B. D.; Weiss, J., Characterization of antimicrobial-bearing liposomes by zeta potential, vesicle size, and encapsulation efficiency. *Food Biophysics* **2007,** 2, (1), 1-9.

13. Roberts, R. F.; Zottola, E. A., Shelf-life of pasteurized process cheese spreads made from cheddar cheese manufactured with a nisin-producing starter culture. *Journal of dairy science* **1993,** 76, (7), 1829-1836.

14. Liolios, C. C.; Gortzi, O.; Lalas, S.; Tsaknis, J.; Chinou, I., Liposomal incorporation of carvacrol and thymol isolated from the essential oil of Origanum dictamnus L. and in vitro antimicrobial activity. *Food Chemistry* **2009,** 112, (1), 77-83.

15. Nongonierma, A. B.; Abrlova, M.; Fenelon, M. A.; Kilcawley, K. N., Evaluation of two food grade proliposomes to encapsulate an extract of a commercial enzyme preparation by microfluidization. *Journal of Agricultural and Food Chemistry* **2009,** 57, (8), 3291-3297.

16. Colas, J. C.; Shi, W.; Rao, V. S. N. M.; Omri, A.; Mozafari, M. R.; Singh, H., Microscopical investigations of nisin-loaded nanoliposomes prepared by Mozafari method and their bacterial targeting. *Micron* **2007,** 38, (8), 841-847.

17. Kosaraju, S. L.; Tran, C.; Lawrence, A., Liposomal delivery systems for encapsulation of ferrous sulfate: Preparation and characterization. *Journal of Liposome Research* **2006,** 16, (4), 347-358.

18. Mozafari, M. R.; Johnson, C.; Hatziantoniou, S.; Demetzos, C., Nanoliposomes and their applications in food nanotechnology. *Journal of Liposome Research* **2008,** 18, (4), 309-327.

19. Mozafari, M. R.; Flanagan, J.; Matia-Merino, L.; Awati, A.; Omri, A.; Suntres, Z. E.; Singh, H., Recent trends in the lipid-based nanoencapsulation of antioxidants and their role in foods. *Journal of the Science of Food and Agriculture* **2006,** 86, (13), 2038-2045.

20. Xia, S.; Xu, S., Ferrous sulfate liposomes: Preparation, stability and application in fluid milk. *Food Research International* **2005,** 38, (3), 289-296.

21. Breukink, E.; Ganz, P.; De Kruijff, B.; Seelig, J., Binding of nisin Z to bilayer vesicles as determined with isothermal titration calorimetry. *Biochemistry* **2000,** 39, (33), 10247-10254.

22. Khosravi-Darani, K.; Pardakhty, A.; Honarpisheh, H.; Rao, V. S. N. M.; Mozafari, M. R., The role of high-resolution imaging in the evaluation of nanosystems for bioactive encapsulation and targeted nanotherapy. *Micron* **2007,** 38, (8), 804-818.

23. Were, L. M.; Bruce, B.; Davidson, P. M.; Weiss, J., Encapsulation of nisin and lysozyme in liposomes enhances efficacy against Listeria monocytogenes. *Journal of Food Protection* **2004,** 67, (5), 922-927.

24. Teixeira, M. L.; dos Santos, J.; Silveira, N. P.; Brandelli, A., Phospholipid nanovesicles containing a bacteriocin-like substance for control of Listeria monocytogenes. *Innovative Food Science and Emerging Technologies* **2008,** 9, (1), 49-53.

25. Massing, U.; Cicko, S.; Ziroli, V., Dual asymmetric centrifugation (DAC)-A new technique for liposome preparation. *Journal of Controlled Release* **2008,** 125, (1), 16-24.

26. Jahn, A.; Vreeland, W. N.; Devoe, D. L.; Locascio, L. E.; Gaitan, M., Microfluidic directed formation of liposomes of controlled size. *Langmuir* **2007,** 23, (11), 6289-6293.

27. Laloy, E.; Vuillemard, J. C.; Dufour, P.; Simard, R., Release of enzymes from liposomes during cheese ripening. *Journal of Controlled Release* **1998,** 54, (2), 213-222.

28. Moussaoui, N.; Cansell, M.; Denizot, A., Marinosomes®, marine lipid-based liposomes: Physical characterization and potential application in cosmetics. *International Journal of Pharmaceutics* **2002,** 242, (1-2), 361-365.

29. Mayneris-Perxachs, J.; Bondia-Pons, I.; Serra-Majem, L.; Castellote, A. I.; Lopez-Sabater, M. C., Long-chain n-3 fatty acids and classical cardiovascular disease risk factors among the Catalan population. *Food Chemistry* **2010,** 119, (1), 54-61.

30. Nicolosi, R. J.; Wilson, T. A.; Lawton, C.; Handelman, G. J., Dietary effects on cardiovascular disease risk factors: Beyond saturated fatty acids and cholesterol. *Journal of the American College of Nutrition* **2001,** 20, (5 SUPPL.).

31. Chen, S. S. C., Soybeans and health. *Journal of the Chinese Nutrition Society* **1994,** 19, (3), 335-345.

32. Taylor, T. M.; Bruce, B. D.; Weiss, J.; Davidson, P. M., Listeria monocytogenes and Escherichia coli O157:H7 inhibition in vitro by liposome-encapsulated nisin and ethylene diaminetetraacetic acid. *Journal of Food Safety* **2008,** 28, (2), 183-197.

33. Wu, Y.; Wang, T., Phospholipid class and FA compositions of modified soybeans processed with two extraction methods. *JAOCS, Journal of the American Oil Chemists' Society* **2003,** 80, (2), 127-132.

34. Folch, J.; Lees, M.; Sloane Stanley, G. H., A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of biological chemistry* **1957,** 226, (1), 497-509.

35. Gbogouri, G. A.; Linder, M.; Fanni, J.; Parmentier, M., Analysis of lipids extracted from salmon (Salmo salar) heads by commercial proteolytic enzymes. *European Journal of Lipid Science and Technology* **2006,** 108, (9), 766-775.

36. Ackman, R., Laboratory preparation of conjugated linoleic acids. *Journal of the American Oil Chemists' Society* **1998,** 75, (9), 1227-1227.

37. Dufour, P.; Vuillemard, J. C.; Laloy, E.; Simard, R. E., Characterization of enzyme immobilization in liposomes prepared from proliposomes. *Journal of Microencapsulation* **1996,** 13, (2), 185-194.

38. Cambiella, A.; Benito, J. M.; Pazos, C.; Coca, J., Interfacial properties of oil-in-water emulsions designed to be used as metalworking fluids. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2007,** 305, (1-3), 112-119.

39. Nespolo, S. A.; Bevan, M. A.; Chan, D. Y. C.; Grieser, F.; Stevens, G. W., Hydrodynamic and electrokinetic properties of decane droplets in aqueous sodium dodecyl sulfate solutions. *Langmuir* **2001,** 17, (23), 7210-7218.

40. Mulders, J. W. M.; Boerrigter, I. J.; Rollema, H. S.; Siezen, R. J.; De Vos, W. M., Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. *European Journal of Biochemistry* **1991,** 201, (3), 581-584.

41. Belhaj, N.; Arab-Tehrany, E.; Linder, M., Oxidative kinetics of salmon oil in bulk and in nanoemulsion stabilized by marine lecithin. *Process Biochemistry* **2010**, (45), 187-195.

42. Simopoulos, A. P., Omega-6/omega-3 essential fatty acids: biological effects. *World review of nutrition and dietetics* **2009,** 99, 1-16.

43. Yashodhara, B. M.; Umakanth, S.; Pappachan, J. M.; Bhat, S. K.; Kamath, R.; Choo, B. H., Omega-3 fatty acids: A comprehensive review of their role in health and disease. *Postgraduate Medical Journal* **2009,** 85, (1000), 84-90.

44. Taylor, T. M.; Davidson, P. M.; Bruce, B. D.; Weiss, J., Liposomal nanocapsules in food science and agriculture. *Critical Reviews in Food Science and Nutrition* **2005,** 45, (7-8), 587-605.

45. Thompson, A. K.; Singh, H., Preparation of liposomes from milk fat globule membrane phospholipids using a microfluidizer. *Journal of Dairy Science* **2006,** 89, (2), 410-419.

46. Takahashi, M.; Uechi, S.; Takara, K.; Asikin, Y.; Wada, K., Evaluation of an oral carrier system in rats: Bioavailability and antioxidant properties of liposome-encapsulated curcumin. *Journal of Agricultural and Food Chemistry* **2009,** 57, (19), 9141-9146.

47. Liu, D. Z.; Chen, W. Y.; Tsai, L. M.; Yang, S. P., The effects of cholesterol on the release of free lipids and the physical stability of lecithin liposomes. *Journal of the Chinese Institute of Chemical Engineers* **2000,** 31, (3), 269-276.

48. Prades, J.; Funari, S. S.; Escriba, P. V.; Barcela, F., Effects of unsaturated fatty acids and triacylglycerols on phosphatidylethanolamine membrane structure. *Journal of Lipid Research* **2003,** 44, (9), 1720-1727.

49. El Jastimi, R.; Edwards, K.; Lafleur, M., Characterization of permeability and morphological perturbations induced by nisin on phosphatidylcholine membranes. *Biophysical Journal* **1999,** 77, (2), 842-852.

50. da Silva Malheiros, P.; Micheletto, Y. M. S.; Silveira, N. P. d.; Brandelli, A., Development and characterization of phosphatidylcholine nanovesicles containing the antimicrobial peptide nisin. *Food Research International* **2010,** 43, (4), 1198-1203.

51. Torchilin, V. P., Recent advances with liposomes as pharmaceutical carriers. *Nature Reviews Drug Discovery* **2005,** 4, (2), 145-160.

Chapitre 6: Evaluation de la microstructure et des caractéristiques physico-chimiqus des films d'emballage bioactifs à base des nanoémulsions contenants des peptides antimicrobiens

La dégradation des peptides actifs par des enzymes protéolytiques présentes dans l'aliment, ou les interactions entre le peptide antibactérien et les constituants alimentaires, peuvent être à l'origine d'une diminution de l'activité antibactérienne. La nisine a été utilisée comme composé actif dans différents polymères (Imran et al., 2010a ; Sebti et al., 2004). L'encapsulation de la nisine dans des liposomes peut être une alternative pour pallier les problèmes liés à l'application directe du composé actif dans l'aliment (Taylor et al., 2008). En raison de la présence simultanée de lipides et d'une phase aqueuse dans la structure des liposomes, les nanoliposomes peuvent être utilisés pour l'encapsulation, la dispersion, la protection, la vectorisation et le relargage de composés solubles dans l'eau ou les lipides (da Silva Maleiroos, 2010 ; Mozafari et al., 2008). La stabilité à long terme de bactériocines encapsulées dans des liposomes a été démontrée, et corrélée à une augmentation de la biodisponibilité (de 29 à 62%) pendant la période de stockage (Degnan et al., 1993).

Ces concepts ont été développés dans les deux chapitres précédents.

Dans ce chapitre, une amélioration de l'emballage actif a été réalisée en fusionnant ces deux concepts que sont la nano encapsulation de la nisine et l'immobilisation dans un biopolymère. Ce concept devrait permettre d'améliorer la biodisponibilité du composé actif et de concevoir la prochaine génération de films biodégradables contenant soit l'agent actif, soit l'agent actif nano-encapsulé, soit les deux.

Ce concept d'emballage nano-actif étant nouveau, il est nécessaire d'étudier son impact, positif ou négatif, sur les fonctions fondamentales d'un emballage. Ainsi, dans l'étude présentée dans ce chapitre, les propriétés significatives de l'emballage (microstructure (topographie et morphologie), propriétés barrière (O_2 et H_2O) et mécaniques, de sorption de l'eau, couleur, et transmission de la lumière) ont été évaluées afin d'estimer et de comparer les caractéristiques des films d'emballages biodégradables non actifs, actifs et nano-actifs. De plus, l'efficacité antibactérienne des différents films a été évaluée vis-à-vis de *Listeria monocytogenes*.

Chapter 6: Microstructure and physico-chemical evaluation of nanoemulsion-based antimicrobial peptides embedded in bioactive packaging films

Stability issues like proteolytic degradation and the potential interaction of the antimicrobial peptide with food components might result in decreased antimicrobial activity. The entrapment of nisin into liposomes might represent an alternative to overcome the problems related to the direct application in food (Taylor et al., 2008). Due to the presence of both lipid and aqueous phases in the structure of lipid vesicles, recently studied nanoliposomes can be utilized in the entrapment, homogenous dispersion, protection, delivery and release of water-soluble and lipid-soluble materials (da silva Malheiros, 2010; Mozafari et al., 2008). Long term stability of liposome-encapsulated bacteriocins had been demonstrated, which correlates to its better bioavailability (29-62% increase) during storage period (Degnan et al., 1993; Laridi et al., 2003). In parallel, to realize the objectives of preservative slow release into food and its surface contamination prevention, the use of nisin as active agent with different biopolymers have been successfully achieved (Imran et al.,2010; Sebti et al., 2003). (These two concepts are discussed in detail previously in thesis chapter 4 and 5)

An improvement of antimicrobial packaging systems was envisaged by fusion the above-mentioned two concepts of improved bioavailability i.e. nisin nanoencapsulation and biopolymer immobilizing to formulate the next generation biodegradable films embedded with either active agent, nano-encapsulated active agent or both of them.

As this concept of nano-active packaging is not reported earlier, so initially it was required to investigate influences, either positive or negative, on fundamental packaging functions. Thus in the present project we had studied the significant packaging attributes [microstructure (topography and morphology), barrier (O_2, H_2O), mechanical, color, light transmission and water sorption properties] to judge and compare the biodegradable non-active, active and nano-active packaging films. Moreover, microbiological study of different treatments was performed to ensure the antimicrobial efficacy against potential food pathogen *Listeria monocytogenes*.

Chapter 6

Microstructure and physico-chemical evaluation of nanoemulsion-based antimicrobial peptides embedded in bioactive packaging films

Muhammad Imran, Anne-Marie Revol-Junelles, Noémie René, Majid Jamshidian, Muhammad Javeed Akhtar, Elmira Arab-Tehrany, Muriel Jacquot, Stéphane Desobry

Laboratoire d'Ingénierie des Biomolécules, ENSAIA–INPL, Nancy Université, 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France

(Food Hydrocolloids, Acceptée 2011)

Abstract

Customized application of antimicrobial peptide (AMP) „nisin" directly into food (neither in active packaging nor encapsulated form) is expensive and associated with loss of activity due to deactivation in complex food systems. The purpose of the present study was to fusion the two concepts for improved bioavailability i.e. AMP nanoencapsulation and biopolymer immobilizing to formulate the next generation biodegradable films embedded with either active agent, nano-encapsulated active agent or both of them. Nanolipoosomes were prepared using soy-lecithin by microfluidizer at 2000 bar with 5 cycles to generate an average size of 151±4 nm with 50±3% encapsulation efficiency. For active films, nisin had demonstrated no negative impact on transparency, thickness and water sorption behavior obtained by GAB model (25°C, 0-0.95 A_w). For nano-active films, the results clearly illustrated that different physico-chemical properties including barrier (oxygen and water vapor permeability), color and transparency (200-900nm) remained comparable to native hydroxypropyl methylcellulose (HPMC) films and were significantly improved than using lecithin directly without nano-scale restructuring. The microstructure studies (topography and morphology) by scanning and transmission electron microscopes (SEM/TEM) revealed different (pore, lamellar, fusion) modes of nisin release from nanoliposomes embedded in HPMC matrix. As microbiological worth, nisin nano-emulsion (encapsulated and free nisin) films were effective against potential food borne pathogen *Listeria monocytogenes*. This innovative concept of biodegradable nano-active films may thus be a preventive system towards improved food safety.

Key words: biodegradable polymer, bioactive peptide, emulsion, controlled release, nano-encapsulation

1. Introduction

„Green consumerism", a trend since the beginning of the previous decade, had emerged due to increasing consumer demand for natural antimicrobial compounds. Such biomolecules are of natural origin, non-toxic for humans, environmentally safe and effective in preserving foods by controlling microorganism"s activity (Mastromatteo, Conte & Del Nobile, 2010). Antimicrobial peptides (AMP) are widely recognized as promising alternatives to the current use of antibiotics (Marcos & Gandia, 2009). Nisin is probably the most utilized AMP in the food industry as food biopreservative world-wide (Ercolini et al., 2010). Nisin is a 3.5 kDa cationic polypeptide produced from *Lactococcus lactis* strains approved for specific uses in foods in more than 40 countries (O'Sullivan, Ross & Hill, 2002). It is the only antimicrobial bacteriocin with the status of generally recognized as safe (GRAS) approved by the Food and Drug Administration (FDA) (Sanjurjo, Flores, Gerschenson & Jagus, 2006). Nisin offers effective control against broad spectrum of Gram positive bacteria especially against the food borne pathogens *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus* (Joerger, 2007). This AMP kills susceptible bacteria through a multi-step process that destabilize the phospholipids bilayer of the cell and creates transient pores (Breukink & de Kruijff, 2006).

Stability issues like proteolytic degradation and the potential interaction of the AMP with food components might result in decreased antimicrobial activity. Use of nisin in its free form (unpackaged or unencapsulated) is expensive and is associated with loss of activity due to degradation or deactivation and emergence of nisin-resistant bacteria strains (Benech, Kheadr, Lacroix & Fliss, 2002; Laridi, Kheadr, Benech, Vuillemard, Lacroix & Fliss, 2003). Significant loss of nisin activity in different foods due to its interactions with complex food components (divalent cations, enzymes, fat …) has already been reported (Davies et al., 1999; Roberts & Zottola, 1993; Taylor, Gaysinsky, Davidson, Bruce & Weiss, 2007).

In the food industry, liposomes have been investigated to deliver proteins, enzymes, vitamins, antioxidants and flavors (Khosravi-Darani, Pardakhty, Honarpisheh, Rao & Mozafari, 2007; Laridi et al., 2003; Mozafari, Johnson, Hatziantoniou & Demetzos, 2008; Taylor, Bruce, Weiss & Davidson, 2008). The entrapment of AMP into liposomes might represent an alternative to overcome the problems related to the direct application of these AMP in food. Due to the presence of both lipid and aqueous phases in the structure of lipid vesicles, recently studied nanoliposomes can be utilized in the entrapment, homogenous dispersion, protection, delivery

and release of water-soluble and lipid-soluble materials (Taylor et al., 2008). As nisin is amphiphilic in nature (Breukink, Ganz, De Kruijff & Seelig, 2000), it is entrapped simultaneously in the core and bilayers of liposome. Thus, it generates the possibility for superior bioavailability of nisin by phospholipid based nano-delivery systems. Long term stability of liposome-encapsulated AMP (nisin, pediocin) had been demonstrated, which correlates to its better bioavailability during storage period (Degnan, Buyong & Luchansky, 1993; Laridi et al., 2003).

As the bacterial load is maximal at food surface, immobilized AMP can also find application for development of bioactive food packaging. Currently, environment conservation awareness has led to a paradigm shift to look for packaging films and processes that are biodegradable. Thus, the concept of biodegradability enjoys both user-friendly and eco-friendly attributes. Biodegradable polymer hydroxypropyl methylcellulose (HPMC) edible films are attractive for food applications because this edible plant derivative had shown to form transparent, odorless, tasteless, oil-resistant, water-soluble films with very efficient oxygen, carbon dioxide, aroma and lipid barriers, but with moderate resistance to water vapor transport (Imran, El-Fahmy, Revol-Junelles & Desobry, 2010a; Villalobos, Chanona, Hernandez, Gutierrez & Chiralt, 2005). To realize the objectives of preservative slow release into food and its surface contamination prevention, the incorporation of nisin in sodium caseinate films has been reported in recent work (Cao-Hoang, Chaine, Grégoire & Waché, 2010).

An improvement of antimicrobial packaging systems is needed in order to prevent the growth of spoilage related microbial species for longer conservation period to enhance the shelf-life and quality of food. In this regard, the aim of the present project was to fusion the above-mentioned two concepts i.e. AMP nanoencapsulation and biopolymer immobilizing to formulate the next generation biodegradable films embedded with either active agent, nano-encapsulated active agent or both of them. Thus, nisin could diffuse/migrate through the liposome (simple model cell membrane) by pore formation as it was demonstrated against bacteria (Breukink & De Kruijff, 1999). Further it can diffuse from the network of film to reach the food in contact, thus present strategy may guarantee the availability of fresh/active nisin protected from its inhibitors in complex food systems (Figure 1).

To realize these objectives, nanoencapsulation of nisin in soy-lecithin by an innovative, rapid, without organic solvents, efficient and industrially applicable method of microfluidic

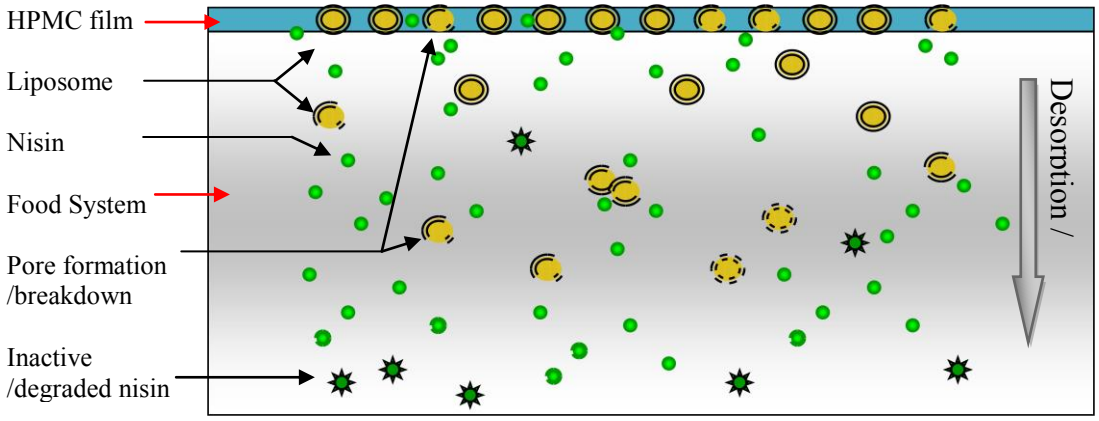


Figure 1: The diffusion/release of liposome-encapsulated nisin embedded in HPMC nano-active films

format was realized. The incorporation of nano-structures in HPMC film forming solution (FFS) may change the native microstructure (topography and morphology), barrier (O_2, H_2O), mechanical, color, light transmission and water sorption properties of formulated packaging films. To analyze the suitability of the composite films, in the present project we had studied the above-mentioned significant attributes to judge and compare the biodegradable non-active, active and nano-active packaging films. Concurrently, the effects of hydrophilic and hydrophobic plasticizers on film physico-chemical characters were studied. Moreover, microbiological study of different treatments was performed to ensure the antimicrobial efficacy against potential food pathogen *Listeria monocytogenes*.

2. Materials and Methods

2.1. Materials

Nisin Z was purchased from Honghao Chemical Co. (Shanghai, China). Nisin used in this study contained >90 % pure nisin (3.84 x 10^6 I.U. per gram and 6.88 % moisture content). HPMC powder with hydroxypropoxyl content ~9% and viscosity ~15 mPa.s (2% in H_20, 25°C) was obtained from Fluka-Biochemika, Japan. Commercial soybean lecithin (SL) was used, extracted according to method describe previously (Wu & Wang, 2003). Glycerol (>97% purity) was used as a plasticizer and was purchased from Merck (Darmstadt, Germany). Millipore nylon filters (0.2 µm) were obtained from Millipore (Cork, Ireland). The liposomal ingredients were kept under nitrogen atmosphere at the recommended storage temperatures (0-4 °C). Ammonium

molybdate was purchased from Fisher Scientific (France). Bicinchoninic acid (BCA) reagents were obtained from Sigma Chemical Co. (Lyon, France).

Microorganism and culture media: *Listeria monocytogenes* CIP 82110^T strain was purchased from collection of institute Pasteur. The bacteria were cultivated in trypticase soy broth (Biokar Diagnostics, Beauvais, France) supplemented with 6 g L^{-1} of bacto-yeast extract (Biokar) (TSB-YE). Incubation was performed at 37 °C overnight. The strain was stored in appropriate culture medium supplemented with glycerol (10%) at -30 °C and propagated twice before use.

2.2. Nisin encapsulation in nanoliposome

High pressure homogenization method (constant cell disruptor system – CCDS, Northants, UK) was preliminary optimized using soy-lecithin (SL) with different concentrations, number of cycles and pressure. From these, optimum concentration (5% w/v), pressure (2000 bar) and number of passes (5 cycles) were used for preparing nanoliposomes. These values were selected by considering the nisin encapsulation efficiency, size and polydispersity index (PDI) of vesicles. Thus, five percent SL was mixed with an aqueous solution containing nisin [3.8 x 10^4 I.U.]. The mixture was stirred (50–60 rpm) for 15 min, diluted with deionized water to obtain 1, 2 or 4 mg/mL of nisin Z concentration (to obtain a final concentration of 1 mg/mL in different film forming solutions) and re-stirred for 15 min by magnetic stirring. Further it was thoroughly mixed by Ultraturax T-25 (Avantek, Strasbourg, France) at 13500 rpm for 3 min. The phospholipid dispersion was then passed through CCDS with a vertical interaction chamber for 5 cycles at given pressure. The homogenization temperature was kept below 10°C by using a flow of cold water flowing (4°C) through a cooling jacket. To recover the liposomes containing nisin, emulsions were passed through Sephadex G-50 (fractionation range for protein 1500-30000 M.W.) column and nisin-encapsulating liposomes were eluted by size exclusion chromatography using eppendorf centrifuge (Eppendorf, Hambourg, Germany).

2.3. Particle size characterization

The mean diameter and particle size distribution of liposomes were determined using dynamic light scattering (DLS) technique employing a Zetasizer Nano-ZS (Malvern instruments, UK). The apparatus is equipped with a 4 mW He/Ne laser emitting at 633 nm, a measurement cell, a photo multiplier and a correlator. Prior to size measurement, the samples were diluted

(1:400) with ultra pure water. The samples were taken in vertical cylindrical cuvettes (10 mm diameter). The scattering intensity was measured at a scattering angle of 173° relative to the source using an avalanche of photodiode detector, at 25°C. Results are presented as an average diameter of the liposome suspension (z-average mean) with the polydispersity index (PDI) which evaluates the size distribution width. In the dispersion, particles are in a constant random Brownian motion that causes the fluctuation of the scattered light intensity with time. Therefore, droplets sizes were obtained from the correlation function calculated using algorithm of the dispersion technology software (DTS). All measurements were carried out at 25°C, with a medium refractive index 1.335. The measurements were performed in five replicates.

2.4. Film Preparation

Film forming solutions (FFS) were prepared by dissolving HPMC in distilled water (final concentration 6 % w/v). The solutions were mixed for 40 min at 65 °C using a heating magnetic stirrer (Fisher Bio-block scientific). Composition of the HPMC biodegradable active film formulations are shown in Table 1. For non-active and active films, selected amounts of glycerol, lecithin and nisin were added during heating and stirring. To obtain the active FFS solutions with nisin, it was dissolved in 10 mL of distilled water and FFS was prepared with the remaining 90 mL. For the nano-active treatments, initially emulsion and liposomes were obtained as described above, and then nano-vesicles solution was added to double concentrated HPMC FFS (1:1) to obtain a final concentration of 6 % for HPMC. The encapsulation efficiency of the CCDS method for nisin entrapment was found 50±3 % with different concentration of nisin (1, 2 or 4 mg/mL). Thus in case of nano-emulsion (encapsulated and free nisin) approximately half of the given nisin concentration is encapsulated and rest is in free form. However, the nanoliposomes obtained from emulsion by size exclusion chromatography have nisin in encapsulated form only. Nisin concentration was adjusted to give a final concentration of 1 mg/mL for active and nano-active films. As a homogenous solution was achieved after mixing, it was degassed at 50-60 °C under vacuum (Yamato®). Films were made by pouring approximately 7 g FFS in the lids of Petri-dishes (Optilux - Nunclon™ Fisher, DK-4000 Roskilde, Denmark) and left to dry them at room temperature (20 °C) and relative humidity (~50%) for 24-48 hours. Films were either stored under similar conditions of drying or at ~ zero relative humidity using phosphorus pentaoxide (P_20_5) depending upon film characterization experiment.

Film characterization

2.5. Microstructure analysis.

2.5.1. Optical microscope. To study the surface properties of non-active and nano-active films, the pre-conditioned films were stained with toluidine blue. Afterwards, film topography was examined by using optical microscope (Olympus, Ax 70) with 100x magnification. Random areas on each film were observed and the representative images were captured and digitized by an Olympus DP 70 (U-CMAD-2, Japan) using the software DP manager version 2.1 (Olympus corporation).

2.5.2. Scanning electron microscope (SEM). Microstructural analyses of cross-sections of dry films (previously conditioned in desiccators with P_2O_5 for at least 15 days) were carried out by cryo-fracturing of films. Film cross sections were prepared by dropping a film into liquid nitrogen followed by fracturing with a pre-chilled razor. Freshly fractured film pieces were retrieved from the liquid nitrogen bath and placed as quickly as possible into a Petri dish containing a piece of filter paper and placed in a desiccators to warm and dry to room temperature. Fractured film pieces were then mounted up on a SEM stub. All samples were then viewed and photographed in a Hitachi S-4800 scanning electron microscope (Hitachi, Japan) at 0.5 to 2 kV. Topographic analyses of upper and lower surfaces, which faced air and Petri plate during dehydration, respectively, were analyzed by using preconditioned films stuck onto a cylindrical aluminum stub by a double-sided tape to observe the morphology of the surfaces as explained above.

2.5.3. Transmission electron microscope (TEM). The microstructure of nanoactive films was analyzed to study the morphological character of the liposomes in the biodegradable coating matrices. The films preconditioned (P_20_5, 20 °C, 15 days) were dehydrated with ethanol for 1 hour. After ethanol treatment, a pre-impregnation in blend of resin Embed 812 and propylene oxide (1:1) was performed. Progressive impregnation steps with increasing amounts of resin blend (Embed 812 and propylene oxide 0.3:0.7, 0.5:0.5, 0.7:0.3) were tested. The samples were finally placed in the latex molds covered with the resin and Reynolds dye and polymerized at 56°C overnight. The ultra-thin sections of <100 nm were achieved by an ultra-microtome, these ultra-thin film pieces were contrasted with uranyl acetate to observe under TEM. The mesh was examined using a Transmission Electron Microscope (Philips CM-20) at an operating voltage of 200 KV.

2.6. Film thickness measurement

The thickness of films was measured using the standard NF Q 03-016 with a manual micrometer (Messmer, London, England) equipped with a head of 1 cm in diameter and with a sensitivity of 2µm. The thickness was measured in ten randomly selected points on each film and then an average value was determined.

2.7. Tensile properties

The tensile characteristics of films consist of tensile strength (TS, MPa), ultimate elongation (UE, percent at break point), and Young‟s modulus (Y, MPa). Maximum tensile strength is the largest stress that a film is able to sustain. Ultimate elongation is the maximum percentage change in the length of a film before breaking. Young‟s modulus, calculated from the slope of the initial linear region of the stress-strain curves, reflects the film stiffness. The tests were performed using the universal testing machine Lloyd instrument (Hants, United Kingdom) on 6 specimens previously stored for 7 days at 20±1 °C and 50±2 % RH. Sample films of approximately 5 cm x 2 cm (analysed area = 3 cm x 2 cm) uniaxially stretched (sensor of force of 5-KN and constant speed of 20 mm/min). The stress-strain curves were computer-recorded and exploited with Nexygen software.

2.8. Water vapour permeability (WVP)

WVP of composite films was investigated with the gravimetric method described in the AFNOR NFH00–030 standard (1974) as reported earlier (Imran et al., 2010a). The film was sealed in a permeation cell containing a desiccant (silica gel). The glass permeation cells were 5.8 cm (i.d.) × 7.8 cm (o.d.) × 3.6 cm deep with an exposed area of 26.42 cm^2. The permeation cells were placed in a controlled temperature and RH chamber. The water vapour transport was determined from the weight variation of the cell with time. Three replicates were made from each film composition. Water Vapour Transmission Rate (WVTR) was determined from the slope of weight gain versus time, once the steady state was reached.

$$WVTR = dm / (A.dt) \qquad (g.h^{-1}.m^{-2}) \qquad (1)$$

$$WVP = [WVTR.X / (\Delta p.3600)] \qquad (g.s^{-1}.m^{-1}.Pa^{-1}) \qquad (2)$$

Where dm is the weight gain of the cup over time (dt), A is the area of exposed film, Δp is the vapour pressure differential across the film, and X is the film thickness.

2.9. Water sorption isotherms

Dynamic vapour sorption system (DVS, SMS Ltd, UK) was used to obtain the sorption isotherms of films. The sample was equilibrated at a constant temperature for different relative humidity values. Film were cut into small pieces (5mm x 5mm) and dried in vacuum desiccators at 20 °C over Phosphorus pentoxide (P_2O_5) for 2 weeks. The programmed relative humidities were from 0 to 95%, divided in 10% increments (10 points). The temperature was set at 25 °C. The samples were considered to be at equilibrium when the value d*m*/d*t* (slope of the change in mass with time) was set to be <0.002 Δ mass %/min. Where Δ mass % is the percent of mass variation of the sample at given time.

The modelisation of the sorption isotherms was done using GAB (Guggenheim-Anderson-de Boer) model. The procedure used for estimating the parameters was non-linear regression (curve fitting), using Origin 6.1 software (Origin Lab corporation, USA).

$$X = X_m .C_{GAB}.K.a_w / [(1 – K. a_w).(1- K. a_w + C. K. a_w)] \quad (3)$$

X is the mass of water adsorbed at a given a_w, Xm is the monolayer value (% db). Constant C_{GAB} is related to the energy associated with the binding between the water molecules and the matrix primary interactions sites or monolayer. K is a temperature-dependent coefficient related to the heat of sorption of the multilayer.

2.10. Oxygen permeability

The basis of this dynamic method is the oxygen transfer through the film. To measure oxygen permeability, the film is placed between two Teflon rings, in a permeation cell. O_2 permeability of preconditioned films (20±1 °C and 50±2 % RH) was determined using a gas chromatographic system (Shimadzu GC-4A; Shimadzu Corp., Kyoto, Japan). Gas concentrations were measured by injecting samples with a gas sampling syringe (Dynatec Pressure Lok, Baton Rouge, LA, USA) into a gas chromatograph equipped with a thermal conductivity detector and two columns (Hayesep for N_2 and molecular sieve for O_2). The flow rate of helium carrier gas was 25 mL min^{-1} and column temperature was 50 °C. This method was based on measurement of

the amount of oxygen diffusing through the film over time. The film was first sealed into a test cell, which was filled with oxygen. At suitable time intervals, gas samples were withdrawn from the lower compartment of the cell via a sampling port and analyzed by gas chromatography (Khwaldia, Banon, Desobry & Hardy, 2004). Oxygen content is reported as percent of detected peaks (O_2 and N_2). The oxygen permeability was determined by using Eq. (4).

$$P\ O_2 = \frac{a \times x \times V}{s \times 60 \times 0.65} \quad (4)$$

Where *a* is the coefficient of the curve slope representing the percentage of oxygen as a function of time, *x* is the thickness of film expressed in m, *V* is the volume of the permeation cell which corresponds to 0.0001 m^3 and *S* is the film surface area exposed i.e. 0.0025 m^2.

2.11. Optical properties

2.11.1. Transparency/light transmission. Film transparency against ultraviolet (UV) and visible light was measured for a wavelength spectrum between 200 and 900nm, using a UV-Visible recording spectrophotometer (Ultrospec 4000 UV/visible, Pharmacia Biotech, UK) according to the procedure given by Fang and others (Fang, Tung, Britt, Yada & Dalgleish, 2002). The transparency of the films was calculated by the equation:

$$\text{Transparency} = -\log T_{600}/x \text{ (Han \& Floros, 1997)} \quad (5)$$

Where T_{600} is the transmittance at 600 nm and x is the film thickness. Three replicates of each treatment were tested.

2.11.2. Color measurement. Edible films and coating‟s color is one of the sensory characteristics, which must be as neutral as possible, so that they are not detected during consumption. To evaluate the color of films, a Minolta CM, CR-210 colorimeter (Minolta, Colombes, France) employing the Hunter and CIE scale was used. The parameters *L** for lightness and chromatic coordinates *a** and *b** are measured. The parameter *L** is the luminosity of the sample between the black and the white, the parameter *a** is the value of variation of the color of the treatment between the green and blue whereas the parameter *b** is the variation of color of the treatment

between the yellow and the red. The whiteness index (WI) of the composite films was also calculated by the following equation (Sanchez-Gonzalez, Vargas, Gonzalez-Martanez, Chiralt & Chafer, 2009).

$$WI = 100 - \sqrt{(100 - L^*)^2 + (a^{*2} + b^{*2})} \quad (6)$$

2.12. Microbiological analysis

The minimal inhibitory concentration (MIC) of nisin was determined by the critical dilution method in 96-well plates (Nunc, Roskilde, Denmark). The target strain *Listeria monocytogenes* CIP 82110^T was prepared in TSB-YE medium to a final OD 660 nm of 0.2. Equal volume of inoculated medium and nisin dilutions were added to the 96 well-plate. The plates were shacked and the initial OD 660 nm was determined with a Titertek Multiscan MCC/340 (Huntsville, AL). The plates were incubated for 24 h at 37 °C. The MIC value (7.8 mg L^{-1}) is the inverse of the highest dilution where no growth is detected. Afterwards, the corresponding MIC dilutions of film forming solutions were incubated with the target strain and its growth kinetics was recorded at 660 nm absorbance for more than 3 days.

To find out the antilisterial activity of films, 1 cm diameter disks were cut from different composite bioactive films and placed on inoculated nutrient medium. The method was previously standardized by adjusting the microbial inoculation rate (0.1% v/v) and the volume of the agar medium layer (12 mL). Dishes were refrigerated at 4 °C for 4 h to allow the process of bacteriocin diffusion without microbial growth and then incubated at 37 °C.

2.13. **Statistical analysis**

Statistical analyses were carried out by using the software KyPlot version 2.0 (Koichi Yoshioka, Department of Biochemistry and Biophysics, Graduate school of Allied health Sciences, Tokyo, Japan). For comparison between HPMC film and films containing plasticizer or active agent, a parametric multiple test (Dunnett test with HPMC film as control) was performed. Furthermore different composite films containing nisin were compared with their respective formulations without nisin using Tukey parametric multiple test.

3. Results and discussion

3.1. Particle size characterization of liposome encapsulating nisin

In most cases, reactive or sensitive material, such as polynucleotides and polypeptides (e.g. nisin), can be turned into stable ingredients through encapsulation or entrapment by nano-carrier systems (e.g. liposome) (Mozafari, 2006).

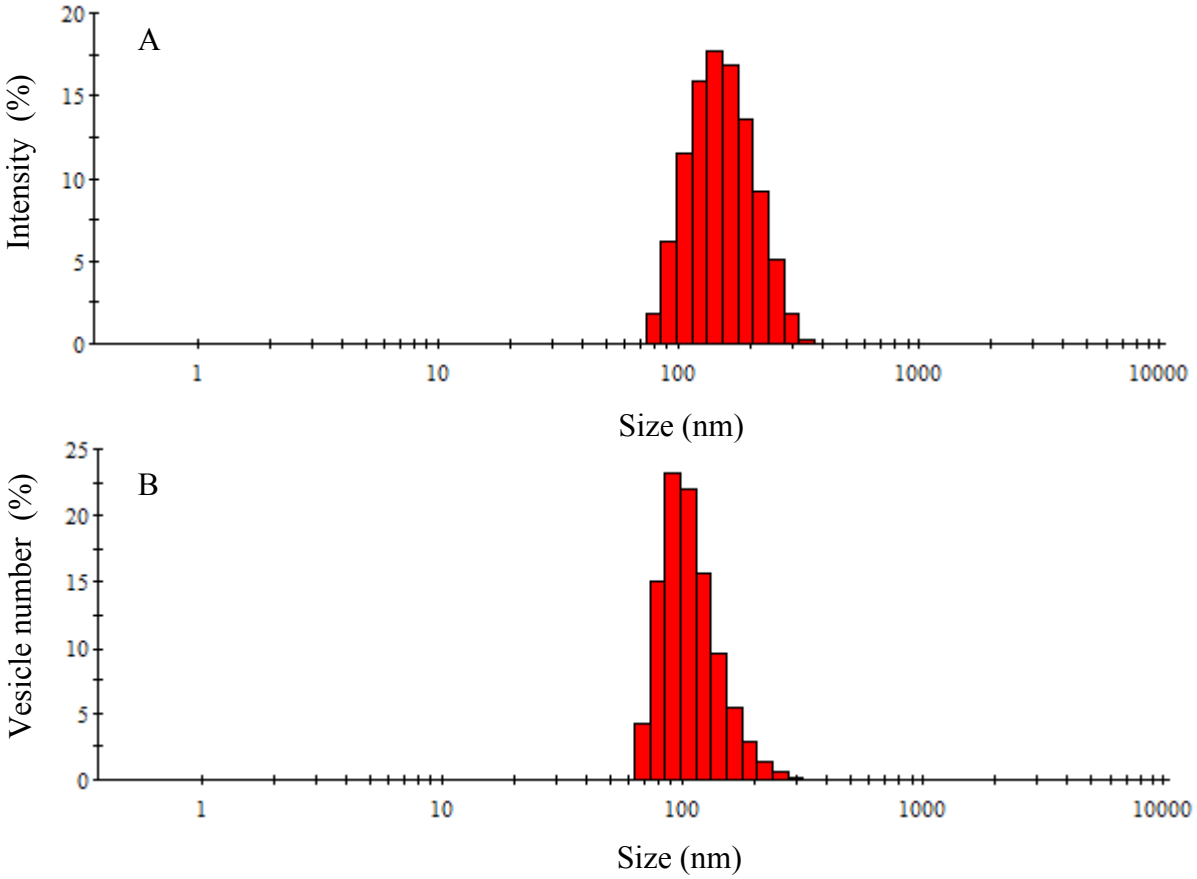


Figure 2: Size distribution of nisin encapsulated nanoliposomes using soy-lecithin (5% w/v) prepared by constant cell disruption system at 2000 bar pressure with five cycles. Size distribution of liposomes at logarithmic scale presented as vesicles intensity percentage (A); and vesicles number percentage (B).

The study of liposome average size, as well as their distribution, is of interest because of its impact on the properties of the films, such as water vapor permeability, mechanical properties and general barrier properties (Fabra, Jiménez, Atarés, Talens & Chiralt, 2009). At 2000 bar

pressure, five passes in CCDS resulted in the average particle size of 151±4 nm for nisin encapsulating liposomes (Figure 2). Particle size distribution was uni-modal with a narrow polydispersity index (PDI) of 0.23 ± 0.02, which had reflected the efficiency of CCDS technique for liposome preparation. Size distribution considering vesicles number percentage was found highest between 80-130 nm. In contrast to µm sized carriers, nano-carriers provide more surface area and have the potential to increase solubility, enhance bioavailability, improve time-controlled release (Mozafari et al., 2008). Thus, nano-sizer results indicated that nanoliposomes containing nisin formulated using soy-lecithin are sufficiently smaller to carryout above-mentioned advantages.

Characterization of the films

Different film formulations characterized in this study are non-active (F_1, F_3, F_5, F_7), active (F_2, F_4, F_6) and nano-active (F_8, F_9) (Table 1). The formulations (F_3, F_4, F_5, and F6) described the effects of two plasticizers glycerol and lecithin, whereas formulations (F_7, F_8, F_9) illustrated the effect of nano-restructuring of soy-lecithin in the form of liposomes. The term nisin emulsion is used to express both forms of nisin (free in solution and encapsulated in liposome).

3.2. Microstructural properties

3.2.1. Surface study

Taking into consideration the lack of miscibility of film forming solution (FFS) components, film microstructure is assumed as a continuous matrix of hydrocolloids, embedding lipid aggregates of different sizes, depending on the film composition and the component interactions developed during both emulsification and film drying steps (Debeaufort & Voilley, 1995). The surface images obtained for control and nano-active films by optical microscope are shown in Figure 3. The films were stained with toluidine blue (TB). In clinical studies, it is increasingly common to stain the cells with TB, which stains more or less whole tissue but granules are more conspicuous. It is a quick stain for light microscopic "orientation sections", which are used afterwards for electron microscopy.

Table 1: Mechanical characteristics (tensile strength TS, young"s modulus Y, ultimate elongation UE) and thickness of non-active, active (nisin) and nano-active (liposome-encapsulated nisin) biodegradable HPMC coatings

	Formulations	Thickness (μm)	Tensile strength TS (MPa)	Young"s modulus Y (MPa)	Ultimate elongation UE (mm)
F_1	HPMC	43.5 ± 1.3	59.0 ± 6.8	2727 ± 361	6.0 ± 3.3
F_2	HPMC + N	47.7 ± 5.6	45.1 ± 6.6**	2133 ± 578	6.2 ± 1.2
F_3	HPMC + G	50.8 ± 2.7*	32.0 ± 3.0***	1229 ± 383**	26.5 ± 2.3***
F_4	HPMC + G + N	59.7 ± 2.0***	30.6 ± 7.3***	1074 ± 066**	28.3 ± 1.1***
F_5	HPMC + Lecithin	75.7 ± 3.4***	22.5 ± 2.8***	1759 ± 326	1.9 ± 0.5*
F_6	HPMC + Lecithin + N	77.9 ± 4.1***	21.1 ± 1.6***	1538 ± 601*	1.8 ± 0.1*
F_7	HPMC + Emulsion	62.8 ± 0.9***	25.7 ± 3.0***	1887 ± 146	0.9 ± 0.1**
F_8	HPMC + N (Emulsion)	63.2 ± 0.8***	21.0 ± 4.0***	1985 ± 389	0.8 ± 0.2**
F_9	HPMC + N (Liposome)	50.4 ± 1.6	37.0 ± 2.5***	2228 ± 657	2.6 ± 0.7

Non-active, F_1, F_3, F_5, F_7; **active**, F_2, F_4, F_6; **nano-active**, F_8, F_9

HPMC, hydroxypropyl methylcellulose; N, nisin 1mg/mL, i.e. ~ 10^4 IU; G, glycerol (30% w/w D.M.); Lecithin, 2.5% w/v; N (Emulsion), Nisin 50% encapsulated and 50% free-form; N (Liposome), nisin 100% encapsulated

Dunnett test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

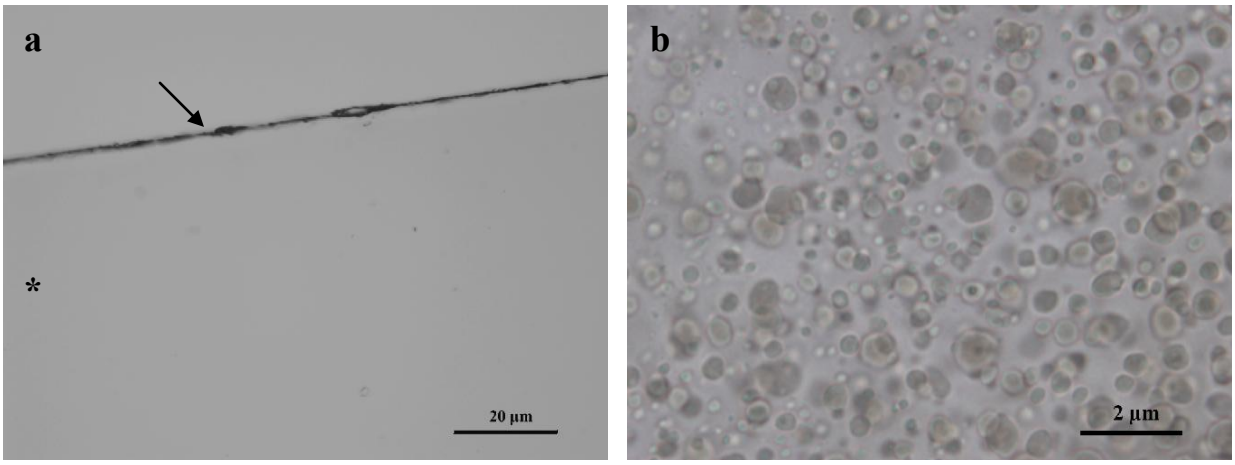


Figure 3: Ultra semi-fine micrographs (osmium staining) of (a) HPMC film and (b) nano-active HPMC film containing liposome encapsulated nisin. * represents the HPMC film while the arrow sign corresponds to film border

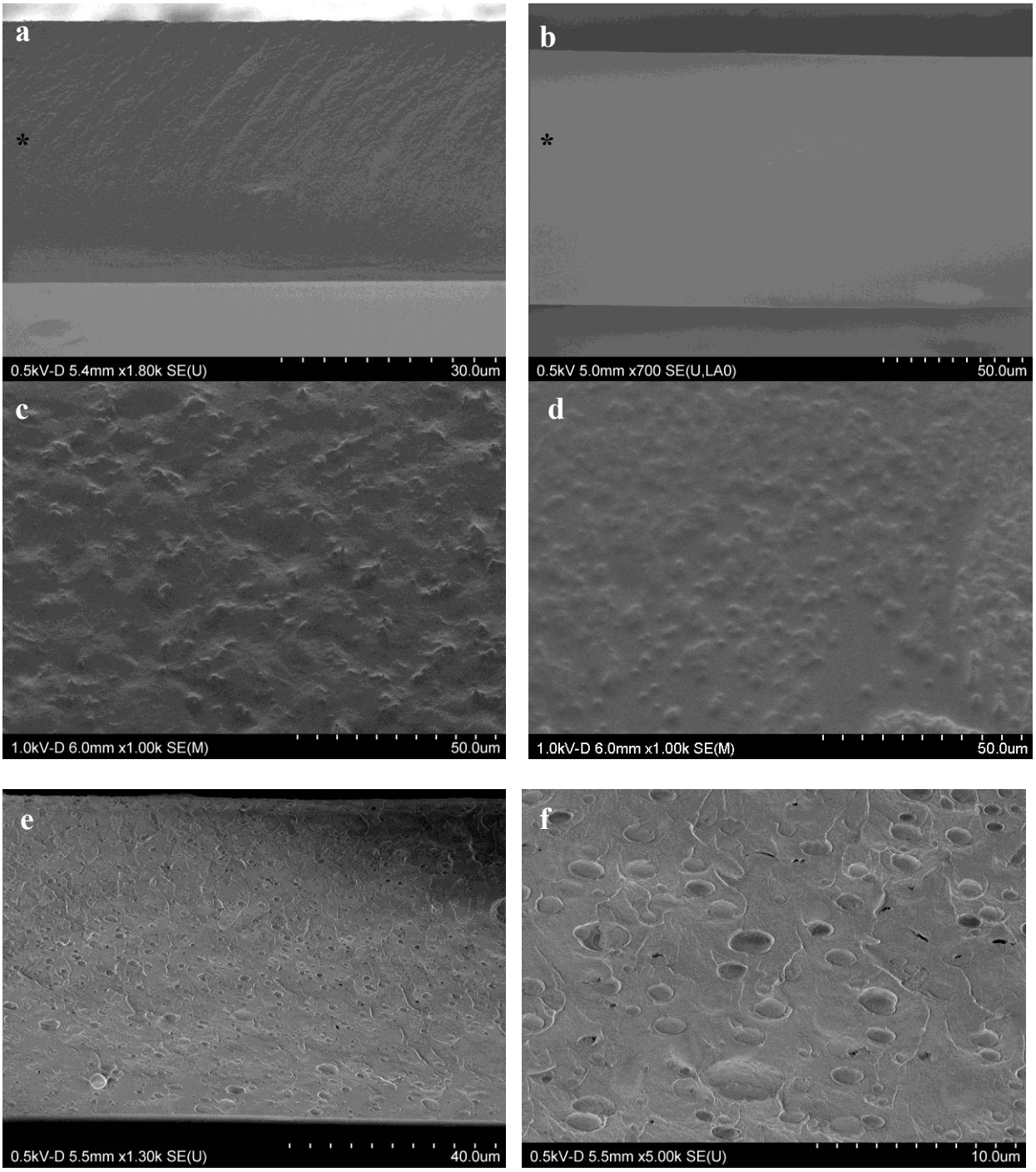
The HPMC film demonstrated clearly a continuous hydrocolloid matrix. However, the captured images for nano-active film (F_9) reflected the inner complexity generated in the film with homogenously dispersed liposomes on the surface of the dried biopolymer film. The size of the liposomes ranged from nano- to micrometer, which could be due to the aggregation/complexation between the liposomes during film drying. The complexity observed is associated with the discontinuities in the refractive index through the material in the different liposome–hydrocolloid interfaces. The size of the nisin encapsulating liposome alone or conjugated in the dried film is the main factor involved in the optical heterogeneity degree of the film matrix. Nevertheless, the representative image of nanoactive film surface structure revealed homogenous dispersion of liposomes embedded in biopolymer HPMC network (Figure 3b).

3.2.2. Topographic & morphologic study (scanning electron microscopy)

The permeability of films can be influenced by the composition, morphology and homogeneity of the coating matrix (Bilbao-Sainz, Avena-Bustillos, Wood, Williams & McHugh, 2010). Scanning electron microscopy (SEM) was used to characterize the topography and morphology of the composite biodegradable films. Figure 4(a, e) represents the cross-sections of the unfilled and liposomes reinforced HPMC films, respectively. The nanoliposomes encapsulating nisin appear lighter than the HPMC continuous matrix and, hence, are easily identifiable in the films. The HPMC cross-section displays a relatively smooth inner-structure (Figure 4a), which is even more smooth with glycerol plasticized nisin containing HPMC film (Figure 4b). Whereas the matrix in the nanocomposite films is rougher (Figure 4e, 6f). The nanoliposomes are evenly distributed within the polymeric matrix; however, hardly any of the nanoparticles are aggregated which shows good repulsion between lecithin liposomes resulting in homogenous dispersibility inside the biopolymeric films.

Considering topographic micrographs, the nanoliposome embedded films showed non-uniform surface with dome-shaped zones and holes (Figure 4 c, d; upper and lower surfaces respectively). These semi-spherical structures represented mainly the liposome structures half-implanted in HPMC matrix, in consequence to the drying process of films. Phospholipid-based lipsomes are well distributed through the film on both upper and lower sides. These physical surfaces may support lower WVP of HPMC-liposome encapsulated nisin (F_9) films compared to those with lecithin incorporation directly without nano-structurization (F_6). Liposome hindered

the transfer of water molecules inside the film; therefore, a homogeneous distribution of active liposme/emulsion would give the film a lower WVP. Similar results were previously mentioned for fat or essential oil incorporation in biodegradable films (Hambleton, Debeaufort, Bonnotte & Voilley, 2009; Sanchez-Gonzalez et al., 2009).



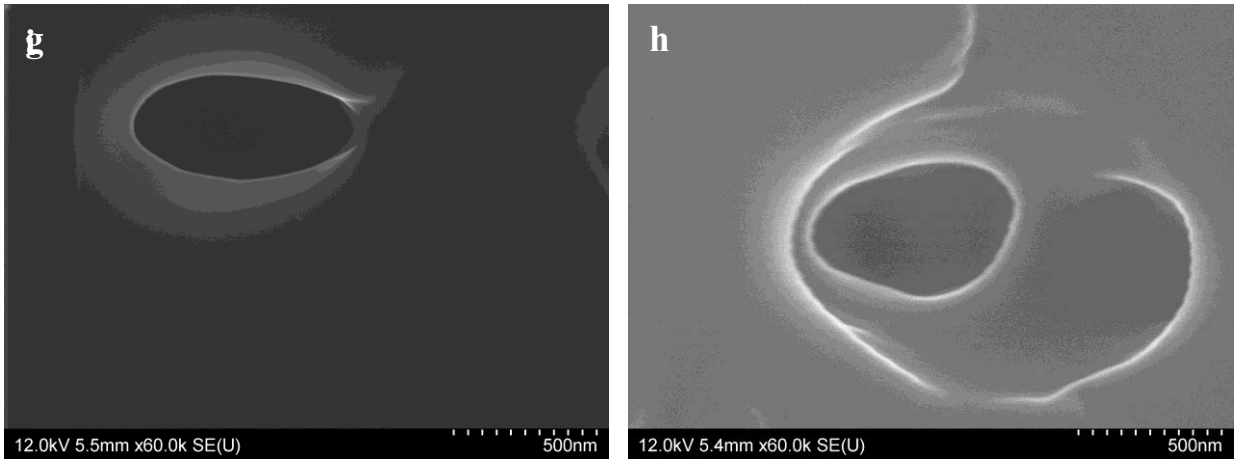


Figure 4: Micrographs of scanning electron microscope (SEM); cross-section view, (a) control HPMC film; (b) plasticized glycerol and nisin containing HPMC film; (e,f) embedded liposomes encapsulating nisin in HPMC film; (g-h) zoom micrographs to observe liposome degradation/lamella opening/ pore-formation by nisin; Surface-view, (c) and (d) upper and lower surface of nano-active films containing encapsulated nisin. * represent the film area as compared to support.

It had been suggested by previous studies that the fat matter is more concentrated at the air side, because during drying there is a film retraction that changes its structure, becoming denser thus the lipid migrates toward the air side (Hambleton et al., 2009). Indeed fat density is lower than the FFS aqueous phase, and thus fat tends to migrate toward the evaporated surface. However, as amphiphilic soya-phospholipids have density (1.04) more or less equivalent to the aqueous phase, it had resulted in similar appearance of liposome at both upper (Figure 4c) and lower (Figure 4d) surfaces. This reveals that very little creaming occurred during the film drying, possibly owing to the highly viscous effect of HPMC, which, furthermore, increased when drying progressed. Most probably the density, viscosity, gravimetric and evaporation pull equilibrated in a way that the dispersion of nisin encapsulating liposomes is quite homogenous in both inner and outer film structure.

This homogenous dispersion and non-floculation of liposmes fulfilled the prerequisite objective to encapsulate the nisin in liposome and further embed it in HPMC matrix to enhance its controlled release. Considering the antimicrobial mechanism of action, nisin is able to form

pores in the liposomal membranes, which had been previously explained for cell model membranes (liposome) and different bacterial species (Breukink et al., 2006; Breukink et al., 2000). Zoom micrographs had revealed interesting micrographs of pore-formation phenomenon in individual liposome embedded in HPMC after 30 days of storage (Figure 4g, h). Similarly, the representative micrograph had exposed the lamellar opening/degradation of the liposomes. Nisin encapsulating lipsomes are slightly enlarged probably due to the deformation forces that act during the polymer chain aggregation during the solvent evaporation. As nisin is amphiphilic in nature, thus it is encapsulated in both core and lamellar phases of liposomes. The slow degradation of multilamellar lipsomes and pore formation by nisin and storage time may possibly, as a consequence, play a role in controlled release of nisin both in the film or once transferred into the food system (Figure 1).

3.2.3. Microstructure (transmission electron microscopy)

For the confirmation of above mentioned microscopic observations, transmission electron microscopy (TEM) was carried out for nisin encapsulating liposomes before and after embedding/drying in HPMC films. TEM permits us to analyze the liposome distribution in emulsified films and to characterize the destabilization phenomena such as aggregation and/or coalescence due to the fabrication process (spread and dry) and due to the addition of the nisin. The micrograph of liposome embedded films (ultra thin slice of < 100nm) had indicated a homogenous dispersion without conjugation occurrence between liposomes inside the film (Figure 5a). HPMC matrix and difference of focused zone had shown different liposomes more or less identifiable but their homogenous distribution is quite obvious. In parallel, the nisin emulsion before drying process of FFS (swollen state) had revealed comparable results of dispersibilty proving sufficient electrophoretic repulsion between soya phospholipid nano-particles (Figure 5b). However, the representative micrograph revealed heterogeneity concerning the lamellarity of liposomes. Generally, the liposomes measuring above 100nm diameter belong to multilamellar group in contrast to unilamellar liposomes (<100nm) (Imran, Revol-Junelles, Martyn, Tehrany, Jacquot & Desobry, 2010b). These multilayer liposomes may improve the controlled release of nisin by providing numerous hindrances as lamellas.

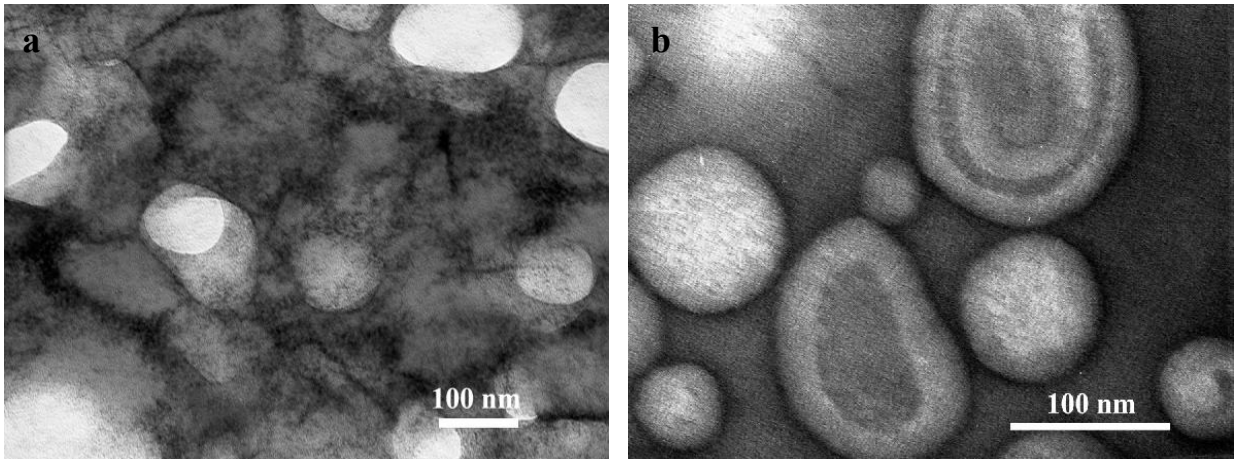


Figure 5: Transmission electron micrographs of (a) nano-encapsulated nisin embedded in HPMC film matrix and (b) nanoliposomes encapsulating nisin observed after fabrication by CCDS

3.3. Film thickness

Film thickness relies primarily on its composition (Table 1); however the use of pure nisin had not influenced the active film thickness as compared to control film (F1), stored at 50% RH and 20°C. These results had shown improvement by producing homogenous active films as compared to the previous study using Nisaplin® (heterogenous mixture of nisin, salt and milk solids)(Imran et al., 2010a). The observed increased thickness of films containing glycerol might be there because glycerol retain higher moisture content at the end of film drying due to its hydrophilic nature (Chen & Lai, 2008). Among the plasticizer, the use of soy lecithin alone or in combination with nisin (F_5, F_6) had significantly increased the film thickness to 77.9 ± 4.1 as compared to F_1 (Dunnett test, $p < 0.001$). However, the incorporation of soy lecithin in the form of nano-emulsion (F_7, F_8) resulted in significant decrease of thickness as compared to F_5 (Tukey test, $p < 0.01$). The active-nano film (F_9) had eventually decreased the size to 50.4 ± 1.6, which is non-significantly different than control film (Dunnett test, $p > 0.05$).

3.4. Tensile properties

Tensile strength (TS) indicates the maximum tensile stress that the film can sustain, ultimate elongation (%UE) is the maximum change in length of a test specimen before breaking, and Young‟s modulus (Y) is a measure of the stiffness of the film (Table 1). Values of mechanical properties obtained for non-active (HPMC) and active (HPMC + nisin) films agreed with previous study using similar 6 % (w/v) concentrated FFS (Imran et al., 2010a), in spite of

the fact that in the present study FFS was prepared in distilled water as compared to 35% alcoholic solution utilized earlier. The incorporation of the lecithin leads to softer, less resistant to break and less stretchable films. However, glycerol had shown an exception by increasing significantly the elongation as compared to control. The nano-active film (F_9) had shown significant improvement for TS as compared to lecithin active film (F_6). The treatment which contains the nano-encapsulated nisin had a better tensile strength and a better percentage of elongation than the treatment containing nano-emulsion nisin. Thus free nisin in HPMC network decreased the TS as shown also by F_2 in comparison to control. But treatment containing free nisin had better tensile strength and a better percentage of elongation than film containing liposomes. This could be explained by discontinuities in the polymer matrix introduced by the lecithin [at macro- (as such) and nano-scale (liposome)] incorporation and by changes in the polymer chain interactions when lipid components are present, which lead to a weak mechanical response. Similarly the stiffness of film differed non-significantly from F_1, when incorporated in the form of nanoliposomes/emulsion. Similar trends had been observed earlier for the plasticizers analyzing the mechanical properties of biopolymer films blended with glycerol and different fatty acids (Andreuccetti, Carvalho & Grosso, 2009; Imran et al., 2010a; Sebti, Ham-Pichavant & Coma, 2002). Thus different behavior can be attributed to the type of plasticizer (hydrophobic, hydrophilic) and particular processing of ingredients resulting in dissimilar interactions with the biopolymer network, which is also affected by the bio-additive nisin incorporated to add functionality to biodegradable coatings.

3.5. Water vapor barrier

The WVP allowed us to estimate the water barrier efficiency of HPMC edible films with or without encapsulated nisin. In the food industry, the difficulty with composite films is the relatively high water vapor permeability. Permeability in packaging is controlled by the diffusivity and solubility of water within the film matrix. Thus implementing food nanotechnology, new organization of firmly linked three dimensional networks can be fabricated to prevent diffusion of water in foodstuffs (de Moura, Aouada, Avena-Bustillos, McHugh, Krochta & Mattoso, 2009). In general, the plasticizers increased the WVP obtained (Table 2).

Table 2: Oxygen permeability, water permeability and water sorption properties (parameter values estimated from the A_w 0-0.95 curves at 25°C fitted by GAB model) of biodegradable composite films. Experimental DVS values were averaged and fitted by GAB model equation 3.

	Formulations	H_2O permeability ($g.m^{-1}.s^{-1}.Pa^{-1}$) ($x10^{-11}$)	O_2 permeability $m^3.m.m^{-2}.s^{-1}.kPa^{-1}$ ($x10^{-17}$)	*Xm*	*C*	*K*
F_1	HPMC	0.77 ± 0.03	7.4 ± 1.6	4.99	2.73	0.88
F_2	HPMC + N	0.87 ± 0.01	7.5 ± 3.6	4.73	3.07	0.90
F_3	HPMC + G	1.45 ± 0.10***	10.3 ± 1.3	8.37	1.49	0.89
F_4	HPMC + G + N	3.2 ± 0.11***	6.7 ± 2.2	10.2	1.21	0.91
F_5	HPMC + Lecithin	0.98 ± 0.10*	9.6 ± 2.0	5.04	1.84	0.81
F_6	HPMC + Lecithin + N	1.78 ± 0.04***	6.9 ± 0.1	4.69	1.98	0.85
F_7	HPMC + Emulsion	0.83 ± 0.03	16.3 ± 4.3**	5.29	1.87	0.83
F_8	HPMC + N (Emulsion)	0.93 ± 0.04	4.8 ± 0.9	4.86	2.11	0.83
F_9	HPMC + N (Liposome)	0.95 ± 0.10	10.8 ± 0.8	4.98	2.29	0.86

Non-active, F_1, F_3, F_5, F_7; **active**, F_2, F_4, F_6; **nano-active**, F_8, F_9

HPMC, hydroxypropyl methylcellulose; N, nisin 1mg/mL, i.e. ~ 10^4 IU; G, glycerol (30% w/w D.M.); Lecithin, 2.5% w/v; N (Emulsion), Nisin 50% encapsulated and 50% free-form; N (Liposome), nisin 100% encapsulated

Dunnett test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

The incorporation of lecithin directly (F_5) slightly increased the WVP. Moreover, nisin addition alongside lecithin significantly increased the WVP. Lipid compounds are recognized to improve the water barrier properties of biopolymer films due to their hydrophobic nature and to reduce the water sorption capacity of the films (Sanchez-Gonzalez, Chafer, Chiralt & Gonzalez-Martinez, 2010). The behavior of lecithin is different from other lipids (essential oils, fatty acids) as it comprises mainly of phospholipids which are amphiphilic in nature. Slight increase in WVP had been previously documented for gelatin-lecithin composite films (Andreuccetti et al., 2009). On the other hand, the incorporation of glycerol provoked a reorganization of the HPMC network which becomes less dense with a larger free volume, facilitating greater mobility and as a consequence greater WVP by the film matrix. Glycerol plasticized film had given significantly higher permeability (Dunnett test, $p < 0.001$), which may be caused by the higher number of available polar (-OH) groups in HPMC-glycerol composite films (Hambleton et al., 2009; Imran et al., 2010a).

However, our study confirmed that structuring lecithin into the form of nanoliposomes (F_7, F_8, F_9) decreased the WVP as compared to direct lecithin incorporation. The nano-sized particles are homogenously distributed which may have reduced the water diffusivity, due to better interaction of the hydrocolloid HPMC and polar-head groups of liposome outer membrane. The liposome/emulsion introduces an increase in the tortuosity factor for water migration in the matrix, thus increasing the distance travelled by H_2O molecules to permeate through the films. The tortuosity factor was higher in these nano-active films, which depends mainly on lipid ratio and particle (liposome) size (Pérez-Gago & Krochta, 2001).

3.6. Water sorption isotherms

Moisture adsorption isotherms (equilibrium moisture content on a dry basis versus water activity) were constructed for non-active, active and nano-active films at 25 °C (Figure 6). The water adsorption curves of HPMC-based films were sigmoid in shape, showing a slower increase in equilibrium moisture content until aw = 0.6, after which the increase in humidity led to a large mass gain, signifying a swelling phenomenon as water activity increased and promoted solubilization. This negligible convexity attained at low water activity was associated with type III sorption isotherm, an attribute of components rich in hydrophilic components and similar behavior was observed by other authors for HPMC-based films (Imran et al., 2010a; Villalobos, Hernandez-Munoz & Chiralt, 2006).

Sorption isotherm behavior specified that, at low a_w, HPMC-based films adsorb moisture with or without plasticizers, which also interacts with water molecules independently. As a_w increased, the water increasingly penetrated the HPMC network, partially dissolving them to form a gel, that initiated higher molecular mobility and component interactions, which had influenced the sorption pattern. The plasticized films containing glycerol (F_3, F_4) had the highest water affinity due to the large amount of hydrophilic groups. The difference in water holding capacity of glycerol containing films as compared to control films can be clearly observed after the initial phase of water molecules fixation at monolayer. The active (F_2, F_6) and nano-active (F_8, F_9) composite films had shown non-significant difference of moisture adsorption compared to pure HPMC films in the complete range of a_w, with least water-binding capacity for nisin encapsulated in nanoparticles emulsion. The lower water-binding capacity could be due to interactions between the liposomes and the hydrophilic sites of the HPMC chain, which substitute

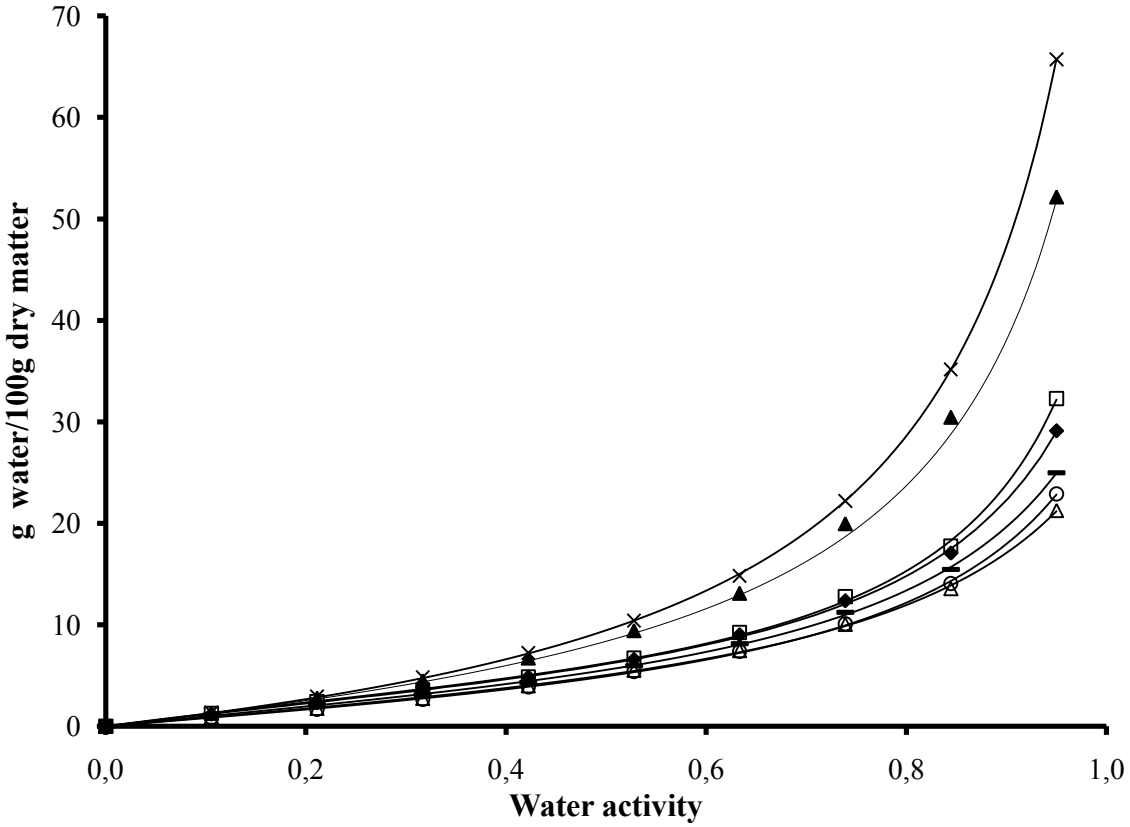


Figure 6: Moisture sorption isotherms of non-active, active (nisin) and nano-active (liposome-encapsulated nisin) biodegradable HPMC coatings at 25 °C and A_w 0-0.95. Experimental DVS values were averaged and fitted by isotherm equation; solid lines represent the GAB model fitted to the data. (♦HPMC film; (□HPMC + nisin; HPMC + glycerol (▲); HPMC + glycerol + nisin (×); HPMC + lecithin + nisin (○); HPMC + nisin-emulsion (△); and HPMC + liposomal nisin (▬)

the HPMC-water interactions that predominate in control films. Water adsorption in the high relative humidity range was identical for nano-emulsion or direct lecithin incorporation. Thus lecithin incorporation (direct or liposome) in HPMC film slightly decrease the moisture adsorption as compared to control film. However, the above-mentioned increase in WVP for HPMC-lecithin-nisin composite film could arise due to non-homogenous dispersion of lecithin as compared to nano-emulsions in HPMC native network, this irregularity could have weaken the film barrier responsible for WVP increase in plasticized active films.

The GAB model was used to fit the DVS data of the composite films. The value of monolayer water content (Xm) is of particular interest, as it refers to strongly adsorbed water to

specific sites and is considered as optimal value at which the film is most stable. Xm value of 4.99±0.19 g/100 g for HPMC film was observed (Table 2) which is close to the previous findings (Imran et al., 2010a; Villalobos et al., 2006). Glycerol increased the Xm values for plasticized films. For active films containing nisin in free or encapsulated form, the values of Xm were quite similar. The constant C, related to the total heat sorption, decreased markedly for glycerol plasticized films. Its decrease suggested that polyol may perhaps occupy sorption sites of polymer and in consequence reduced the bonding energy. The nano-active films, however, had higher bonding energy for water molecules as exhibited by control also.

3.7. Oxygen permeability

The addition of plasticizers increased the oxygen permeability (OP), as lecithin and the glycerol can modify the basic structure of the HPMC film network (Table 2). When nisin is mixed with glycerol there is a slight decrease in the OP, which is even lower than control film. These modifications are non-significant (Dunnett test, $p > 0.05$), because the changes in lateral chain gap of HPMC is not sufficient to alter oxygen molecule diffusion. In fact, the molecular diameter of oxygen is about 11 A° while that of H_2O is 2.4 A° (Hambleton et al., 2009). Even if plasticization of HPMC network occurs, most probably it is not sufficient to favor oxygen diffusion. The film containing the nisin in nano-emulsion form (free and encapsulated simultaneously) indicated the lowest permeability to oxygen. However, the film containing total nisin encapsulated in nanolipsosomes had a little elevated OP. Thus the presence of nisin may render the HPMC composite film matrix more compact and improve the barrier against the O_2, since the formulation F_8 indicated significantly lower OP as compared with F_7 (Tukey test, $p < 0.05$). Considering the barrier potential of cellulose derivatives against O_2 and H_2O molecules, similar tendency had been observed earlier when OP was not changed significantly for carboxymethylcellulose-based edible films when essential oils were added, whereas WVP was modified (Bifani, Ramirez, Ihl, Rubilar, Garcia & Zaritzky, 2007).

3.8. Optical properties

3.8.1. Transparency attributes

The color and transparency are pertinent properties since they have a direct impact on the consumer acceptability and quality of the packaged product. Indeed, a consumer will choose a

food product with a transparent package through which the food is visible to judge its visual quality, rather than food that has an opaque package. It is observed that HPMC film has given excellent transparency of film, while lecithin incorporation significantly decreased light transmittance (Figure 7).

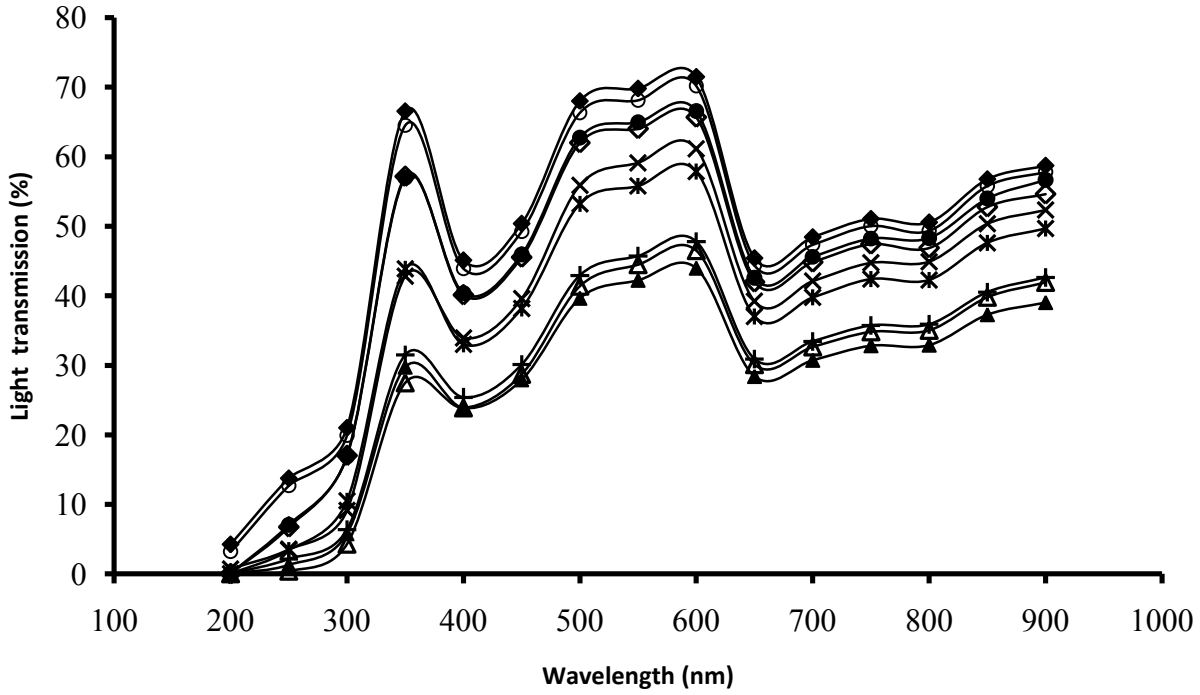


Figure 7: Transmission of light (%T) in UV, visible and NIR spectra through biodegradable films. Non-active films, (◆) HPMC film, (○) HPMC + glycerol, (▲) HPMC + lecithin, (×) HPMC + liposome; active-films, (◇) HPMC + nisin, (●) HPMC + glycerol + nisin, (△) HPMC + lecithin + nisin; nano-active films, (+) HPMC + nisin-emulsion (50% encapsulated), (✳) HPMC + liposomal nisin (100% encapsulated). Mean of triplicate analysis.

The treatment containing glycerol had lower transmission than the control film and it is further reduced non-significantly when nisin is added to HPMC film. Generally plasticizer are incorporated in composite films to improve the light transmission, however HPMC native films demonstrates such an exceptional transparent feature that composite film having plasticizers or active agents are relatively less transparent (Imran et al., 2010a) (Table 3). Indirectly, this supports the idea of using HPMC as biopolymer films for food. In nano-active films, the nanoliposome/emulsion incorporation reduced the film transparency; however the transparency

value at 600 nm had shown that it is significantly higher (Tukey test, $p < 0.001$) as compared to the use of lecithin at the macro scale (direct incorporation). It can be suggested that the structurization of lecithin into nano-scale liposomes for nisin encapsulation increases the transparency aspect. Comparing the transmittance of UV region with visible region, we observed that the percentage of transmittance was lower at 210 nm, signifying that composite films of HPMC have a good preventive ability against middle ultraviolet MUV radiations (200-300 nm).

Table 3: Whiteness index (WI), *a* and *b* color values and light transparency of composite active films as affected by nisin, plasticizer or both at macro and nano-scale

	Formulations	Transparency - log (T_{600}×X)	Color Whiteness Index (WI)	Chromatic coordinates *a**	*b**
F_1	HPMC	2.22 ± 0.02	32.0 ± 0.1	-0.18 ± 0.01	-0.38 ± 0.03
F_2	HPMC + N	2.13 ± 0.01	32.0 ± 0.1	-0.2 ± 0.01	-0.42 ± 0.05
F_3	HPMC + G	2.14 ± 0.01	31.7 ± 0.1	-0.18 ± 0.01	-0.37 ± 0.07
F_4	HPMC + G + N	2.05 ± 0.01***	32.2 ± 0.2	-0.21 ± 0.02	-0.39 ± 0.02
F_5	HPMC + Lecithin	1.76 ± 0.09***	32.1 ± 0.5	-0.06 ± 0.03***	1.7 ± 0.23***
F_6	HPMC + Lecithin + N	1.78 ± 0.01***	30.7 ± 0.3	-0.2 ± 0.01	0.39 ± 0.03***
F_7	HPMC + Emulsion	1.99 ± 0.01***	31.6 ± 0.4	-0.07 ± 0.05***	1.33 ± 0.21***
F_8	HPMC + N (Emulsion)	1.86 ± 0.03***	31.4 ± 2.3	-0.33 ± 0.04***	1.3 ± 0.38***
F_9	HPMC + N (Liposome)	2.06 ± 0.01***	27.6 ± 1.1	-0.23 ± 0.02	0.8 ± 0.01*

Non-active, F_1, F_3, F_5, F_7; **active**, F_2, F_4, F_6; **nano-active**, F_8, F_9

HPMC, hydroxypropyl methylcellulose; N, nisin 1mg/mL, i.e. ~ 10^4 IU; G, glycerol (30% w/w D.M.); Lecithin, 2.5% w/v; N (Emulsion), Nisin 50% encapsulated and 50% free-form; N (Liposome), nisin 100% encapsulated

Dunnett test, * p < 0.05 ; ** p < 0.01 ; *** p < 0.001

3.8.2. Color attributes

Addition of various compounds that structurally bind with film forming solution could change the native color of the film (Rhim, Gennadios, Handa, Weller & Hanna, 2000). The parameters L^*, a^* and b^* had been measured corresponding luminosity, variation between green and blue, and yellow and red respectively. The L^* parameter values ranged between 29 and 32 (data not shown), which is not significant variation. Similarly, the whiteness index (WI) due to the changes in diffuse reflectance provoked by light scattering had not shown significant variations. However, the parameter b^*, which gives the variation of color from yellow to red is

more critical: as one can observe data values range from -0.37±0.07 to 1.7±0.23 (Table 3). Treatments having a positive value contained lecithin, and therefore had a yellow color. The restructuring of the lecithin into nanoliposomes encapsulating nisin significantly decreased the value of the parameter *b* (Tukey test, $p < 0.001$). Moreover, as nisin gives a white color, it masks the yellow color of film. Therefore active films containing nisin were less yellow than non-active composite treatments.

3.9. Antimicrobial effectiveness

As an initial test for the antilisterial activity, different film forming solutions (corresponding MIC) were tested on inoculated tryptic soya broth (TSB-YE) medium. As expected, HPMC film forming solution alone had not shown antimicrobial action against *Listeria monocytogenes* as compared to control with no FFS added (Figure 8). Active HPMC film forming solution with nisin inhibited the listerial development up to 24 hours but afterwards there was gradual growth of bacteria due to nisin deactivation and bacterial resistance. The nano-active FFS with liposome encapsulated nisin had demonstrated lesser antimicrobial activity as bacterial growth started before 10 hours of incubation. Nevertheless the bacterial growth was reduced by half (peak values) as compared to control. This reduction in pathogen growth was due to nisin controlled release from liposome and probably as a result of active nano-liposomes interactions with bacteria. However nanoemulsion film forming solution (Figure 8A) with free and encapsulated nisin had indicated better control of pathogen as compared to 100% encapsulated nisin and free nisin. This effect is possibly due to the fact that free nisin controlled the initial bacterial growth burst and eventually fresh nisin release from liposome and nano-liposome interactions with *Listeria monocytogene*s could have improved the antimicrobial potential. The interaction of liposome with target cells can occur by adsorption onto the cell surface, fusion with the cell membrane, release of drug by micro-pinocytosis, or due to a specific or nonspecific endocytosis (Torchilin, 2005). To exhibit the actual potential of these nano-active films for improving control over *Listeria monocytogene*s, different films of 1 cm^2 were tested on inoculated tryptic soya agar (TSA) medium. As expected, non-active HPMC had not given any

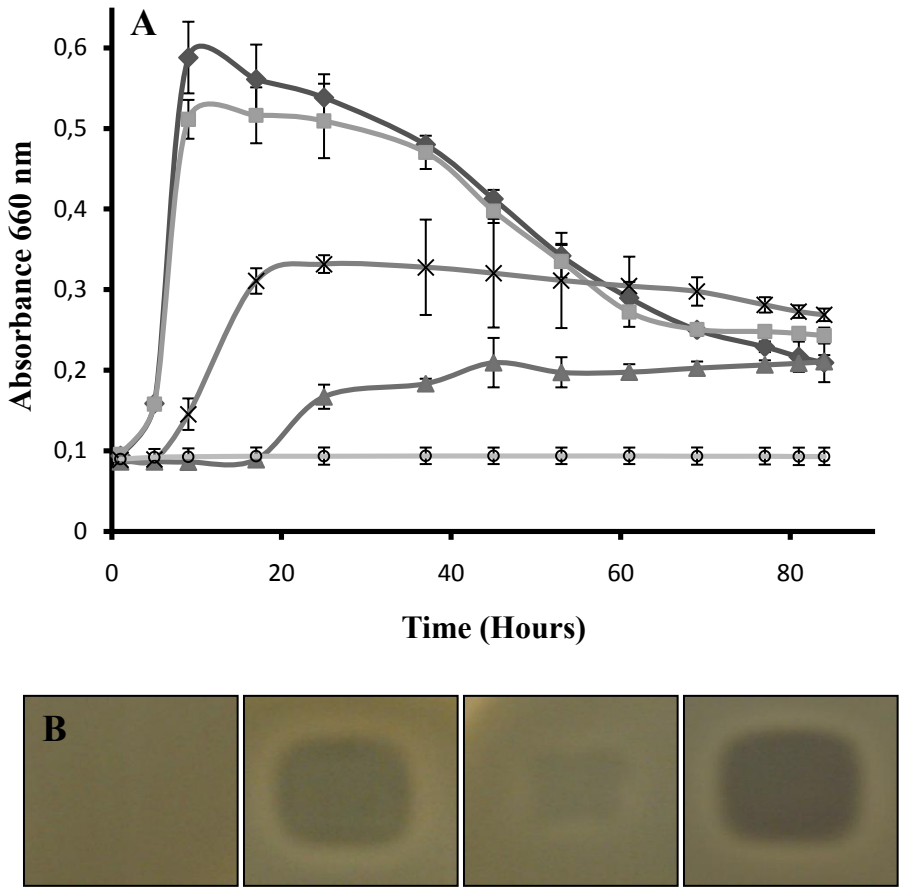


Figure 8: (A)- Growth kinetics of *Listeria monocytogenes* incubated with different film forming solutions at MIC dilutions for more than 3 days at 37 °C. The film forming solutions; (◆) control, (■) HPMC, (▲) HPMC+nisin, (×) HPMC+liposome encapsulated nisin, and (o) HPMC+ nisin emulsion (free and encapsulated nisin). **(B)-** The inhibition zone of the growth of *L. monocytogenes* CIP 82110^{T} inoculated in TSA-YE by nisin containing active films: from left to right – non-active HPMC film; nisin active HPMC film; liposome encapsulated nisin embedded HPMC film; nisin emulsion (free and encapsulated nisin) embedded HPMC film at 37°C with over-night incubation.

inhibition zone (Figure 8B) against *Listeria monocytogenes*. Active HPMC film with nisin produced inhibition zone slightly larger than actual covered size by film due to release and diffusion of nisin in the nutrient medium. The nano-active films had demonstrated different activity, as nanoemulsion film with free and encapsulated nisin had indicated better control of

pathogen as compared to 100% encapsulated nisin (Figure 8B). Thus, both the antibacterial tests in broth and solid medium had shown that the strategy of incorporating encapsulated and free nisin either directly in growth medium or embedded in bioactive films in-contact with nutrient medium could control efficiently the outburst of food pathogen.

4. Conclusion

Due to novel, formerly unknown, properties attributed to engineered nanoparticles many new products are introduced in the agro-food area. Liposomes are recognized as an excellent carrier for controlled release and target delivery of numerous active agents (Mozafari, 2006; Mozafari et al., 2008). However, one major limitation is that they have a tendency to leak and lose encapsulated components over time, moreover, gradual coalescence and destability may occur in food system (Laye, McClements & Weiss, 2008). Thus innovative approach of fusion of two concepts for improved bioavailability i.e. AMP nanoencapsulation and biopolymer immobilizing to formulate the next generation biodegradable films embedded with either active agent, nano-encapsulated active agent or both of them had resolved liposome delivery limitations. For active films, nisin had demonstrated no negative impact on transparency, thickness and water sorption behavior. However, the presence of plasticizer glycerol significantly improved the film elastic capacity, but negatively altered the permeability and tensile strength. For nano-active films, the results clearly showed that different physic-chemical properties including barrier (oxygen and water vapor permeability), color and transparency remained similar to native HPMC films and significantly improved than using lecithin directly without nano-scale restructuring. However, the tensile strength and percentage elongation were decreased significantly compared to the control film of HPMC. The results of sorption isotherms of water and GAB modelisation have shown a slight improvement in resistance to water adsorption when nisin was embedded as nano-emulsion in HPMC matrix. The observation of the structure and morphology of the film by SEM and TEM established the new concept of biodegradable packaging containing nano-encapsulated nisin may help to improve the slow release of the active agent. The antimicrobial results suggested that incorporation of nisin in nano-emulsion form (encapsulated and free) can possibly be an overall effective approach to control food pathogen without compromising the basic physico-chemical attributes of composite HPMC biodegradable films.

Acknowledgements

The authors acknowledge the technical assistance of Alain Kohler, Jacqueline Chanél, Dr. Jaafar Ghanbaja and Dr. Catherine Strazielle for their help to accomplish scanning and electron microscopy at SCMEM and Faculté de Medecine, Nancy, France.

References

Andreuccetti, C., Carvalho, R. A., & Grosso, C. R. F. (2009). Effect of hydrophobic plasticizers on functional properties of gelatin-based films. *Food Research International*, *42*(8), 1113-1121.

Benech, R. O., Kheadr, E. E., Lacroix, C., & Fliss, I. (2002). Antibacterial activities of nisin Z encapsulated in liposomes or produced in situ by mixed culture during Cheddar cheese ripening. *Applied and Environmental Microbiology*, *68*(11), 5607-5619.

Bifani, V., Ramirez, C., Ihl, M., Rubilar, M., Garcia, A., & Zaritzky, N. (2007). Effects of murta (Ugni molinae Turcz) extract on gas and water vapor permeability of carboxymethylcellulose-based edible films. *LWT - Food Science and Technology*, *40*(8), 1473-1481.

Bilbao-Sainz, C., Avena-Bustillos, R. J., Wood, D. F., Williams, T. G., & McHugh, T. H. (2010). Composite edible films based on hydroxypropyl methylcellulose reinforced with microcrystalline cellulose nanoparticles. *Journal of Agricultural and Food Chemistry*, *58*(6), 3753-3760.

Breukink, E., & De Kruijff, B. (1999). The lantibiotic nisin, a special case or not? *Biochimica et Biophysica Acta - Biomembranes*, *1462*(1-2), 223-234.

Breukink, E., & de Kruijff, B. (2006). Lipid II as a target for antibiotics. *Nature Reviews Drug Discovery*, *5*(4), 321-323.

Breukink, E., Ganz, P., De Kruijff, B., & Seelig, J. (2000). Binding of nisin Z to bilayer vesicles as determined with isothermal titration calorimetry. *Biochemistry*, *39*(33), 10247-10254.

Cao-Hoang, L., Chaine, A., Grégoire, L., & Waché, Y. (2010). Potential of nisin-incorporated sodium caseinate films to control Listeria in artificially contaminated cheese. *Food Microbiology*, 1-5.

Chen, C. H., & Lai, L. S. (2008). Mechanical and water vapor barrier properties of tapioca starch/decolorized hsian-tsao leaf gum films in the presence of plasticizer. *Food Hydrocolloids*, *22*(8), 1584-1595.

Davies, E. A., Milne, C. F., Bevis, H. E., Potter, R. W., Harris, J. M., Williams, G. C., Thomas, L. V., & Delves-Broughton, J. (1999). Effective use of nisin to control lactic acid bacterial spoilage in vacuum-packed bologna-type sausage. *Journal of Food Protection*, *62*(9), 1004-1010.

de Moura, M. R., Aouada, F. A., Avena-Bustillos, R. J., McHugh, T. H., Krochta, J. M., & Mattoso, L. H. C. (2009). Improved barrier and mechanical properties of novel hydroxypropyl methylcellulose edible films with chitosan/tripolyphosphate nanoparticles. *Journal of Food Engineering*, *92*(4), 448-453.

Debeaufort, F., & Voilley, A. (1995). Methyl cellulose-based edible films and coatings I. Effect of plasticizer content on water and 1-octen-3-ol sorption and transport. *Cellulose*, *2*(3), 205-213.

Degnan, A. J., Buyong, N., & Luchansky, J. B. (1993). Antilisterial activity of pediocin AcH in model food systems in the presence of an emulsifier or encapsulated within liposomes. *International Journal of Food Microbiology*, *18*(2), 127-138.

Ercolini, D., Ferrocino, I., La Storia, A., Mauriello, G., Gigli, S., Masi, P., & Villani, F. (2010). Development of spoilage microbiota in beef stored in nisin activated packaging. *Food Microbiology*, *27*(1), 137-143.

Fabra, M. J., Jiménez, A., Atarés, L., Talens, P., & Chiralt, A. (2009). Effect of fatty acids and beeswax addition on properties of sodium caseinate dispersions and films. *Biomacromolecules*, *10*(6), 1500-1507.

Fang, Y., Tung, M. A., Britt, I. J., Yada, S., & Dalgleish, D. G. (2002). Tensile and barrier properties of edible films made from whey proteins. *Journal of Food Science*, *67*(1), 188-193.

Hambleton, A., Debeaufort, F., Bonnotte, A., & Voilley, A. (2009). Influence of alginate emulsion-based films structure on its barrier properties and on the protection of microencapsulated aroma compound. *Food Hydrocolloids*, *23*(8), 2116-2124.

Han, J. H., & Floros, J. D. (1997). Casting antimicrobial packaging films and measuring their physical properties and antimicrobial activity. *Journal of Plastic Film and Sheeting*, *13*(4), 287-298.

Imran, M., El-Fahmy, S., Revol-Junelles, A. M., & Desobry, S. (2010a). Cellulose derivative based active coatings: Effects of nisin and plasticizer on physico-chemical and antimicrobial properties of hydroxypropyl methylcellulose films. *Carbohydrate Polymers*, *81*(2), 219-225.

Imran, M., Revol-Junelles, A. M., Martyn, A., Tehrany, E. A., Jacquot, M., & Desobry, S. (2010b). Active food packaging evolution: Transformation from micro- to nanotechnology. *Critical reviews in food science and nutrition*, *50*(9), 1-24. Article in press.

Joerger, R. D. (2007). Antimicrobial films for food applications: A quantitative analysis of their effectiveness. *Packaging Technology and Science*, *20*(4), 231-273.

Khosravi-Darani, K., Pardakhty, A., Honarpisheh, H., Rao, V. S. N. M., & Mozafari, M. R. (2007). The role of high-resolution imaging in the evaluation of nanosystems for bioactive encapsulation and targeted nanotherapy. *Micron*, *38*(8), 804-818.

Khwaldia, K., Banon, S., Desobry, S., & Hardy, J. (2004). Mechanical and barrier properties of sodium caseinate-anhydrous milk fat edible films. *International Journal of Food Science and Technology*, *39*(4), 403-411.

Laridi, R., Kheadr, E. E., Benech, R. O., Vuillemard, J. C., Lacroix, C., & Fliss, I. (2003). Liposome encapsulated nisin Z: Optimization, stability and release during milk fermentation. *International Dairy Journal*, *13*(4), 325-336.

Laye, C., McClements, D. J., & Weiss, J. (2008). Formation of biopolymer-coated liposomes by electrostatic deposition of chitosan. *Journal of Food Science*, *73*(5).

Marcos, J. F., & Gandia, M. (2009). Antimicrobial peptides: To membranes and beyond. *Expert Opinion on Drug Discovery*, *4*(6), 659-671.

Mastromatteo, M., Conte, A., & Del Nobile, M. A. (2010). Combined use of modified atmosphere packaging and natural compounds for food preservation. *Food Engineering Reviews*, *2*(1), 28-38.

Mozafari, M. R. (2006). Bioactive entrapment and targeting using nanocarrier technologies: an introduction. *In: Mozafari, M.R. (Ed.), Nanocarrier Technologies: Frontiers of Nanotherapy. Springer, The Netherlands*, 1-16.

Mozafari, M. R., Johnson, C., Hatziantoniou, S., & Demetzos, C. (2008). Nanoliposomes and their applications in food nanotechnology. *Journal of Liposome Research*, *18*(4), 309-327.

O'Sullivan, L., Ross, R. P., & Hill, C. (2002). Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie*, *84*(5-6), 593-604.

Pérez-Gago, M. B., & Krochta, J. M. (2001). Lipid particle size effect on water vapor permeability and mechanical properties of whey protein/beeswax emulsion films. *Journal of Agricultural and Food Chemistry*, *49*(2), 996-1002.

Rhim, J. W., Gennadios, A., Handa, A., Weller, C. L., & Hanna, M. A. (2000). Solubility, tensile, and color properties of modified soy protein isolate films. *Journal of Agricultural and Food Chemistry*, *48*(10), 4937-4941.

Roberts, R. F., & Zottola, E. A. (1993). Shelf-life of pasteurized process cheese spreads made from cheddar cheese manufactured with a nisin-producing starter culture. *Journal of dairy science*, *76*(7), 1829-1836.

Sanchez-Gonzalez, L., Chafer, M., Chiralt, A., & Gonzalez-Martinez, C. (2010). Physical properties of edible chitosan films containing bergamot essential oil and their inhibitory action on Penicillium italicum. *Carbohydrate Polymers*, *Article in press*.

Sanchez-Gonzalez, L., Vargas, M., Gonzalez-Martanez, C., Chiralt, A., & Chafer, M. (2009). Characterization of edible films based on hydroxypropylmethylcellulose and tea tree essential oil. *Food Hydrocolloids*, *23*(8), 2102-2109.

Sanjurjo, K., Flores, S., Gerschenson, L., & Jagus, R. (2006). Study of the performance of nisin supported in edible films. *Food Research International*, *39*(6), 749-754.

Sebti, I., Ham-Pichavant, F., & Coma, V. (2002). Edible bioactive fatty acid-cellulosic derivative composites used in food-packaging applications. *Journal of Agricultural and Food Chemistry*, *50*(15), 4290-4294.

Taylor, T. M., Bruce, B. D., Weiss, J., & Davidson, P. M. (2008). Listeria monocytogenes and Escherichia coli O157:H7 inhibition in vitro by liposome-encapsulated nisin and ethylene diaminetetraacetic acid. *Journal of Food Safety*, *28*(2), 183-197.

Taylor, T. M., Gaysinsky, S., Davidson, P. M., Bruce, B. D., & Weiss, J. (2007). Characterization of antimicrobial-bearing liposomes by zeta potential, vesicle size, and encapsulation efficiency. *Food Biophysics*, *2*(1), 1-9.

Torchilin, V. P. (2005). Recent advances with liposomes as pharmaceutical carriers. *Nature Reviews Drug Discovery*, *4*(2), 145-160.

Villalobos, R., Chanona, J., Hernandez, P., Gutierrez, G., & Chiralt, A. (2005). Gloss and transparency of hydroxypropyl methylcellulose films containing surfactants as affected by their microstructure. *Food Hydrocolloids*, *19*(1), 53-61.

Villalobos, R., Hernandez-Munoz, P., & Chiralt, A. (2006). Effect of surfactants on water sorption and barrier properties of hydroxypropyl methylcellulose films. *Food Hydrocolloids*, *20*(4), 502-509.

Wu, Y., & Wang, T. (2003). Phospholipid class and FA compositions of modified soybeans processed with two extraction methods. *JAOCS, Journal of the American Oil Chemists' Society*, *80*(2), 127-132.

Chapitre 7: Marquage fluorescent du lantibiotique nisine Z: Purification, caractérisation et l'évaluation du mécanisme de l'action contre *Listeria*

L'utilisation de la nisine comme alternative à l'emploi de conservateurs chimiques dans les aliments ainsi que l'augmentation du taux d'intoxications alimentaires sont deux des multiples raisons expliquant l'incroyable augmentation des travaux de recherche portant sur la nisine observée au cours de ces dix dernières années.

Les applications récentes de la nisine incluent soit son incorporation dans un emballage actif soit son intégration dans des systèmes de transport, et ceci afin de prolonger sa biodisponibilité (chapitres 3, 4 et 5). Dans ces deux cas de figure, les études de quantification de la cinétique de relargage nécessitent de pouvoir quantifier de façon précise la nisine. La localisation précise du mécanisme d'action de la nisine *in vivo* nécessite de pouvoir la localiser. Le marquage de la nisine par un composé fluorescent peut être une solution novatrice pour réaliser ces objectifs.

L'objectif majeur de l'étude présentée dans ce chapitre est d'optimiser la procédure de marquage par un composé fluorescent d'une préparation commerciale de nisine Z, préalablement purifiée, au niveau de son extrémité C-terminale, de réaliser sa purification et sa caractérisation par chromatographie liquide couplée à un spectromètre de masse (LC/MS). Afin de vérifier l'impact du marquage sur les potentialités antibactériennes de la molécule, le mécanisme d'action (mesure des fuites de K^+ et impact sur le potentiel membranaire) et le mode d'action de la nisine et de la nisine marquée ont été évalués vis-à-vis de *Listeria monocytogenes*, *L. innocua* et *L. ivanovii*. Par ailleurs, des études de microscopie ont été réalisées afin d'illustrer une application potentielle de la nisine marquée, qui est la compréhension de la localisation au niveau membranaire de son mécanisme d'action.

Chapter 7: Fluorescent labeling of the lantibiotic peptide nisin Z: Purification, characterization and assessment of anti-listerial mechanism of action

Applications of nisin as safe alternative for chemical reagents in food preservation (> 40 countries for over 50 years) and escalating rate of food borne illness outbreaks from ready to eat food are one of the many reasons for dramatic increase of research focusing nisin during past decade. In view of this perspective, nisin can play a vital role to bestow an elementary form of innate immunity to food stuffs, helping processors to enlarge their control over food pathogens long after manufacture during shelf-life (Cotter *et al.*, 2005).

The current trends of nisin applications include either in the form of active packaging or encapsulated in nano-carrier systems to ensure prolonged bioavailability (See thesis chapter 3, 4, 5). But, the prospective study of nisin detection, quantification (release from packaging and nano-delivery systems) and *in vivo* mechanism of action against potential pathogens requires precise localization and quantification approach.

The fluorescent labeling of nisin variant Z can be an innovative solution to the above-mentioned requirements, which is not reported till date. The main objective of the present study was to optimize the labeling procedure of the commercial purified nisin Z with fluorescent marker at its C-terminal, purification of labeled product and its characterization through recently developed method of liquid chromatography/mass spectrometry. Subsequently to verify the antimicrobial potential, the comparison of mechanism of action (membrane destabilization by potassium efflux and $\Delta\Psi$) and antimicrobial activity of labeled and unlabelled nisin was verified against *Listeria monocytogenes, Listeria innoccua* and *Listeria ivanovii*. Furthermore microscopical investigations against this lethal pathogen were carried out as initial application of labeled nisin to understand its bactericidal role.

Chapter 7

Fluorescent Labeling of the Lantibiotic Peptide Nisin Z: Purification, Characterization and Assessment of Anti-listerial Mechanism of Action

Muhammad Imran [1], Anne-Marie Revol-Junelles [1], Marlies de Bruin [2], Cedric Paris [1], Eefjan Breukink [2], Stéphane Desobry [1*]

[1] Laboratoire d'Ingénierie des Biomolécules, ENSAIA–INPL, Nancy Université, 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France

[2] Department of Biochemistry of Membranes, Bijvoet Centre for Biomolecular Research, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH, Utrecht, Netherlands

(Food Microbiology, En cours de soumission)

Abstract:

The peptide-derived antimicrobial nisin, the most studied class I bacteriocin, has been extensively used as a food bio-preservative. In recent years, the labeling of biomolecules by fluorescent markers has emerged as innovative methodology for bio-analytical purposes in food microbiology, medicine and pharmaceutics due to the advantages of precision, rapidity, wide detection limits, no extraction cost, in vivo recognition… In the present study, fluorescent labeled nisin Z fabricated had a molecular weight of 3717.3 Da determined by mass spectrometry, which was confirmed by ionic charge states $[M+3H]^{3+}$ (m/z 1240.1) and $[M+2H]^{2+}$ (m/z 1859.4) of labeled peptide as a result of bonding between carboxyl and amine groups of nisin Z and 5-(aminoacetamido) fluorescein (AAA-flu), respectively. The efficiency of labeling process was revealed by thin layer chromatography analyses while bicinchoninic acid assay demonstrated 10% yield of labeled product as compared to initial nisin concentration in the reaction mix. Comparison of intracellular K^+ leakage and transmembrane electrical potential ($\Delta\Psi$) against three listerial strains between unlabeled and labeled nisin demonstrated non-significant difference, which implies that purified nisin Z had endured the labeling process without any loss to its activity. Furthermore, *in vivo* activity of labeled nisin under confocal laser microscope revealed its localization at the septum of listerial cell division where the membrane-bound cell wall precursor lipid II is maximal suggesting specific pore-formation as bactericidal mechanism. Thus, nisin Z fluorescent labeling reported here possibly will provide the basis of new strategies in future to detect and quantify the nisin in complex foods, packaging, nano-delivery and *in vivo* systems.

Key words: fluorescent labeling, antimicrobial peptide, potassium efflux, lipid II, LCMS, *Listeria monocytogenes*

1. Introduction:

Discovered in 1928, one year prior to Alexander Fleming‟s discovery of penicillin, nisin is one of the oldest known antibacterial agents produced by *Lactococcus lactis* and has been extensively used as a food preservative (Delves-Broughton, 1996; Chatterjee *et al.*, 2005). It is considered as a member of class I bacteriocin (lantibiotics) due to its post-translationally modified structure from precursor peptides [prepeptide which contains 57 amino acid residues] (Lubelski *et al.*, 2008). The lantibiotics, family of lanthionine-containing peptide antibiotics, are subdivided into type A and B groups depending upon their straight-chain or globular structure, respectively. Nisin belongs to type A with overall positive charge [+4], its structure possessing 34 amino acids has amphipathic properties (Breukink and De Kruijff, 1999).

The escalating antibiotic-resistance dilemma has emphasized the urgent need for novel antimicrobial agents. Lantibiotic nisin is promising candidate to alleviate this setback due to its unique pore-forming activity against bacteria (Hsu *et al.*, 2004). The potent bactericidal activity (MICs nanomolar range) of nisin against Gram-positive bacteria and its low toxicity in humans explain the use of nisin as a preservative in the food industry or as an antibiotic in health care (Delves-Broughton, 1996; Breukink and de Kruijff, 2006). Nisin was approved as generally recognized as safe (GRAS) for food use by joint Food and Agriculture organization/ world health organization (FAO/WHO) in 1969. Later in 1983, this bacteriocin was added to the European food additive list as number E234 for regulatory purposes (EU, 2009). The widespread application of nisin against drug resistant strains and food borne pathogens *Clostridium botulinum* and *Listeria monocytogenes* (Jamuna *et al.*, 2005; Jamuna and Jeevaratnam, 2009), safe alternative for chemical reagents in food preservation (> 40 countries for over 50 years) and escalating rate of food borne illness outbreaks from ready to eat (RTE) food are one of the many reasons for dramatic increase of research focusing nisin during past decade. In view of this perspective, nisin can play a vital role to bestow an elementary form of innate immunity to food stuffs, helping processors to enlarge their control over food pathogens long after manufacture during shelf-life (Cotter *et al.*, 2005).

In spite of prolonged application of nisin as a bio-preservative in food sector, no reports of emerging resistance have thus far reported. This may be due to its exceptional double mode of action against pathogens. The primary mechanism, which requires micromolar concentrations,

nisin binds to the anionic lipids (Breukink and De Kruijff, 1999). The accumulation of nisin in the outer lipid monolayer of the target membrane drives aggregation of nisin monomers, which is followed by formation of short-lived pore-like structures. Relaxation of the pore-like structure can lead to translocation of the peptides across the lipid bilayer (Van Kraaij *et al.*, 1998; Breukink and de Kruijff, 2006). The secondary mechanism of action of nisin, which operates in the presence of Lipid II in the membrane results in stable pores because the affinity of nisin for Lipid II is much higher (2×10^7 M^{-1}) than the affinity of nisin for membranes containing anionic lipids (about 1,800 M^{-1}) [see in review (Breukink and de Kruijff, 2006)].

The different modes of membrane pore formation have generated the idea of encapsulating nisin in micro/nano-delivery systems to provide its controlled release in active form. In recent times, nanocarrier systems for the protection and delivery of bioactive agents are under intensive research and development by the pharmaceutical, cosmetic, and food industries (Mozafari *et al.*, 2008). Furthermore, the well established therapeutic potential against gastrointestinal infections and relatively low cytotoxicity towards goblet cells and enterocytes is leading the present research towards site-specific delivery of nisin formulations (Maher *et al.*, 2009). However, little information is available on the precise quantification of nisin release into food/biological system to assure the control of food spoilage and food borne illness.

The future study of nisin detection, quantification and *in vivo* mechanism of action against potential pathogens requires precise localization and quantification approach. The mechanism of action has been studied either by fluorescent dyes, fluorescent indicators and insertion of fluorescent probes (tryptophan variants) in nisin (Breukink *et al.*, 1998; Budde and Jakobsen, 2000). Thus labeling of biomolecules by radioactive or fluorescent markers is one of the most frequent methodologies used for bio-analytical purposes in medicine, pharmacy and cellular biology (Sameiro and Gonalves, 2009). The fluorescent labeling of nisin variant Z is not reported till date. Further, this concept is yet to be applied in food preservation to improve its quality and shelf-life features. The main objective of the present study was to optimize the labeling procedure of the commercial purified nisin Z with fluorescent marker at its C-terminal, purification of labeled product and its characterization through recently developed method of liquid chromatography/mass spectrometry. Subsequently to verify the antimicrobial potential, the comparison of mechanism of action (membrane destabilization by potassium efflux and $\Delta\Psi$) and antimicrobial activity of labeled and unlabelled nisin was verified against *Listeria*

monocytogenes, Listeria innoccua and *Listeria ivanovii*. Furthermore microscopical investigations against this lethal pathogen were carried out as initial application of labeled nisin to understand its bactericidal role by confocal fluorescence microscopy.

2. Materials and methods:

2.1. Materials

Nisin Z used in this study contained >90 % pure nisin [Honghao Chemical Co. (Shanghai, China), the formulation contains 3.84 x 10^6 I.U. per gram and 6.88 % moisture content]. N, N dimethylformamide (DMF), 1-Hydroxy-7-Azabenzotriazole (HOAt), N- (3-dimethylaminopropyl) -N'-ethylcarbodiimide hydrochloride (EDC), trifluoroacetic acid (TFA) and Bicinchoninic acid (BCA) reagents were obtained from Sigma Chemical Co. (Steinheim, Germany). 5-(aminoacetamido) fluorescein (AAA-flu) and thin layer chromatography silica gel 60 RP-18 plates were purchased from Invitrogen (Oregone, USA) and Merck (Darmstadt, Germany) respectively. Nigericin and valinomycin (Sigma-Aldrich) were prepared in chloroform or in dimethylsulfoxide, respectively, to a final concentration of 10 mM. The ΔΨ was measured qualitatively with the fluorescent probe 3,3'-dipropylthiadicarbocyanine iodide [DiSC3(5)] (Sigma-Aldrich). All chemicals were of analytical grade.

Bacterial strains and culture conditions: *Listeria strains (L. monocytogenes* CIP 82110^T, *L. innocua* CIP 12511, *L. ivanoii* CIP 12510) were obtained from the public collection [Collection de l'Institute Pasteur (CIP)]. The strains were cultivated in trypticase soy broth (Biokar Diagnostics, Beauvais, France) supplemented with 6 g L^{-1} of bacto-yeast extract (Biokar) (TSB-YE). Incubation was performed at 37 °C overnight. All strains were stored in appropriate culture medium supplemented with glycerol (10%) at -30 °C and propagated twice before use.

2.2. Pretreatments to improve bacteriocin purification

The commercial semi-purified nisin Z with more than 90 percent purity was subjected to two purification steps to remove the impurities. In the first treatment step; 0.5-1 g nisin Z was dissolved in 200 mL water, then 200 mL chloroform was added. The white emulsion obtained after strong shaking was subjected to centrifugation at 3500 rpm for 5 min. The white solid (nisin) between two layers was separated and lyophilized (Lyophilizer - Avantek, France).

Subsequently to extract the pure nisin, lyophilized pellet was dissolved in HPLC mobile phase A. The mobile phase A and B were prepared containing water:acetonitrile:TFA as 90:10:0.1 % (v/v) and 10:90:0.06 % (v/v) respectively. Semi-preparative liquid chromatography was performed using BIOCAD® 700E perfusion chromatography work station. Chromatographic separation was performed on Apollo C-18 (250mm x 22mm x 5 μm) reverse phase column (GRACE®, Darmstadt, Germany). The column was equilibrated with mobile phase A, and 5 mL of the samples (dissolved in mobile phase A) were injected. Elution was performed by 45-min programme at a flow rate of 5 ml/min as follows: a linear gradient ranging from 10 % to 35 % (v/v) mobile phase B for 10 min, 35 % to 42 % (v/v) mobile phase B in 20 min, maintained up to 100 % B for 5 min, and column was re-equilibrated with 10 % B for 10 min in the end. After multiple HPLC runs, the fractions corresponding to major peak of nisin (retention time 15.1 min) were collected, lyophilized and stored at –20 °C until further use.

2.3. Synthesis of fluorescent labeled-nisin

The fluorescent marker 5-(aminoacetamido)fluorescein (AAA-flu) was coupled with carboxyl group of nisin Z via a HOAt/EDC coupling in 100 μl DMF. The reaction mix containing 50 nmol peptide, 50 nmol AAA-flu and 60 nmol of both EDC and HOAt was incubated overnight at 25 °C. The ratio of AAA-flu to nisin Z was varied between 10:1 and 1:10 for improving the labeling efficiency. The labeled nisin was purified from the reaction mixture, after evaporation of the DMF, using reversed phase (C18) HPLC as described above for bacteriocin purification. Fractions containing labeled nisin were collected, lyophilized and stored at –20 °C until further use.

2.4. TLC analysis

To verify and compare the labeling productivity after overnight incubation, the reaction mixtures with different AAA-flu to nisin Z ratio were analyzed by thin-layer chromatography (TLC silica plates, 60 RP-18 F, Merck) using water:acetonotrile:TFA (50:50:0.05%, vol/vol/vol) as the solvent. Samples were spotted on TLC plate (5- by 10 cm), air dried and run until the solvent front was roughly 1-2 cm from the top. Chromatography was performed in 15 × 20 × 4 cm vertical glass chambers pre-equilibrated with 100 mL of developing solvent and containing one sheet (15 × 20 cm) of filter paper to maintain a constant solvent atmosphere. Labeled spots

or lanes were visualized under ultraviolet while protein spots were identified by ninhydrine (1%) spray.

2.5. LCMS characterization of labeled nisin

Liquid chromatography mass spectrometry (LCMS) was performed using Thermo electron HPLC system (Thermo electron corporation, Waltham, MA, USA) equipped with Finnigan LTQ MS (Thermo electron corporation, USA). Chromatographic separation was performed on RP-18 (150 mm x 2.1 mm x 5 μm) Alltima C-18 reverse phase column (Alltech) equipped with pre-column RP-18 (7.5 mm x 2.1 mm x 5μm) at 25°C. Elution was performed by 45-min programme at a flow rate of 0.2 ml/min as described before. MS detection was performed during the elution programme by PDA-MS coupled detectors. The conditions of ESI-MS were as follows: ionization mode, ESI (electro spray ionization); polarity, positive; Source gas (A.U.), shealth (40), auxiliary (5), sweep (5); spray voltage, 5 KV; capillary temperature, 275 °C; capillary voltage, 34 V; tube lens voltage, 220 V. The ion optics parameters were optimized by automatic tuning using nisin Z solution directly infused into the MS source. The full scan chromatograms were taken in a range from m/z 50 to 2000. Furthermore, zoom scans for multiple charge ions (2^+, 3^+) were carried out to obtain the charge state of nisin. The data acquisitions were performed using Xcalibur 1.4 software.

2.6. Efficiency of labeling

The concentration of labeled or unlabeled nisin was determined using the bicinchoninic acid (BCA) assay. In this assay, the BCA reagent was added to bacteriocin protein i.e. nisin. After incubation at 37 °C for 30 min, the absorbance at 562 nm was measured using a UV–Vis spectrophotometer (Ultrospec 4000 UV/visible, Pharmacia Biotech, UK). Bovine serum albumin containing 1 mg/ml protein was diluted and used as the protein standard.

2.7. Mechanism of action

2.7.1. ΔΨ measurement assay

Most of the bacteriocin act by creating pores in the plasma membrane through which there is leakage of cytoplasmic components which breakdown the transmembrane potential. To

evaluate the ΔΨ, cells were harvested in the log growth phase (O.D660= 0.6), washed twice with ice-cold 50 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) buffer containing 0.6 mM of KCl, glucose 0.2%, adjusted at pH 7.0 with KOH 40%, resuspended in the same buffer to 1/100 of their initial volume, and stored on ice.

The ΔΨ was qualitatively measured with the fluorescent probe 3,3'-dipropylthia-dicarbocyanine iodide [(DiSC3(5); Sigma-Aldrich]. Glucose-energized cells (to a final population of 2.5×108 cfu mL^{-1}) were added to quartz cuvette containing HEPES buffer and DiSC3(5) (5 μM). Next, after 2 min nigericin (1.5 nM) was added, which dissipates the pH gradient (ΔpH), than nisin (1× MIC) or valinomicyn (1.5 nM) were added. Fluorescence measurements were performed with a spectroflorometer FLX (Safas-Monaco, Monaco) at 643 and 670 nm for excitation and emission, respectively (Herranz *et al.*, 2001a; Herranz *et al.*, 2001b).

2.7.2. Potassium efflux determination

The release of intracellular content allows evaluation of membrane destabilizing activity by different antimicrobial compounds. In contrast to fluorescent markers, the ubiquity of inorganic potassium ions allowed us to avoid preliminary loading of the cell with high concentrations of fluorescent dyes to determine membrane destabilization by labeled or unlabeled nisin Z. Stationary-phase cells were harvested by centrifugation (10,000×g at 4°C, 15 min). The pellets were washed three times with tryptone-salt (TS 0.9%) supplemented with glucose (1%). Cells were resuspended in TS-glucose to a final population of 2.5×10^8 CFU mL^{-1}. Nisin or labeled nisin or the same volume of buffer without the peptide was added to a final concentration corresponding to MIC. Cells were incubated with labeled or unlabelled nisin for 60 min at 30°C. The cells were harvested by centrifugation (10,000×g at 4°C, 15 min), and the concentration of the potassium of the supernatant was determined with the atomic absorption spectrophotometer 1100 (Perkin-Elmer, Courtaboeuf, France).

2.8. Transmission electron microscope

As sample preparation, overnight cultures of *L. monocytogenes* CIP 82110 were collected by centrifugation at 4°C for 1-3 min at 11000 rpm. Then the cells were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH. 7.2 (CAC), and kept on ice for 1-2 h.

Afterwards, the bacterial cells were washed three times in CAC, fixed in 1% osmium tetroxide and 5% potassium bichromate (1/1) in CAC at room temperature, and washed 3 times with Milli-Q water. Contrast coloring was performed with 0.4% uranylacetate, and rehydration with a 100, 96, 70 and 50% ethanol solution series. The bacterial cells were embedded in Epon synthetic resin and sliced on ultra-microtome. The thin sliced cells were examined using a transmission electron microscope (TEM: Philips CM-20) at an operating voltage of 200 KV.

2.9. Confocal laser scanning microscope (localization of nisin at *Listeria* surface)

Different cultures of *L. monocytogenes* CIP 82110, *L. innocua* CIP 12511 and *L. ivanoii* CIP 12510 grown overnight were diluted 1:30 and incubated again at 37°C for about 3-4 h to an optical density (660nm) between 0.6 - 0.8. After that, fluorescein-labeled nisin (0.5 µg mL^{-1}) was added to 0.5 ml culture and the cells were incubated under above-defined conditions for another 10 min. The cultures were centrifuged at 10000 rpm for 3-5 minutes, and then the cell pellet was washed in saline buffer, fixed in 4% paraformaldehyde (PFA) and kept at room temperature for 15-30 min. After three washes in saline buffer, the cells were resuspended and 4 µL bacterial suspensions were added on 1% low-melting-point agar. Cells were inspected with an Olympus Fluoview FV10i confocal laser scanner microscope (excitation wavelength 488 nm, detection between 500 and 550 nm), and images were obtained by FV10i 1.2 software.

2.11. Statistical analysis

Statistical analyses were carried out by using the software KyPlot version 2.0. For comparison a parametric multiple Tukey test ($p \leq 0.05$) was performed.

3. Results and discussion

3.1. Bacteriocin purification

Commercial pure nisin was purchased from Honghao chemicals Co. (Shanghai, China) and characterized initially by LCMS. The relative area percentage on PDA and MS total ion chromatogram (TIC) chromatogram indicated that nisin purity was higher than 90 %. The mass spectrum for the largest peak showed that this molecule was detected as m/z 1111.34 and 1666.33, which corresponded to $[M+3H]^{+3}$ and $[M+2H]^{+2}$, respectively. Therefore, the molecular

mass of the bacteriocin was determined to be 3331 which agreed completely with molecular mass of nisin Z. However, two tiny shoulder peaks corresponded to m/z 1116.66 and 1045.28 respectively for ions equivalent to $[M+3H]^{+3}$. These are most probably the degenerated or similar peptides produced during fabrication of nisin Z at industrial scale. To obtain the pure nisin Z, two steps purification process as explained above was carried out by semi-purified C-18 column (Figure 1). Impurities other than protein were easily separated by chloroform extraction which was further purified for specific peptide by semi-preparative HPLC. Thus the single major peak in the nisin Z formulation corresponding to 3331 molecular mass was purified for subsequent labeling experimentation.

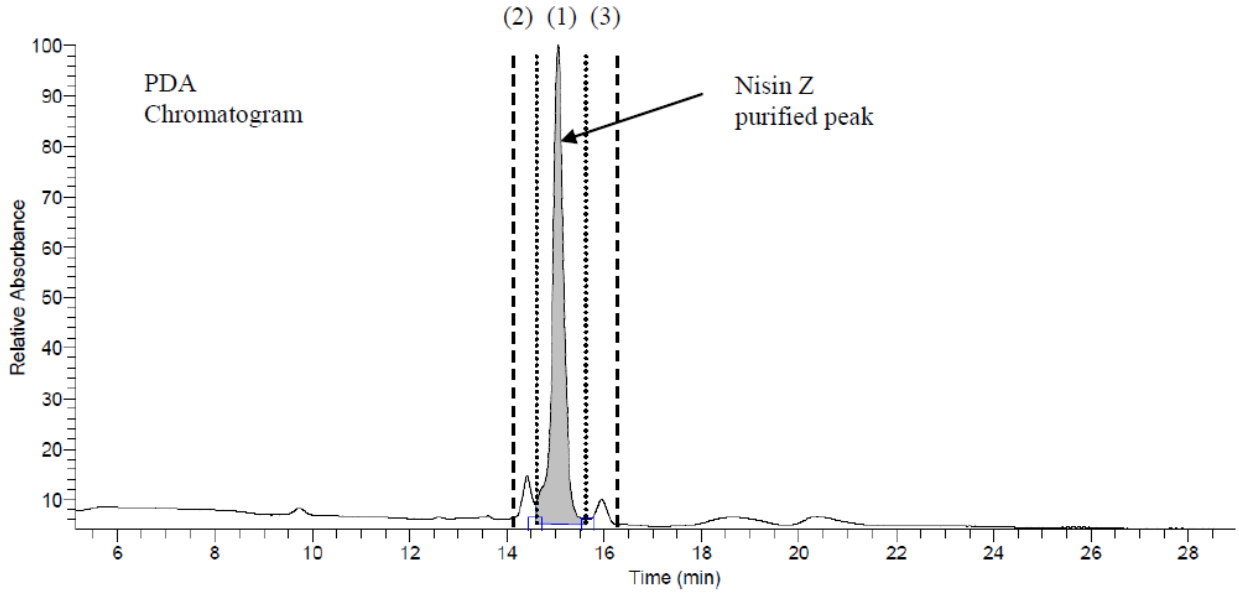


Figure 1: Purification of nisin Z major peak 1 by semi-preparative liquid chromatography. (1): nisin Z identified peak by LCMS detected as $[M+3H]^{3+}$ (m/z 1111.34) and $[M+2H]^{2+}$ (m/z 1666.33) ions (2, 3): tiny shoulder peaks of impurities corresponding to m/z 1116.66 and 1045.28 as $[M+3H]^{3+}$ ions.

3.2. Fluorescent labeling of nisin

The C-terminal fluorescent labeling of nisin was selected over N-terminal alteration to direct the extent of labeling to a 1:1 molar ratio. The primary structure of nisin includes only one carboxylic acid at the C-terminal with no internal glutamic acid residues. On the other hand, nisin

has four amine groups which are reportedly implicated in the bacterial membrane perturbation (Breukink and De Kruijff, 1999) and therefore modification through amine fictionalization was eliminated. The modification of nisin at N-terminal with polyethylene glycol was reported using relatively harsh organic solvents (DMSO) at pH 8, though it had ruined the antimicrobial activity of nisin (Guiotto *et al.*, 2003). In the present study, the AAA-flu was attached to C-terminal of nisin Z using standard coupling procedure. EDC is commonly utilized as a carboxyl activating agent for amide bonding with primary amines. EDC and HOAt have been used in peptide synthesis and cross linking proteins to specific agents as cross-linker and coupling agents respectively. Thus probing of nisin with AAA-flu produced fluorescently labeled-nisin with a covalent bond at C-terminal (Figure 2).

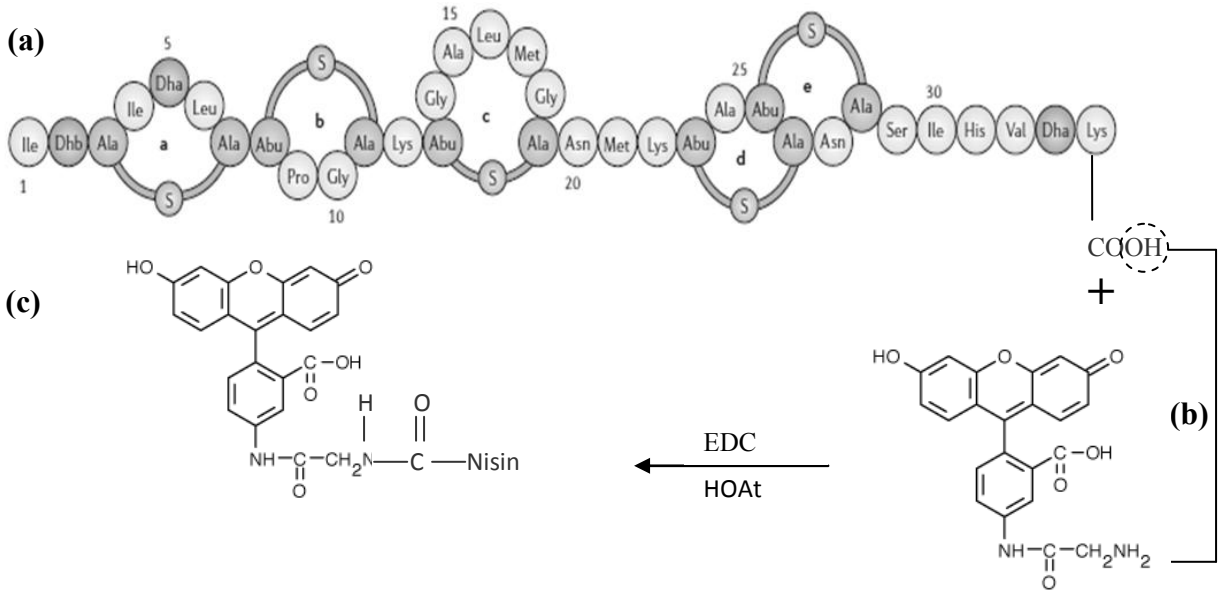


Figure 2: Schematic representation of reaction illustrating the labelling of nisin at the carboxyl terminal with one step EDC/HOAt reaction in DMF buffer; a) in the structure of nisin Z, the five lanthionine rings are are marked a-e from the amino terminus; b) nisin formed a covalent bond with AAA-flu; c) fabrication of fluorescently labelled nisin catalysed by cross-linker and coupling agent

Thin layer chromatography (TLC) affinity assay was developed to observe the nisin labeling. The basics of this assay are that nisin Z is more or less stationary on TLC plate and the binding of nisin with AAA-flu will stop the migration of AAA-flu. In this experiment, AAA-flu migrated away from the baseline in the AAA-flu lane 1; whereas fluorescent marker was held down in the lane 3 due to bonding with nisin Z. The assay was carried out under UV light and ninhydrin staining which highlight the fluorescent marker and peptide presence respectively (Figure 3 A, B). Thus the lane 1 and 2 in both figures represent the fluorescent label (10 nmol) and nisin (5 nmol) observed by either under UV or ninhydrin staining, respectively. Whereas the lane 3 revealed a band at nisin position with both techniques, hence illustrating the presence of fluorescently labeled nisin (prevent migration of fluorescent marker). These results offer a direct way of observing the fabrication of labeled nisin.

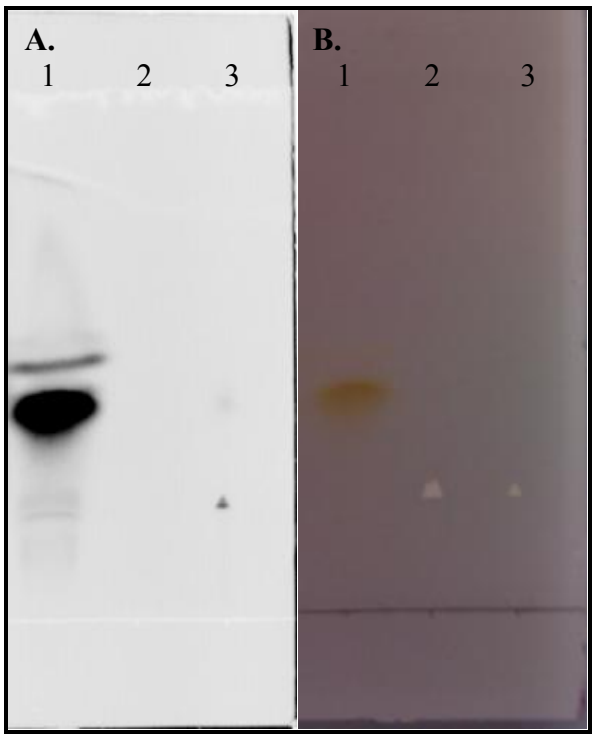
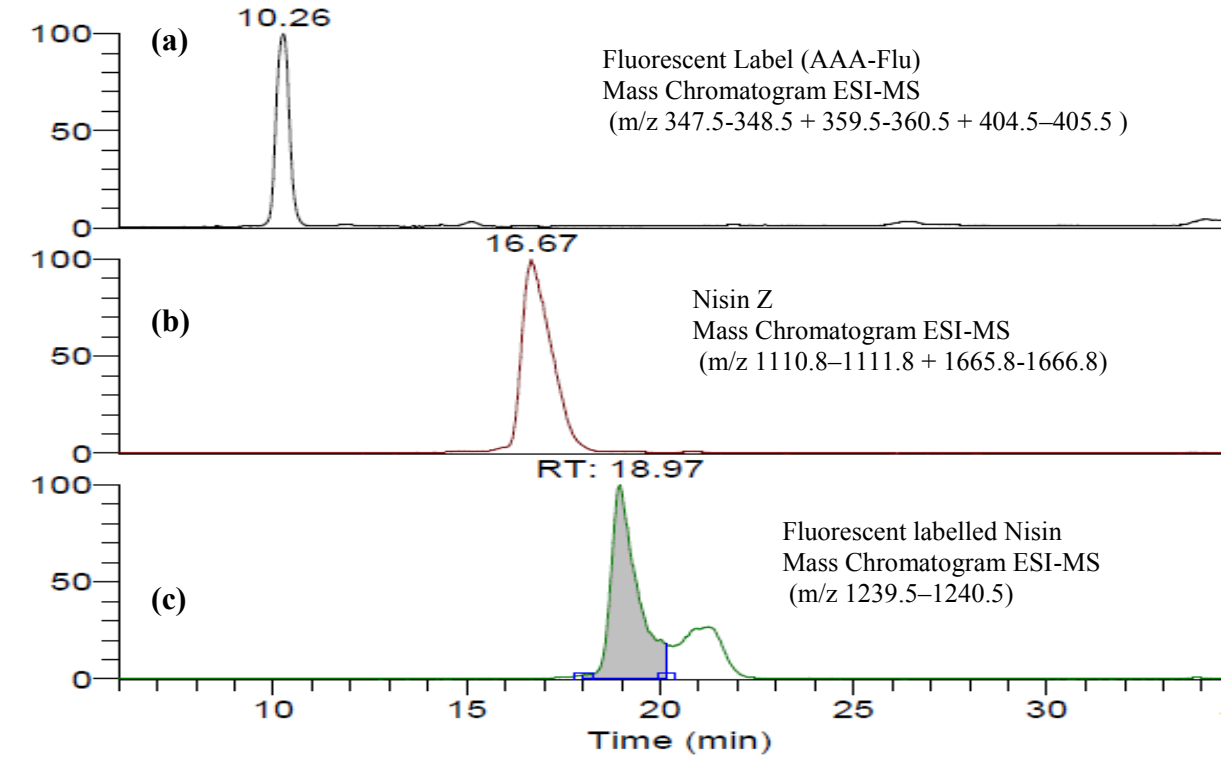


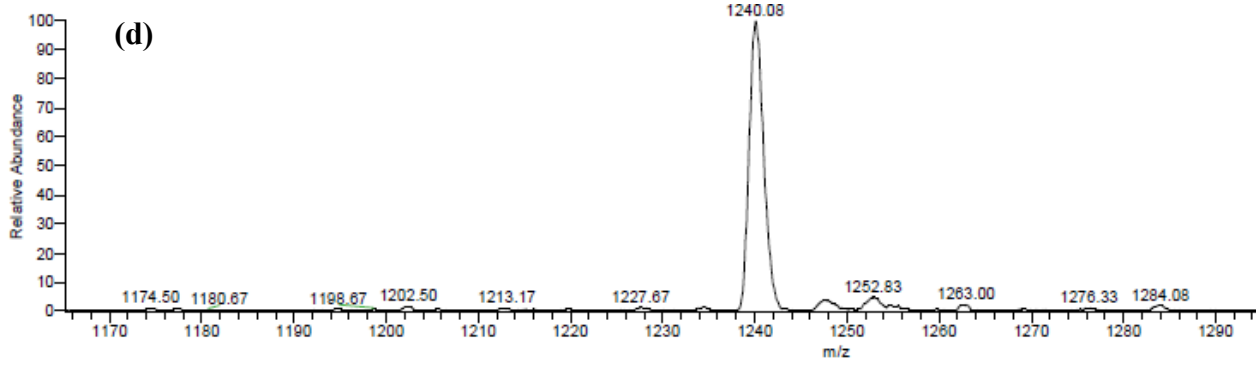
Figure 3: Thin layer chromatography assay of labeled nisin fabrication (A) TLC plate observed under UV light: Lane 1, AAA-flu 10 nmol; Lane 2, Nisin Z 5 nmol; Lane 3, labelled nisin 1.65 nmol. (B) Same TLC plate observed after ninhydrin (1%) staining for peptide nisin.

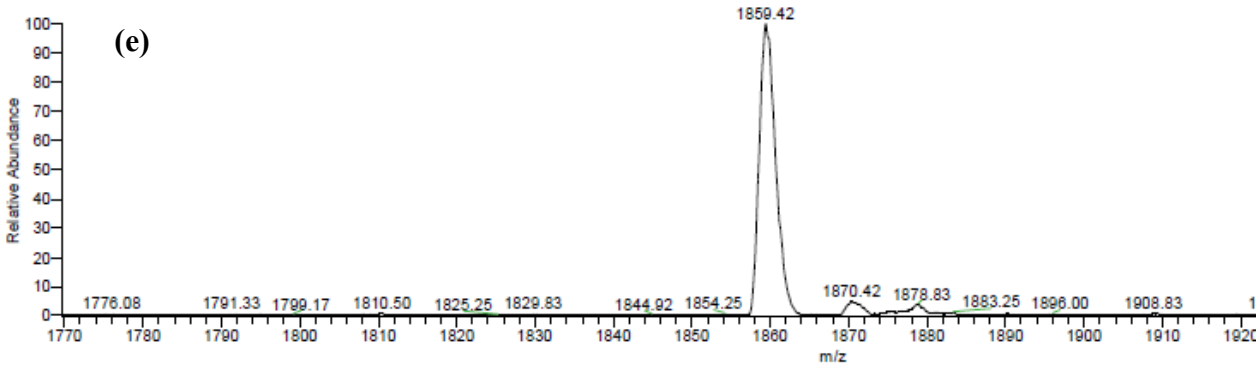
The fluorescent labeled nisin was successfully separated from EDC, HOAt, unreacted nisin and AAA-flu by reverse phase chromatography. To confirm that nisin was labeled, the molecular weight (MW) of the labeled nisin was established by LCMS. Nisin Z alone had a MW of 3331 Da, which was comparable to the results reported earlier (Mulders *et al.*, 1991). Bacteriocin-derived ions were scanned by extracting m/z 50 – 2000. Peak detected at 16.6 min in the mass chromatogram had been identified as nisin. The mass spectrum at the retention time showed that this molecule was detected as m/z 1111.34 and 1666.33, which corresponded to $[M+3H]^{+3}$ and $[M+2H]^{+2}$, respectively (Figure 4b). The AAA-flu was observed in electro-spray ionization (ESI) mass spectrum in three states of m/z as 347.5-348.5 + 359.5-360.5 + 404.5–405.5 (Figure 4a).

The fluorescently labeled nisin Z prepared had a molecular weight of 3717.3 Da by mass spectroscopy (Figure 4c). Zoom scan were done to confirm charge state of $[M+3H]^{3+}$ (m/z 1240.1) and $[M+2H]^{2+}$ (m/z 1859.4) ions of labeled peptide (Figure 4 f, g). The expected molecular weight of fluorescently labeled nisin Z fabricated by a reaction mix of nisin Z (3331 Da) and AAA-flu (404.38 Da) would be 3735.38 Da which reduces to 3717.38 with the loss of water molecule due to bond between carboxyl and amine groups of nisin and AAA-flu respectively. The % error between the theoretical and experimental value is negligible, which indicates successful labeling of nisin.

To determine the efficiency of labeling process, the concentration of labeled nisin in collected peaks corresponding to the product was measured by bicinchoninic acid assay for proteins. The results demonstrated 10 % yield of labeled product as compared to the total amount of nisin utilized in the reaction mixes, which were injected for purification on reverse-phase semi-preparative HPLC system.


(a)
10.26
Fluorescent Label (AAA-Flu)
Mass Chromatogram ESI-MS
(m/z 347.5-348.5 + 359.5-360.5 + 404.5–405.5)
(b)
16.67
Nisin Z
Mass Chromatogram ESI-MS
(m/z 1110.8–1111.8 + 1665.8-1666.8)
(c)
RT: 18.97
Fluorescent labelled Nisin
Mass Chromatogram ESI-MS
(m/z 1239.5–1240.5)
Time (min)


(d)
Relative Abundance
1240.08
1174.50
1180.67
1198.67
1202.50
1213.17
1227.67
1252.83
1263.00
1276.33
1284.08
m/z


(e)
Relative Abundance
1859.42
1776.08
1791.33
1799.17
1810.50
1825.25
1829.83
1844.92
1854.25
1870.42
1878.83
1883.25
1896.00
1908.83
m/z

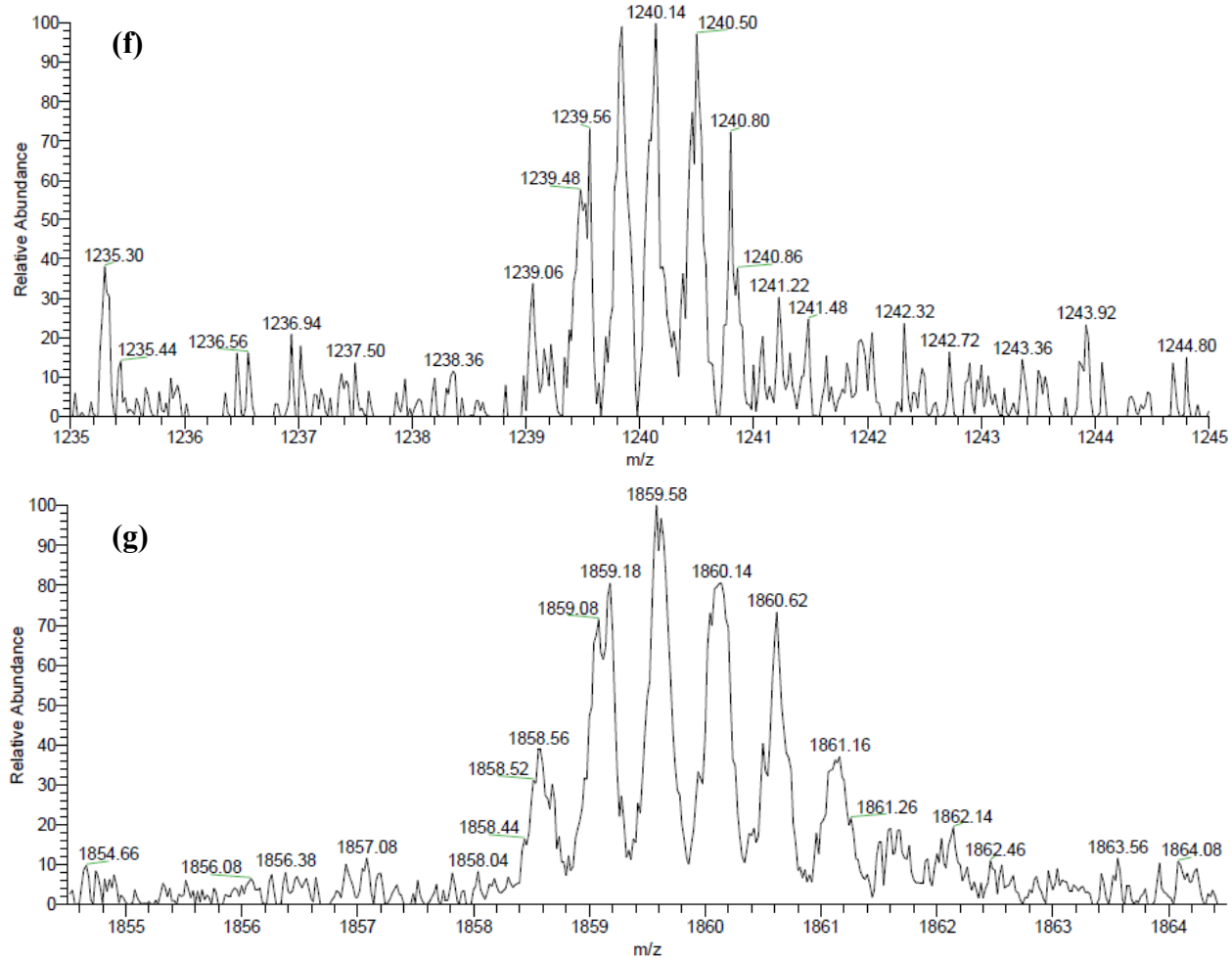


Figure 4: Detection of fluorescently labelled nisin Z by liquid chromatography / mass spectrometry. (a): Mass Chromatogram ESI-MS of fluorescent Label (AAA-Flu) molecular mass 404 (b) nisin Z peak at the retention time (16-18 min) showed detection as $[M+3H]^{3+}$ (m/z 1111.34) ions (c) fluorescently labelled nisin identified peak illustrated product detection as $[M+3H]^{3+}$ (m/z 1240.1) ions (d, e) mass spectrum illustrating fluorescent labelled nisin as $[M+3H]^{3+}$ (m/z 1240.1) and $[M+2H]^{2+}$ (m/z 1859.4) ions (f, g) zoom scan to confirm charge state $[M+3H]^{+3}$ and $[M+2H]^{2+}$ ions of labelled peptide

3.3. Comparison of labeled and unlabelled nisin

To verify that fluorescent labeling of nisin had not rendered any negative effect; the cell membrane perturbation and pore formation (intracellular K release) of labeled and unlabeled nisin were investigated against *L. monocytogenes* CIP 82110, *L. innocua* CIP 12511 and *L. ivanoii* CIP 12510.

The leakage of intracellular K^+ was investigated to recognize the transformed nisin capability of pore formation to induce efflux of internal vital ions pool. Control cells and cells treated with the bacteriocin (labeled and unlabeled nisin) were incubated, and the extracellular K^+ concentrations were measured by flame photometry. The addition of nisin (MIC value) to glucose-energized cells caused a dramatic loss of cellular K^+ (Figure 5). Measurement of the extracellular K^+ content indicated that the bacteriocin had induced massive leakage of K^+ from the cells, as the concentration outside had increased. The same experiment was carried out with labeled nisin; the K^+ content was significantly higher than control cells ($p \leq 0.001$). However, the results had demonstrated non-significant difference from unlabeled nisin ($p > 0.05$), so labeled nisin was revealed to make listerial strains permeable to inorganic K^+ ions by pore formation phenomenon (Figure 5).

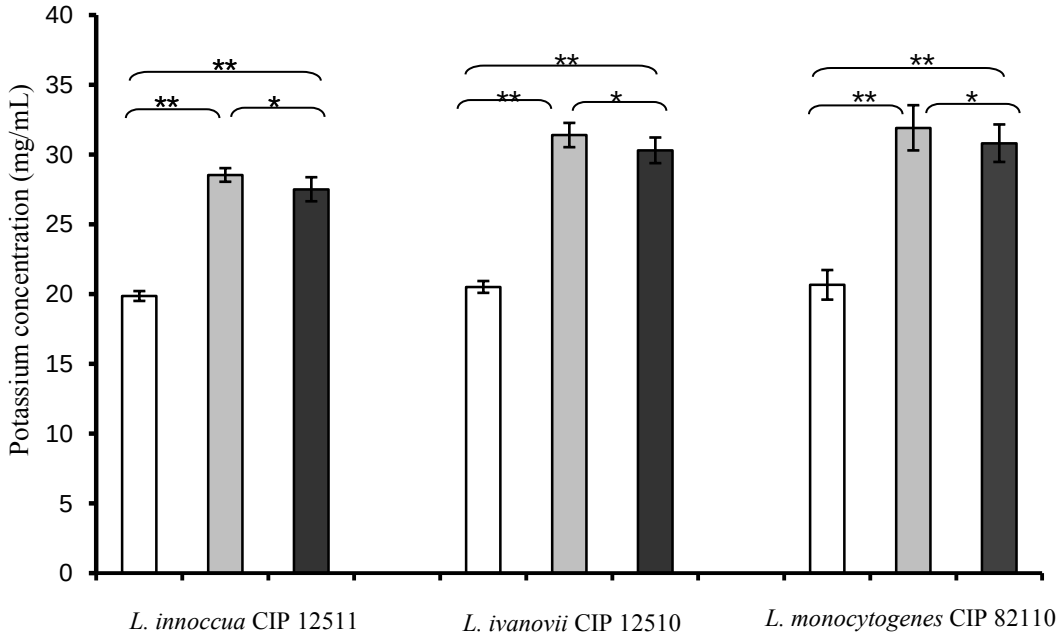


Figure 5: Measurement of the potassium concentration in the culture supernatant using atomic absorption spectrophotometery. Open bars without nisin, grey bars with nisin, black bars with fluorescent labelled nisin at the MIC values 0.66 µg/mL, 0.33 µg/mL and 0.78 µg/mL for *L. innocua* CIP 12511, *L. ivanoii* CIP 12510 and *L. monocytogenes* CIP 82110 respectively. Statistical analysis with Tukey test: single asterisk non significant difference ($p > 0.05$); double asterisk significant difference ($p \leq 0.001$).

In parallel, transmembrane electrical potential (ΔΨ) was observed with the fluorescent probe 3,3-dipropylthia-dicarbocyanine iodide [$DISC_3(5)$]. This probe measures the electrical potential gradient disruption across the cytoplasmic membrane of intact cells. Assays were carried out in the presence of nigericin to avoid the production of a transmembrane pH gradient (ΔpH). Generation of a ΔΨ (inside negative) subsequent to addition of the glucose to the cells resulted in quenching of the fluorescent signal. In the presence of the K^+/H^+ exchanger nigericin (1.5 nM), the cells were capable to maintain a maximum ΔΨ, which was dissipated by the addition of the K^+ ionophore valinomycin (1.5 nM) (data not shown). The addition of nisin to cells of *L. innocua* CIP 12511 and *L. ivanoii* CIP 12510 maintaining a maximum ΔΨ led to immediate depolarization of the cytoplasmic membrane as shown by the increase in fluorescence values (Annex 1 a, c). After the LCMS verification of nisin labeling, transmembrane electrical potential dissipation could be an indication of intact bacteriocin potential of labeled nisin intended for its mechanism of action against pathogenic bacteria. The signal obtained for ΔΨ value by using similar quantity of labeled nisin against cells of *L. innocua* CIP 12511 and *L. ivanoii* CIP 12510 had confirmed that labeled nisin potential was unharmed (Annex, chapitre 7).

All listerial strains tested had revealed bactericidal activity of labeled and unlabeled nisin on tryptic soy agar plate with serial dilutions of bacteriocins (data not shown). As observed with other lactic acid bacteriocins, the primary site of action of nisin appears to be the cytoplasmic membrane. The present facts also imply that the bacteriocin induces cell death by making sensitive cell membrane permeable, allowing for the efflux of K^+ ions. These act resulted in the dissipation of the ΔΨ components of the proton motive force. Nisin, a member of class I bacteriocins, appear to form stable pores that were all found to dissipate the transmembrane potential. It was reported earlier that bacteriocins from lactic acid bacteria acted by the common mechanism of depleting proton motive force (Chatterjee *et al.*, 2005). Thus the above results confirmed that nisin had endured the labeling process without any loss to its activity of bacterial membrane destabilization.

3.4. Fluorescently labeled nisin applications:

Due to broad-spectrum against Gram-positive bacteria, nano-molar MICs, no toxicity in humans and GRAS status; nisin is imperative as a preservative in the food industry. Thus fluorescein labeled nisin produced in the present study has significant value as it can be employed

to detect, calculate and quantify the nisin in complex food systems with precision and down-to very low concentration which is out of scope for traditional peptide quantification techniques. Considering food shelf-life, the diffusion and migration of labeled-nisin from food packaging and nano-delivery systems (peptide-encapsulation) can assist in predicting accurately the bioavailability of nisin during conservation (see chapter 8). Furthermore, the fluorescent labeled nisin could help to understand the mechanism of action of bacteriocin against pathogens e.g. listerial strains as discussed below.

3.4.1. Localization of labeled nisin at Listerial surface:

Different models have been suggested for the mechanism of pore formation by lantibiotics. These include the `barrel-stave' mechanism, which involves the initial accumulation of the peptide at the membrane surface through ionic interactions with the phospholipids head groups, thus nisin insertion is followed by aggregation. It is unidentified how many monomers are necessary to form a pore, but pore-formation is believed to be a dynamic process with peptides joining and leaving the pore complex (Garcera et al., 1993). Secondly, a wedge model has also been projected for short-lived pore formation by nisin. It states that the positively charged C-terminus, together with the bound lipids, enter into the membrane forming a wedge-like pore composed of multiple nisin molecules (Moll et al., 1997). However, more recently, an alternative model involving hijacking of lipid II for pore formation is reported. According to this model, nisin binds with cell wall precursor lipid II as a docking molecule for subsequent pore-formation, which had increased the membrane permeabilization activity of nisin by three order of magnitude (Wiedemann et al., 2001; Hsu et al., 2004; Breukink and de Kruijff, 2006).

Previous studies had revealed that the lipid II molecules are in excess at the division sites of bacteria to play their role of cell wall precursor. To study the fluorescent labeled nisin interaction with lipid II in different *Listeria spp.*, cell division pattern was studied with transmission electron microscope. As manifested by other rod-shaped bacteria, listerial strains also exhibited the cell division plane in the longitudinal centre of the cells (Figure 6). Afterwards, the different strains were incubated with labeled nisin and observed under confocal microscope. The listerial strains were sensitive to nisin, thus the nisin-lipid II patches were located near cell division sites of bacteria undergoing division process (Figure 7). These results are in accordance with the previous studies using fluorescently labeled vancomycin (antibiotic binding to lipid II

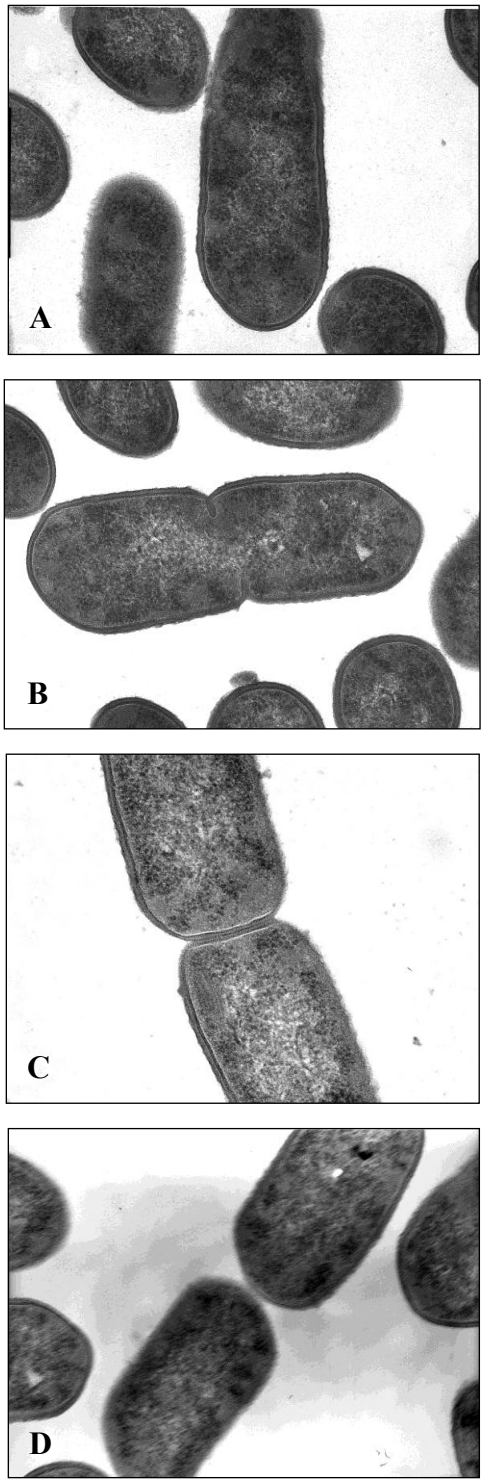


Figure 6: Transmission electron micrographs of *Listeria monocytogenes* CIP 82110, representative cells are clearly visible at different stages of division (A to B).


A
B
C

Figure 7: Localization of fluorescently labeled nisin on *Listeria spp.* by confocal microscope. The bacterial cells including *L. ivanoii* CIP 12510 (A), *L. monocytogenes* CIP 82110 (B) and *L. innocua* CIP 12511 (C) were incubated for 10 minutes with fluorescein labeled nisin (0.5 µg/mL) at 37°C. The arrow signs represent the nisin - lipid II binding at division sites of bacteria undergoing or already divided. The insets show the zoom images of representative bacteria for better visualization.

precursor) at the cell division sites of *Bacillus subtilis* (Daniel and Errington, 2003). The intensity signal of labeled nisin in *L. monocytogenes* (Figure 7B) was comparatively less as compared to other strains of *L.innocua* and *L. ivanovii*. A possible explanation for this effect could be the relatively restricted access of the peptide to lipid II based on how cell wall synthesis and bilayer fashion is functionally organized in different strains more or less susceptible to nisin. It has been reported earlier that higher nisin concentrations were required to dissipate both components of the proton motive force in different strains with dissimilar susceptibility to antimicrobial peptide mainly depending upon phospholipids composition in membrane (Verheul et al., 1997).

4. Concluding remarks

Fuelled primarily by the success of nisin i.e. virtual lack of toxicity for humans, nano-molar range broad-spectrum antimicrobial activity and exceptional resistance to thermal treatments, this bacteriocin is widely employed as a natural preservative in food industry. We consider it realistic that optimization, purification and characterization of nisin Z fluorescent labeling will not only form the basis of new strategies to detect and quantify the nisin bioavailability in food complex systems, food packaging (release kinetics) and nano-delivery systems (peptide encapsulation) but also facilitate us to understand the mechanism of action against food-borne pathogens. The fluorescently labeled nisin Z prepared had a molecular weight of 3717.3 Da by mass spectroscopy, confirmed by charge state of $[M+3H]^{3+}$ (m/z 1240.1) and $[M+2H]^{2+}$ (m/z 1859.4) ions of labeled peptide as a result of bond between carboxyl and amine groups of nisin Z and AAA-flu respectively. The efficiency of labeling process was revealed by

TLC analyses while bicinchoninic acid assay demonstrated 10 % yield of labeled product as compared to initial nisin concentration in reaction mix. Comparison of intracellular K^+ leakage and transmembrane electrical potential ($\Delta\Psi$) against three listerial strains between pure and labeled nisin demonstrated non-significant difference, which implies that nisin had endured the labeling process without any loss to its activity. As one of the applications of fluorescein labeled nisin, confocal microscopic study against *L. monocytogenes, L.innocua* and *L. ivanovii* successfully demonstrated the peptide-lipid II interaction at the cell-division sites as possible mechanism of action against this food borne pathogen. To this end, use of fluorescent labeled nisin Z in food applications might be of considerable advantage over using traditional detection/quantification/mechanistic studies. In these respects, natural antimicrobial peptides, both labeled and unlabeled, merit further in-depth scientific attention.

Acknowledgements:

We appreciatively acknowledge the excellent technical assistance of Dr. Jacquline Chanél, Dr. Dominique Dumas, Dr. Christophe Stenger and Jean-Michel Girardet considering TEM, CLSM and semi-prep HPLC.

References:

Breukink, E., De Kruijff, B., 1999. The lantibiotic nisin, a special case or not? Biochimica et Biophysica Acta - Biomembranes 1462, 223-234.

Breukink, E., de Kruijff, B., 2006. Lipid II as a target for antibiotics. Nature Reviews Drug Discovery 5, 321-323.

Breukink, E., Van Kraaij, C., Van Dalen, A., Demel, R.A., Siezen, R.J., De Kruijff, B., Kuipers, O.P., 1998. The orientation of nisin in membranes. Biochemistry 37, 8153-8162.

Budde, B.B., Jakobsen, M., 2000. Real-time measurements of the interaction between single cells of Listeria monocytogenes and nisin on a solid surface. Applied and Environmental Microbiology 66, 3586-3591.

Chatterjee, C., Paul, M., Xie, L., van der Donk, W.A., 2005. Biosynthesis and mode of action of lantibiotics. Chemical Reviews 105, 633-683.

Cotter, P.D., Hill, C., Ross, R.P., 2005. Food microbiology: Bacteriocins: Developing innate immunity for food. Nature Reviews Microbiology 3, 777-788.

Daniel, R.A., Errington, J., 2003. Control of cell morphogenesis in bacteria: Two distinct ways to make a rod-shaped cell. Cell 113, 767-776.

Delves-Broughton, J., 1996. Applications of the bacteriocin, nisin. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology 69, 193-202.

EU, 2009. Food Additives in Europe (including E-number listing). (http://www.reading.ac.uk/foodlaw/additive.htm)

Garcera, M.J.G., Elferink, M.G.L., Driessen, A.J.M., Konings, W.N., 1993. In vitro pore-forming activity of the lantibiotic nisin. Role of nmotive force and lipid composition. European Journal of Biochemistry 212, 417-422.

Guiotto, A., Pozzobon, M., Canevari, M., Manganelli, R., Scarin, M., Veronese, F.M., 2003. PEGylation of the antimicrobial peptide nisin A: Problems and perspectives. Farmaco 58, 45-50.

Herranz, C., Chen, Y., Chung, H.J., Cintas, L.M., Hernandez, P.E., Montville, T.J., Chikindas, M.L., 2001a. Enterocin P Selectively Dissipates the Membrane Potential of Enterococcus faecium T136. Applied and Environmental Microbiology 67, 1689-1692.

Herranz, C., Cintas, L.M., Hernandez, P.E., Moll, G.N., Driessen, A.J.M., 2001b. Enterocin P causes potassium ion efflux from Enterococcus faecium T136 cells. Antimicrobial Agents and Chemotherapy 45, 901-904.

Hsu, S.T.D., Breukink, E., Tischenko, E., Lutters, M.A.G., De Kruijff, B., Kaptein, R., Bonvin, A.M.J.J., Van Nuland, N.A.J., 2004. The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. Nature Structural and Molecular Biology 11, 963-967.

Jamuna, M., Babusha, S.T., Jeevaratnam, K., 2005. Inhibitory efficacy of nisin and bacteriocins from Lactobacillus isolates against food spoilage and pathogenic organisms in model and food systems. Food Microbiology 22, 449-454.

Jamuna, M., Jeevaratnam, K., 2009. Antibacterial efficacy of nisin and pediocins against food spoilage and pathogenic organisms in broth and a vegetarian food. Journal of Food Science and Technology 46, 563-568.

Lubelski, J., Overkamp, W., Kluskens, L.D., Moll, G.N., Kuipers, O.P., 2008. Influence of shifting positions of Ser, Thr, and Cys residues in prenisin on the efficiency of modification reactions and on the antimicrobial activities of the modified prepeptides. Applied and Environmental Microbiology 74, 4680-4685.

Maher, S., Vilk, G., Kelleher, F., Lajoie, G., McClean, S., 2009. Chemical modification of the carboxyl terminal of nisin a with biotin does not abolish antimicrobial activity against the indicator organism, kocuria rhizophila. International Journal of Peptide Research and Therapeutics 15, 219-226.

Moll, G.N., Clark, J., Chan, W.C., Bycroft, B.W., Roberts, G.C.K., Konings, W.N., Driessen, A.J.M., 1997. Role of transmembrane pH gradient and membrane binding in nisin pore formation. Journal of Bacteriology 179, 135-140.

Mozafari, M.R., Johnson, C., Hatziantoniou, S., Demetzos, C., 2008. Nanoliposomes and their applications in food nanotechnology. Journal of Liposome Research 18, 309-327.

Mulders, J.W.M., Boerrigter, I.J., Rollema, H.S., Siezen, R.J., De Vos, W.M., 1991. Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. European Journal of Biochemistry 201, 581-584.

Sameiro, M., Gonalves, T., 2009. Fluorescent labeling of biomolecules with organic probes. Chemical Reviews 109, 190-212.

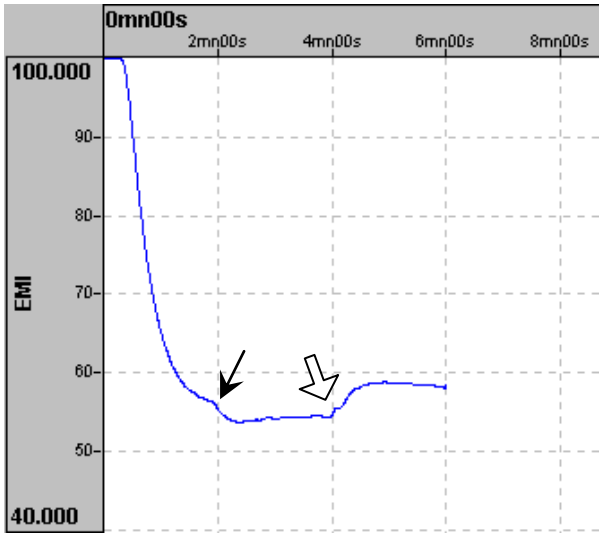
Van Kraaij, C., Breukink, E., Noordermeer, M.A., Demel, R.A., Siezen, R.J., Kuipers, O.P., De Kruijff, B., 1998. Pore formation by nisin involves translocation of its C-terminal part across the membrane. Biochemistry 37, 16033-16040.

Verheul, A., Russell, N.J., Van 'T Hof, R., Rombouts, F.M., Abee, T., 1997. Modifications of membrane phospholipid composition in nisin-resistant Listeria monocytogenes Scott A. Applied and Environmental Microbiology 63, 3451-3457.

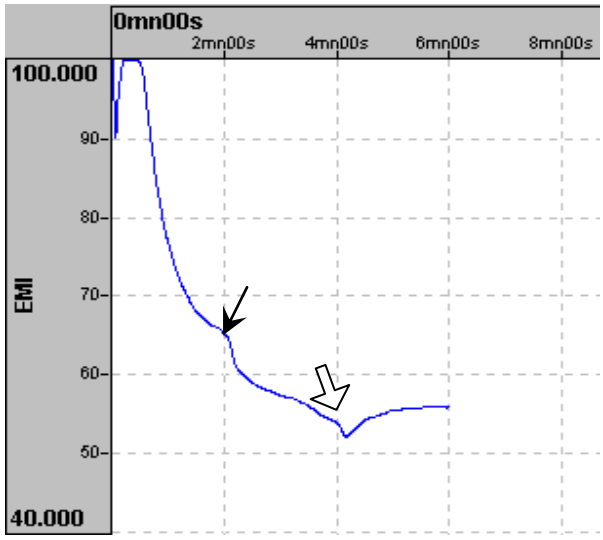
Wiedemann, I., Breukink, E., Van Kraaij, C., Kuipers, O.P., Bierbaum, G., De Kruijff, B., Sahl, H.G., 2001. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. Journal of Biological Chemistry 276, 1772-1779.

Annex 1 (Chapitre 7): Comparison of activity of labelled and unlabelled nisin against *L. innocua* CIP 12511 and *L. ivanoii* CIP 12510. Fluorescence of probe 3,3'-dipropylthia-dicarbocyanine iodide [(DiSC3(5)] was measured to determine the ΔΨ. Glucose-energized cells were added to quartz cuvette containing HEPES buffer and DiSC3(5) (5 µM). After 2 min nigericin (1.5 nM) was added (black arrow), which dissipates the pH gradient (ΔpH). After 4 min nisin or fluorescent labelled nisin at the MIC values 0.66 µg/mL and 0.33 µg/mL for *L. innocua* (a, b) and *L. ivanoii* (c, d) respectively were added (open arrow). The ΔΨ induced DiSC3(5) fluorescence change reflects the ability of bacteriocin to impact membrane potential.

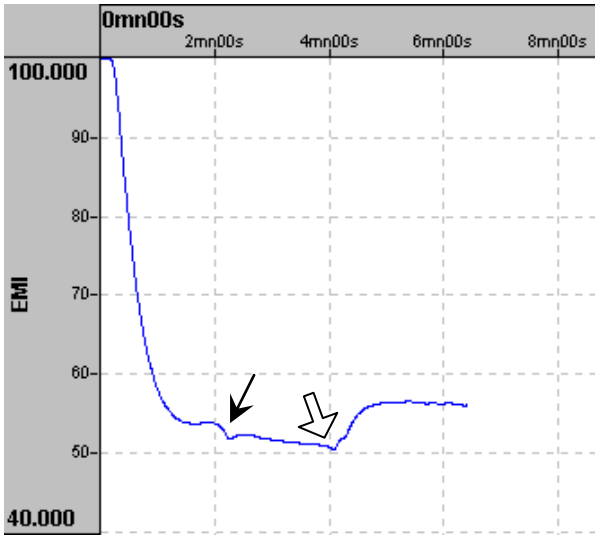
a.



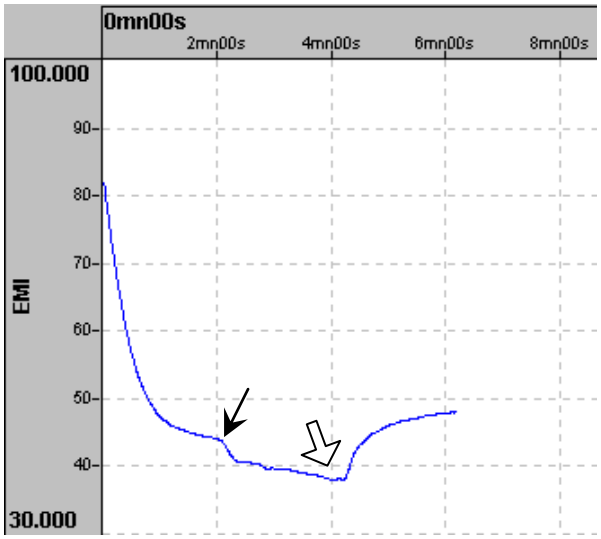
b.



c.



d.



Chapitre 8: Libération contrôlée de la nisine à partir de bio-membranes: Comparaison entre chitosane, hydroxypropyl méthylcellulose, caséinate de sodium et acide poly-lactique et applications en emballage

Les composés antibactériens peuvent être soit incorporés directement dans l'aliment lors de sa formulation, soit appliqués à la surface des aliments. Ces deux voies d'utilisation ont cependant une efficacité limitée en raison d'une perte rapide d'activité. Cette perte d'activité peut être due à une diminution de la concentration liée à un effet de dilution dans la matrice alimentaire et aux interactions négatives avec les composés de l'aliment (Guiga et al., 2010). Pour éviter ces inconvénients, l'enrobage du composé actif dans la matrice du polymère d'emballage est une solution intéressante (Imran et al., 2010b, Mastromatteo et al., 2010). Les valeurs des coefficients de diffusion et de partage (D et K) des composés antibactériens depuis le film d'emballage vers l'aliment apportent des informations sur l'efficacité du film à agir comme transporteur pour le composé antibactérien et sur la durée d'efficacité du composé actif dans le bio-polymère et dans l'aliment, vis-à-vis des micro-organismes (Frassen et al., 2004). La justesse de la prédiction des taux de diffusion dépend fortement de la sensibilité/efficacité de la méthode de quantification car les concentrations de nisine désorbée ou migrante peuvent être très basses (valeurs de D comprises entre 10^{-2} et 10^{-11} m^2s^{-1}) (Guiga et al., 2009 ; Sebti et al., 2003). Les méthodes conventionnelles de quantification de la nisine sont approximatives, quand elles sont liées à la réponse d'un micro-organisme, et peuvent être délicates à mettre en œuvre dans le cas du marquage par anticorps. Dans ce contexte, l'utilisation de composé marqué est une des méthodologies la plus couramment employée (Sameiro and Gongalves, 2009).

En raison des problèmes environnementaux liés aux déchets d'emballage, de nombreux groupes de chercheurs s'intéressent aux polymères verts comme alternative biodégradable au plastiques synthétiques (Jamshidian et al., 2010). Ainsi, l'objectif des travaux présentés ici est d'évaluer et de comparer les taux de relargage de la nisine marquée par un composé fluorescent à partir de différents polymères biodégradables de différentes origines et compositions incluant des dérivés de la cellulose tel que l'Hydroxy Propyle Méthyle Cellulose (HPMC), des dérivés de protéines animales tels que des caséinates de sodium (CS), des dérivés de cytosquelette de crustacés tels que le chitosane (CTS) et des dérivés de monomères d'acide lactique tel que l'acide poly-lactique (PLA). Cette approche permettra de comparer l'efficacité de ces différents films d'emballage biodégradables à être des vecteurs de composés actifs.

Chapter 8: Controlled release of an antimicrobial bio-preservative nisin from bio-membranes: Comparison between HPMC, sodium caseinate, poly-lactic acid and chitosan for packaging applications

Antimicrobial agents are either directly incorporated into the food formulations during their preparation, or applied on their surface. Both of these operations offer a limited efficiency as they result in a rapid loss of antimicrobial activity due to a quick decline of active concentration at their surface, resulting from dilution into bulk foods and interactions with food components (Guiga et al., 2010). To answer this constraint, embedding the active agent in the polymeric network bioactive packaging is an interesting advancement (Imran et al., 2010b, Mastromatteo et al., 2010).

The study of mass migration (diffusion and partition coefficients D, K) values for antimicrobials in the packaging films to food system provide information about how long the antimicrobials are efficient against target microorganisms at the surface of the films and how effectively the selected films act as antimicrobial carriers (Franssen et al., 2004). The efficacy of predicting the diffusion rates depends mainly on the efficacy/sensitivity of antimicrobial agent quantification method, as nisin desorption or migration concentration can be exceptionally low (value of D between 10^{-2} to 10^{-11} m^2s^{-1}) (Guiga et al., 2009, Sebti et al., 2003). The conventional methods for nisin quantification are either approximate (depend on response of target bacteria) or necessitate longer time of analysis (nisin antibodies production). In this regard labeling is one of the most common methodologies used for bioanalytical purposes (Sameiro and Gonçalves, 2009).

Due to environmental problems derived from the packaging wastes, various research groups are looking toward green polymers as biodegradable alternatives to synthetic plastic packages (Jamshidian et al., 2010). Thus, the objective of the present study was to evaluate and compare the release rates of fluorescently labeled nisin from different biodegradable polymers with distinct origin including cellulose derivative hydroxypropyl methylcellulose (HPMC), dairy animal‟s protein based sodium caseinate (SC), marine animal‟s exoskeleton based chitosan (CTS), and bio-derived monomers based poly-lactic acid (PLA) to compare their aptness as active agent carriers for biodegradable packaging films.

Chapter 8

Controlled release of an antimicrobial bio-preservative nisin from bio-membranes: Comparison between HPMC, sodium caseinate, poly-lactic acid and chitosan for packaging applications

Muhammad Imran, Anne-Marie Revol-Junelles, Majid Jamshidian, Muhammad Javeed Akhtar, Amira Klouj, Stéphane Desobry

Laboratoire d'Ingénierie des Biomolécules (LIBIO), ENSAIA–INPL, Nancy Université, 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France

(Journal of controlled release, Soumise 2011)

Abstract:

One of the critical factors concerning food spoilage is the microbial growth at food surface, which can be inhibited by maintaining a high concentration of antimicrobials at food surfaces and limiting their diffusion into the food core. The objective of the present study was to evaluate and compare the release rates of fluorescently labeled nisin from different biodegradable polymers [hydroxypropyl methylcellulose (HPMC), chitosan (CTS), sodium caseinate (SC) and polylactic acid (PLA)] at 4 and 40 °C to judge their relative capacity and efficiency as antimicrobial carriers. Fick's second law was applied to determine the diffusion coefficient (D) of nisin, which indicated that D value for CTS ($1.97\pm0.1 \times 10^{-13}$ cm^2s^{-1}) ~ PLA < SC < HPMC ($49.3\pm1.5 \times 10^{-13}$ cm^2s^{-1}) at 4°C. The increase in temperature had significantly increased the nisin release from SC and HPMC films due to glass transition and hydrophilic nature. Subsequently, power law model was applied to diffusion data to explore the mechanism of nisin release. The values of partition coefficient (K) followed the similar trend but were < 1 except for HPMC at 40°C, which implies that at equilibrium less percentage of the migrating substance nisin was liberated in food simulating liquid. Concerning antimicrobial potential, a superior inhibitory effect against *L. monocytogenes* and *Staphylococcus aureus* was observed with HPMC, CTS and SC in association with nisin.

Keywords: Slow release, Diffusion coefficient, Fluorescent labeling, Partition coefficient, Microstructure, *Listeria monocyogenes*

1. Introduction

Since its inception for the pharmaceutical applications, the concept of „controlled release" is usually intended to optimize preventive or therapeutic regimen by providing slow and continuous delivery of drugs with greater convenience. To endow the key functionality of controlled release in food industry, the interest in food packaging with antimicrobial properties has increased strikingly, due to the fact that these systems are able to control the microbiological decay of perishable food products [1]. Nisin, the commercially available antimicrobial, is probably the most used bio-preservative in food industry worldwide. Nisin is an amphiphilic cationic peptide belonging to the class I bacteriocins, produced by certain strains of *Lactococcus lactis* [2]. This ribosomally synthesized peptide possesses a relatively broad spectrum of antibacterial activity against Gram positive food borne pathogens and spoilage organisms. Moreover, nisin belongs to lantibiotics (lanthionine-containing peptide antibiotics) as it contains the unusual amino acids. Lantibiotics are generally considered potentially useful as food bio-preservatives, although the Food and Drug Administration (FDA) has recognized only nisin (E234) as an approved food additive due to its non-toxicity [3-5].

Antimicrobial agents are either directly incorporated into the food formulations during their preparation, or applied on their surface. Both of these operations offer a limited efficiency as they result in a rapid loss of antimicrobial activity due to a quick decline of active concentration at their surface, resulting from dilution into bulk foods and interactions with food components [6]. Consequently, in the complex food systems, nisin effectiveness can be reduced by food attributes such as high pH, high fat content, large particle size, non-uniform distribution of nisin, and its inactivation by proteolytic enzymes [7, 8]. One of the critical factors affecting food spoilage is the microbial growth at food surface. Microbial inhibition can be improved by maintaining a high concentration of preservatives at food surfaces and by limiting their diffusion into the food core [9]. To attain this objective, extensive research has been realized to retain the active agent in the polymeric network and control its release from bioactive packaging [1, 7].

Predicting nisin diffusion from packaging to the model food system is necessary to control its antimicrobial activity, since nisin needs to reach microorganisms to exercise its potential [10]. Likewise, diffusion coefficient (D) and partition coefficient (K) values for antimicrobials in the packaging films provide information about how long the antimicrobials are efficient against target microorganisms at the surface of the films and how effectively the

selected films act as antimicrobial carriers [11]. The efficacy of predicting the values of D and K depends mainly on the efficacy/sensitivity of antimicrobial agent quantification method, as nisin desorption or migration concentration can be exceptionally low (value of D between 10^{-2} to 10^{-11} m^2s^{-1}) [12, 13]. The conventional methods for nisin quantification includes, diffusion bioassay using test organisms e.g. *Micrococcus luteus* [14, 15]; bicinchoninic acid BCA method by reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium strongly influenced by four amino acid residues (cysteine or cystine, tyrosine, and tryptophan) [12]; high performance liquid chromatography [16]; or indirect competitive immunoassay ELISA using polyclonal rabbit antiserum raised against purified nisin-keyhole limpet hemocyanin [10]. These quantification methods are either approximate (depend on response of target bacteria) or necessitate longer time of implementation (nisin antibodies production). In the recent past, detection and quantification based on fluorescence techniques has received special attention and notable progress has been made in both fluorescence instrumentation and synthesis of new fluorophores. In this regard labeling is one of the most common methodologies used for bioanalytical purposes. The fluorescence intensity of the conjugate (e.g. fluorescein) allows for its easy detection at submicromolar concentrations such as those usually required in sensitive quantification or microscopy studies. The fluorescently labeled proteins are useful regarding in vivo imaging, screening, diagnostic proteomics, and single biomolecule spectroscopy. This strategy for the selective and efficient incorporation of a low molecular weight fluorophore (MW ≈ 10% of biomolecule) into proteins at defined sites deliver the final labeled product with similar characteristics of migration and mechanism of action [17]. To avoid the above-mentioned constraints, in the present study, fluorescein labeled nisin was fabricated to study diffusion rates in model food system by rapid fluorescence spectrometry.

Owing to the health concern of the consumer and the environmental problems derived from the packaging wastes, various research groups are looking toward green polymers as biodegradable alternatives to synthetic plastic packages [18]. To acquire this „green-tag" by using bioactive preservative in biodegradable packaging material, researchers has studied the incorporation of different bioactive agents in eco-friendly polymers including, nisin in cellulose derivative based films [13]; potassium sorbate in chitosan film [19]; propolis in polylactic acid (PLA) [20]; thymol and lysozyme in protein based zein and whey protein films respectively [21, 22]… However, there is no published work on the release of nisin, from packaging to model

food system, comparing different biopolymers to judge the relative capacity and efficiency of various biopolymers as antimicrobial carriers.

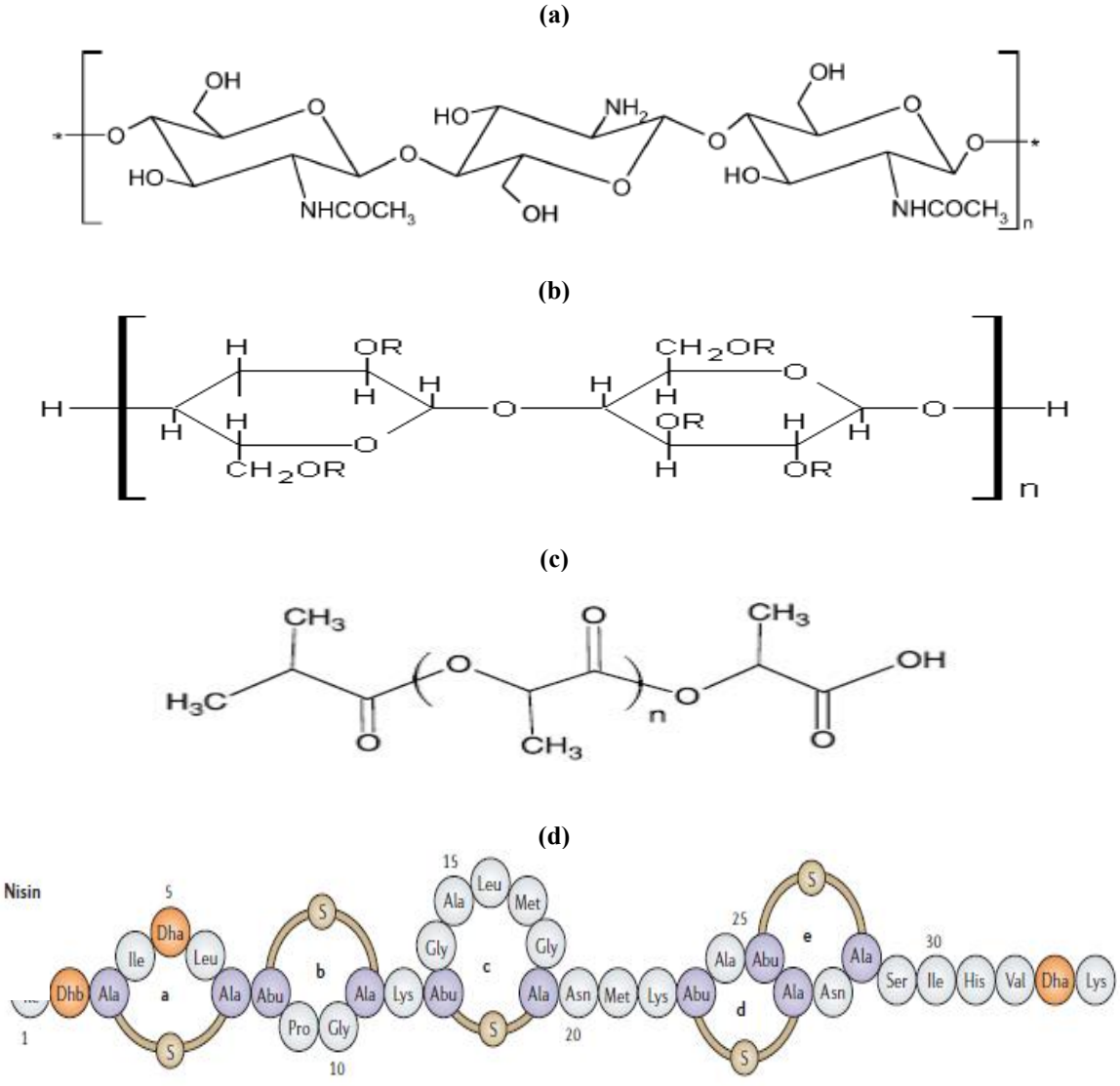


Figure 1: Structure details of biopolymers; (a-c), chitosan (poly beta-1,4-D-glucosamine), HPMC, PLA repectively; and active agent nisin Z (d). [Reference [18, 23, 24]]

A major research gap is the lack of packaging materials that can provide the release of active compounds at rates suitable for a wide variety of food packaging applications [20]. The objective of the present study was to evaluate and compare the release rates of fluorescently labeled nisin from different biodegradable polymers with distinct origin and composition (Figure 1). Cellulose derivative hydroxypropyl methylcellulose (HPMC), dairy animal‟s protein based sodium caseinate (SC), marine animal‟s exoskeleton based chitosan (CTS), and chemically synthesized from bio-derived monomers based poly-lactic acid (PLA) were selected as packaging membranes due to their improved physical, mechanical, barrier (water, gas), transparency, and biodegradability properties [19, 20, 25-27]. In addition to the effect of biopolymer type, the influence of temperature (4°C and 40°C) on nisin release was measured in water-ethanol as food simulating liquid (FSL). Based on the release kinetics obtained, the suitability of HPMC, SC, CTS and PLA as retention matrix for nisin concerning the objective of developing bioactive packaging is discussed.

2. Materials and methods

2.1. Materials

Chitosan with the degree of deacetylation 75-85 % (medium molecular weight, 200-800 cP viscosity, soluble in 1% acetic acid aqueous solution) and sodium caseinate was purchased from Sigma-Aldrich (Sigma chemicals, St-Louis, MO, USA). PLA polymer (2002D, NatureWorks^{TM}, MN, USA) was used as a thermoplastic biopolymer. HPMC powder with hydroxypropoxyl content ~9% and the viscosity ~15 mPa.s (2% in H_20 at 25°C), was obtained from Fluka-Biochemika, Japan. Glycerol (>97% purity) was used as a plasticizer and was purchased from Merck (Darmstadt, Germany). Nisin was purchased from Honghao Chemical Co. (Shanghai, China). Nisin used in this study contained > 90 % pure nisin (according to the manufacturer, the formulation contains 3.84 x 10^6 I.U. per gram and 6.88 % moisture content). N, N dimethylformamide (DMF), 1-Hydroxy-7-Azabenzotriazole (HOAt), N- (3-dimethylaminopropyl) -N'-ethylcarbodiimide hydrochloride (EDC), trifluoroacetic acid (TFA), ethanol (> 99.5% purity) and bicinchoninic acid (BCA) reagents were obtained from Sigma Chemical Co. (Steinheim, Germany). The label 5-(aminoacetamido) fluorescein (AAA-flu) and

thin layer chromatography silica gel 60 RP-18 plates were purchased from Invitrogen (Oregone, USA) and Merck (Darmstadt, Germany) respectively.

Microorganism and culture media: *L. monocytogenes* CIP 82110^T and *Staphylococcus aureus* CIP 4.83 strains were purchased from collection of Pasteur Institute. The bacteria were cultivated in trypticase soy broth (Biokar Diagnostics, Beauvais, France) supplemented with 6 g L^{-1} of bacto-yeast extract (Biokar) (TSB-YE). Incubation was performed at 37 °C overnight. The strain was stored in TSB-YE supplemented with glycerol (10%) at -30 °C and propagated twice before use. Agar medium was prepared by addition of 12 g L^{-1} of bacteriological agar.

2.2. Fluorescent labeling of nisin

Preliminary experiments revealed that purified nisin was a pre-requisite for the realization of the labeling purpose. Thus semi-purified commercial nisin Z with more than 90 percent purity was subjected to high performance liquid chromatography (HPLC) to remove the impurities (tiny shoulders around the bacteriocin corresponding major peak). The mobile phase A and B were prepared containing water: acetonitrile: TFA as 90:10:0.1% (v/v) and 10:90:0.06% (v/v) respectively. Semi-preparative liquid chromatography was performed using Waters 2690 HPLC separations module (Alliance®, USA). Chromatographic separation was performed on Apollo C-18 (250mm x 22mm x 5 µm) reverse phase column (GRACE®, Darmstadt, Germany). The column was equilibrated with mobile phase A, and 0.5 mL of the sample (dissolved in mobile phase A) was injected. Elution was performed by 45 minutes programme at a flow rate of 5 ml/min as follows: a linear gradient ranging from 10% to 35% (v/v) mobile phase B for 10 min, 35% to 42% (v/v) mobile phase B in 20 min, maintained up to 100% B for 5 min, and column was re-equilibrated with 10% B for 10 min in the end. The fractions corresponding to major peak of nisin were collected, lyophilised and stored at –20 °C until further use.

The protocol of peptide labelling reported earlier for nisin A [28] was optimised for Z variant of peptide. The fluorescent marker 5-(aminoacetamido) fluorescein (AAA-flu) was coupled with carboxyl group of nisin Z via a HOAt/EDC coupling in 100 µl DMF. The reaction mix containing 50 nmol peptide, 50 nmol AAA-flu and 60 nmol of both EDC and HOAt was incubated overnight at room temperature. The ratio of AAA-flu to nisin Z was varied between 10:1 and 1:10 for improving the yield, however 3:1 ratio provided maximum labelled product. The labeled nisin was purified from the reaction mixture, after evaporation of the DMF, using

reversed phase (C18) HPLC as described above for bacteriocin purification. Fractions containing labelled nisin were collected, lyophilised and stored at –20 °C until further use.

The concentration of labeled nisin was determined using the bicinchoninic acid (BCA) assay. In this assay, the BCA reagent was added to bacteriocin protein i.e. nisin. After incubation at 37 °C for 30 min, the absorbance at 562 nm was measured using a UV–Vis spectrophotometer (Ultrospec 4000 UV/visible, Pharmacia Biotech, UK). Bovine serum albumin containing 1 mg/ml protein was diluted and used as the protein standard.

2.3. Film fabrication

2.3.1. HPMC film preparation

Film forming solution (FFS) was prepared by dissolving HPMC in distilled water (final concentration 6 % w/v) by modifying the procedure reported earlier [26]. The solutions were mixed for 40 min at 65 °C using a heating magnetic stirrer (Fisher Bio-block scientific). The concentration of a mixture of labelled nisin and nisin (1:10) was adjusted to give a final concentration of 1 mg/mL nisin for active films (8 mg/dried film, 0.137 mg nisin cm^{-2} based on film exposed area). For a homogenous solution, active FFS was then subjected to ultrasonic treatment with bench-top ultrasonic cleaner Branson-1510 (Danbury, CT, USA). Films were made by pouring approximately 8 g FFS in the Teflon 90×110 mm Petri dishes (Welch, USA) and left to dry them at room temperature for 24-48 hours. Entire process of film fabrication was accomplished in the absence of light to avoid degradation of AAA-Flu. Films were either stored at 20±2 °C and approximately 50% or zero relative humidity using magnesium nitrate or phosphorus pentaoxide (P_20_5), respectively, depending upon film characterization experiment.

2.3.2. Chitosan film preparation

Chitosan FFS was prepared by dissolving 1 g chitosan in 100 mL of 1% aqueous acetic acid solution at 25-30°C to achieve stoichiometric protonation of the NH_2 sites [19]. The solution was stirred by magnetic agitation (600-700 rpm) during 24 hours until the chitosan was fully dissolved. The FFS was filtered successively through 0.8, and 0.45 mm Millipore membranes. The diffusion compound, mixture of labelled nisin and nisin (1:10), was added to the initial solution during stirring for making active chitosan films at 0.32 mg/ml nisin (8 mg/dried film,

0.137 mg nisin cm^{-2} based on film exposed area). For a homogenous solution, active FFS was then subjected to ultrasonic treatment with bench-top ultrasonic cleaner Branson-1510 (Danbury, CT, USA). Then about 25 g of solution was poured into Petri dishes and dried at room temperature during 72-75 hours.

2.3.3. Sodium caseinate film preparation

Sodium caseinate (5% w/v) was dissolved in distilled water under continuous stirring (500 rpm) at 50 °C for 2 hours. After total dissolution, glycerol was added as the plasticizer at a concentration of 20% (w/w) which is necessary to overcome the brittleness and to improve the extensibility of sodium caseinate films [25]. The mixture of labelled nisin and nisin (1:10) solution was then added to the sodium caseinate solution to reach a final concentration of bacteriocin of 1.4 mg/ml (8 mg/dried film, 0.137 mg nisin cm^{-2} based on surface area of active films). For a homogenous solution, active FFS was then subjected to ultrasonic treatment. Films were cast with 5.7 g portions of sodium caseinate solutions, poured into Teflon 90×110 mm Petri dishes (Welch, USA) and then dried at room temperature for 48-72 h.

2.3.4. PLA film preparation

FFS of 3.5% w/w of PLA was prepared in chloroform and afterwards 0.8% w/w of mixture of labelled nisin and nisin (1:10) based on PLA FFS was added (0.8 mg/ml), and let the solution mixed during 1 hour for complete distribution of nisin in polymer matrix. For a homogenous solution, active FFS was then subjected to ultrasonic treatment with bench-top ultrasonic cleaner Branson-1510 (Danbury, CT, USA). Then 10 g of FFS was casted in Teflon 90×110 mm Petri dishes (Welch, USA), and the extra chloroform was evaporated in a dark place under hood during 48 h at 20°C. For a complete drying, the Petri dishes were kept in a hermetic container containing P_2O_5 powder. The final film thickness was 50 ± 1 µm measured by mechanical profilometer.

2.3.5. Film thickness measurement

The thickness of different biopolymers films was measured using the standard NF Q 03-016 with a manual micrometer (Messmer, London, England) equipped with a head measuring 1

cm in diameter and a sensitivity of 2µm. The thickness was analyzed in 10 randomly selected points on each film and then an average value was calculated.

2.4. Experimental procedure for kinetics of nisin release

The procedure of diffusion test were modified from the protocol explained earlier [15]. Films of 58 cm^2 surface area and with 50.5 ± 1 µm average thickness were cut into 6 × 1.5 cm pieces, all the pieces were immersed vertically into 10 ml water-ethanol (10:90 v/v) food simulating liquid (FSL). The use of suitable water-ethanol FSL was requisite to avoid the film hydrolysis due to their biodegradable nature. To avoid bleaching/degradation of labeled nisin by light, colored vials (6.5 × 1.6 cm) were employed with tightly closed septum to prevent the escape of FSL. The diffusion test was performed at 4 and 40 °C to analyze the effect of temperature. A cold storage room with a controlled temperature was used to perform experiments at 4 °C. For the diffusion at 40 °C, the diffusion vials were placed in an incubator (Memmert, Germany). The initial temperature of FSL was set to the selected temperatures before the nisin containing biopolymer films were immersed in the vials. For each temperature, at least three replicates of experiment were performed. A volume of 200 µL was taken at predetermined time intervals. The change in volume due to sampling was considered while determining the nisin concentration in the diffusion vials. The concentration of nisin diffused in FSL was determined by Xenius XM spectrofluorimeter (Safas, Monaco) using 490 and 520 nm as excitation and emission wavelengths respectively.

2.5. Diffusion coefficient (D) and partition coefficient (K) measurement

Fick‟s second law was considered to investigate the diffusion phenomenon of nisin, the bio-active agent with antimicrobial properties, from HPMC, CTS, SC and PLA to in water-ethanol as food simulating liquid (FSL) (Equation 1).

$$\frac{\partial C_F(x,t)}{\partial t} = D\,\frac{\partial C_F(x,t)}{\partial x^2} \qquad (1)$$

Where $C_F(x,t)$ is the concentration of nisin in the film at position x and time t , and D is the diffusion coefficient of nisin through the packaging film ($cm^2\ s^{-1}$). The diffusion coefficient of nisin (D) was determined from the experimental data using a relationship derived from the Fick‟s

second law for a plane sheet with the following assumptions: (1) the initial concentration of nisin is uniform across the film, (2) the initial concentration of nisin in the food simulating liquid is zero, (3) the amount of nisin diffused in water is same as amount released from the particular film, and (4) D is not concentration dependent and is only affected by FSL temperature (Equation 2) [29].

$$\frac{M_t}{M} = 1 - \frac{8}{\pi^2} \sum_{n=0} \frac{1}{(2n+1)^2} \exp\left[\frac{-2(n+1)^2 \ ^2Dt}{L^2}\right] \quad (2)$$

Where Mt and M are the mass of nisin released from the film at time t and at infinite time respectively. L is the thickness of the film.

For short times (Mt/M < 2/3), equation 2 can be simplified to the equation 3 and apparent diffusivity can be determined with equation 4 [22, 29].

$$\frac{M_t}{M} = 4\left(\frac{Dt}{4L^2\pi}\right)^{\frac{1}{2}} \quad (3)$$

$$D = \left(\frac{S \times L}{2}\right)^2 \times \pi \quad (4)$$

Where, S is the slope of a plot representing Mt/M a gainst $t^{1/2}$.

To investigate the mechanism of diffusion, following power law model was useful [19].

$$\frac{M_t}{M} = kt^n \quad (5)$$

Where „k" is a constant that describes the macromolecular network and „n" is a diffusion exponent attribute of the release mechanism. According to this law, for n = 0.5, the diffusion transport mechanism follows a Fickian law; for n = 1.0 (Case II), the solute transport is directly proportional to time; for 0.5 < n <1.0, anomalous (non-Fickian) transport is the predominant mechanism and, for n > 1.0 (Super Case II), the transport is such that the solute is released in the later stages [19, 29].

In general, partition coefficient is defined as the ratio of the migrant equilibrium concentration in the food stimulant (Cs,) to the film polymer ($C_{F,}$).

$$K = \frac{C_{S,}}{C_{F,}} \quad (6)$$

Using these coefficients, we can determine the partition coefficient for a food simulant/polymer system.

$$K = \frac{M_{S,}\ /V_S}{M_{F,}\ /V_F} \quad (7)$$

Where $M_{S,}$ and $M_{F,}$ are the amounts of nisin (mg) in the solution and the film (respectively), while V_S is the volume of the simulant and film (cm^3).

2.6. Microstructure determination

Microstructural analyses of cross-sections of the dry films (previously conditioned in desiccators with P_2O_5 for at least 15 days) were carried out by cryo-fracturing of films. Film cross sections were prepared by dropping a film into liquid nitrogen followed by fracturing with a prechilled razor. The freshly fractured film pieces were retrieved from the liquid nitrogen bath and placed as quickly as possible into a Petri dish containing a piece of filter paper and placed in a desiccator to warm and dry to room temperature. Fractured film pieces were then mounted up on a SEM stub. All samples were then viewed and photographed in a Hitachi S-4800 scanning electron microscope (Hitachi, Japan) at 0.5 to 2 kV.

2.7. Comparison of different films bioactivity

To find out the antibacterial activity of films, 1 cm diameter disks were cut from different composite bioactive films and placed on inoculated nutrient medium. The method was previously standardized by adjusting the microbial inoculation rate (0.1% v/v) and the volume of the agar medium layer (12 mL). Dishes were refrigerated at 4 °C for 4 h to allow the process of bacteriocin diffusion without microbial growth and then incubated at 37 °C. Data was expressed as growth inhibitory zone diameter (cm) and measured at the nearest 1 mm for three replicates.

2.8. Statistical analyses

Statistical analyses were carried out by using the software KyPlot version 2.0 (Koichi Yoshioka, Department of Biochemistry and Biophysics, Graduate school of Allied health Sciences, Tokyo, Japan).

3. Results and discussion

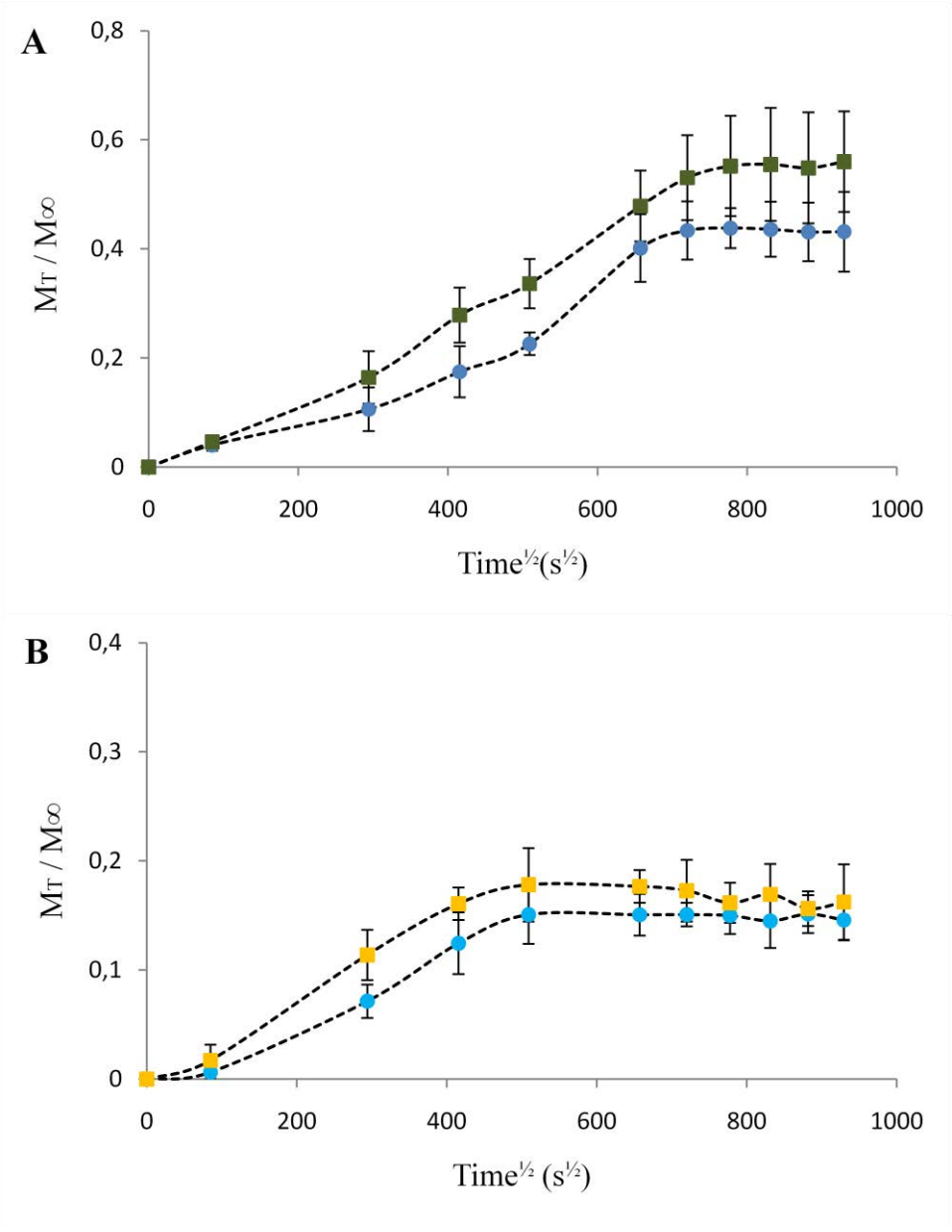
To explore the possibility of using biodegradable films as antimicrobial carriers, FDA approved only bacteriocin „nisin" was incorporated in HPMC-, CTS-, SC- and PLA-based active coatings. Nisin release from four matrices of different origin (plant fiber, sea crustaceans, animal protein, and chemical synthesis) was quantified by using fluorescently labeled-nisin through rapid method of fluorescence spectrometry. Different biopolymers were chosen in order to consider the influence of matrices" nature on release of antimicrobial nisin. Hence, it was possible to study the influence of both biopolymer role and temperature on nisin release at 4°C and 40°C. We studied several biopolymer-based films, to find out a good embedding matrice by predicting nisin release rates in food simulating liquid. Therefore at a given time nisin availability at minimal inhibitory concentration inside food and at food surface would help to predict effective control of pathogens and thus food shelf-life.

3.1. Nisin release kinetics

The original amounts of the fluorescently labeled nisin mix in four different films were the same as a result excellent solubility of nisin in film forming solutions (FFS) and due to little concentration per cm^2 (0.137 mg nisin cm^{-2}).In general, the release of a bioactive compound from a biopolymeric network occurs in two stages. In the primary step, water molecules penetrate/diffuse into the film polymeric complex from the outer solution. Thus the meshes of the polymeric network become increasingly wider, allowing the active agent to diffuse through the film into the outer solution until a thermodynamic equilibrium between the two phases is reached [16, 21]. Thus, the nisin released kinetic rely on the subsequent event: (i) food simulating liquid (FSL) penetration/diffusion; (ii) biomacromolecular network relaxation; (iii) diffusion of the active compound through the polymeric array to the FSL. In the present study it is assumed that both FSL diffusion and biomacromolecular matrix relaxation occur more rapidly than the active compound diffusion through the different film networks. Hence, nisin release kinetic can be

described be means of the Fick"s Second Law, intended for a plane sheet with constant boundary condition and uniform initial concentration.

Nisin release kinetics for the different biopolymers are reported in plot of fractional nisin migration (mass released) with time at 4° and 40°C (Figure 2). As observed, labeled nisin release behaviors differ comparing the four matrices. As expected, the amount of nisin released in the outer solution increased until equilibrium is reached between the film and FSL.



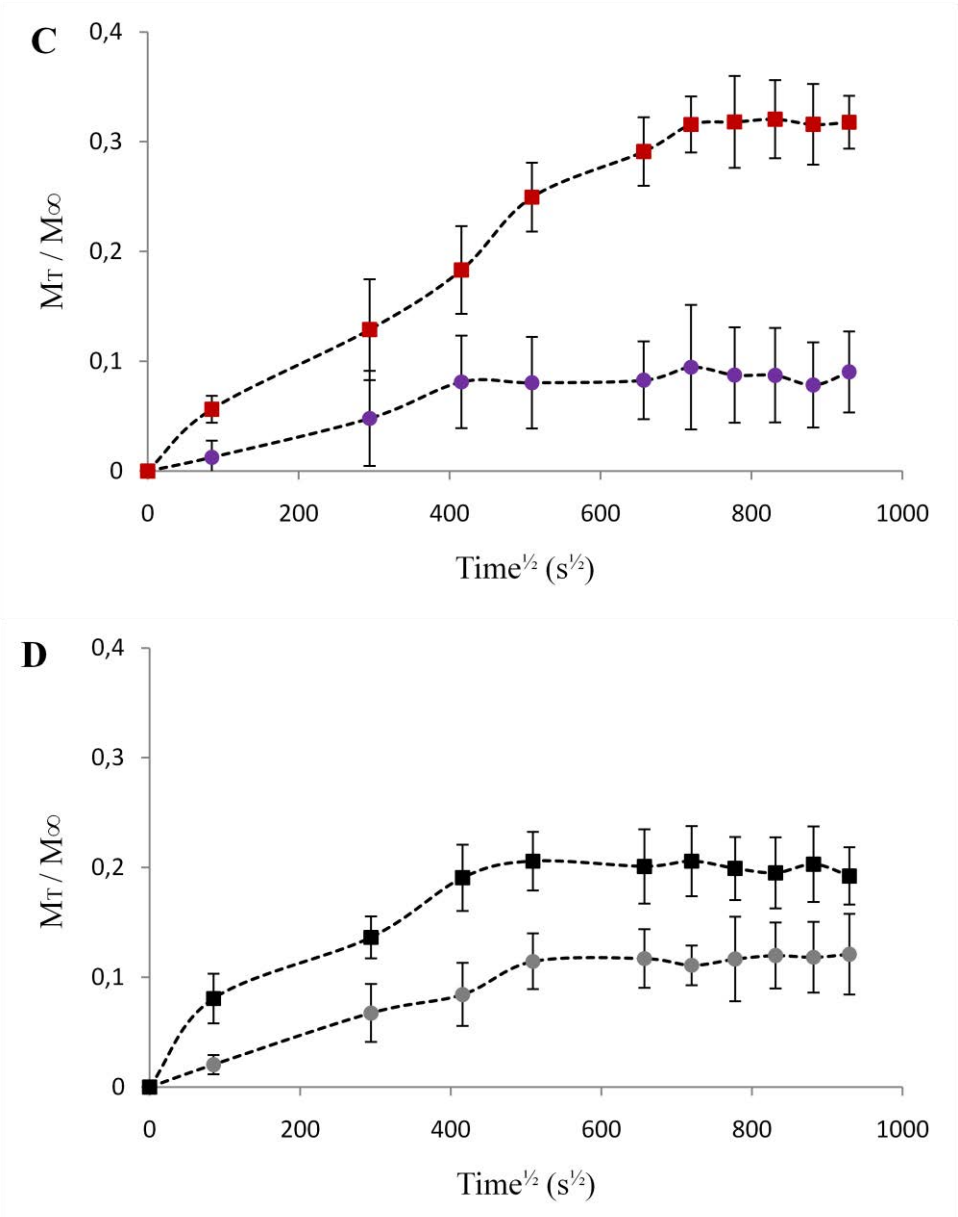


Figure 2: Diffusion of labeled nisin from biopolymeric films into water-ethanol (10:90 v/v) stimulant. Study of the influence of temperature (4°C ● and 40°C ■) and biopolymer type (A, HPMC; B, CTS; C, SC; and D, PLA) on release kinetics is graphically represented.

Higher amounts of nisin were released from active HPMC films at all times and temperatures compared to other film types. Concerning active HPMC films, 40 and 55% of nisin were released from the films at 4° and 40°C temperature respectively until equilibrium was reached after 160 h. In the case of CTS and PLA films, nisin diffusion was restricted at both temperatures and only < 20% of initial amount (M_0) in films had released into the aqueous-ethanol FSL and kinetics seemed to reach at equilibrium before 100 h. The diffusion of an

antimicrobial compound in one or between several phases depends on the characteristics of the active compound, solubility, partition coefficients, temperature, physico-chemical properties of biopolymers and active agent – biopolymer interactions. Among these, temperature and additive-biopolymer interaction strongly influence the respective kinetic release [8, 30]. In case of SC-active films, the release of nisin increased with temperature. After reaching thermodynamic equilibrium the nisin amount released were 8% and 32% of M_0 at 4° and 40°C, respectively. The glass transition temperature of plasticized sodium caseinate films at lower water activity occurs around 40°C enabling the characteristic transition to flexible/rubbery state which may explain the higher release of nisin from SC biopolymer at higher temperature.

Thus, the results indicated relatively less ability of nisin retention by HPMC active films, as a faster release and easier diffusion of nisin from HPMC was observed than other biodegradable polymers at all times and all temperatures. A possible explanation for difference observed between nisin release rates is the stability of film structure. In the presence of aqueous-ethanol FSL, the film structure firmness modifies in a different way relative to biopolymer nature, which allows an easier or controlled release of nisin. Concerning the influence of the temperature, nisin diffusion increased for HPMC and PLA but a significant augment was detected for SC-active films. Variation of temperature could result in dissimilar interaction, arrangement and crystallinity of active peptide and biopolymer molecules. Thus, obstruction of nisin movement in film matrix and subsequent desorption could fluctuate.

3.2. Diffusion coefficient: Influence of biopolymer nature and temperature

Previous studies indicated that film thickness has profound influence on release rates of active molecules embedded in edible polymers, because nisin travels a shorter distance through the thinner film to reach the aqueous solution [30]. In order to avoid the outshine of biopolymer nature effect on nisin diffusion by variation in thickness, film forming solution quantity was optimized to achieve 50±1 μm thickness for active- HPMC, CTS, SC and PLA films (Table 1). Thus, the effects attributed to the variability of nisin diffusion comprised on physico-chemical properties of different polymers or temperature variations.

Table 1: Diffusion coefficients (D) of nisin release from active films (HPMC, CTS, SC, PLA) at 4° and 40°C in water-ethanol simulant

Film type	Average thickness (µm)	Temperature (°C)	D ($\times 10^{-13}$) cm^2s^{-1}
HPMC	50.1 ± 0.8	4°	49.3 ± 1.50 [a]
		40°	97.8 ± 3.20 [b]
CTS	50.4 ± 1.4	4°	1.97 ± 0.08 [c]
		40°	1.99 ± 0.10 [c]
SC	50.2 ± 0.9	4°	1.27 ± 0.05 [c]
		40°	18.1 ± 0.39 [d]
PLA	50.5 ± 1.5	4°	2.01 ± 0.08 [c]
		40°	2.02 ± 0.11 [c]

[a to d] means with different superscripts indicate significant difference ($p < 0.001$)

Afterwards, the power law model was applied to diffusion data to explore the mechanism of release. The initial part of release curve (Mt/M < 0.6) was fitted using the power law model. Diffusion exponents (n) were calculated by plotting ln(Mt/M) against ln(t), for HPMC, CTS, SC and PLA films containing equivalent amount of nisin per film area at 4 °C (Figure 3A) and at 40°C (Figure 3B). The values of n were acquired as the slopes of the straight lines fitted to the data and the diffusion constant (k) as the intercept (Table 2).

Table 2: Parameters of power law model for nisin diffusion from active films (HPMC, CTS, SC, PLA) in water-ethanol simulant at 4° and 40°C

	4°C			40°C		
Biopolymers	*N*	*k*	R2	*n*	*k*	R2
HPMC	0.55	8.28	0.954	0.57	8.21	0.995
CTS	0.46	7.8	0.967	0.56	8.85	0.924
SC	0.48	8.52	0.959	0.41	6.56	0.984
PLA	0.42	7.52	0.97	0.34	5.94	0.951

n, diffusion coefficient of power law model; *k*, constant of power law model

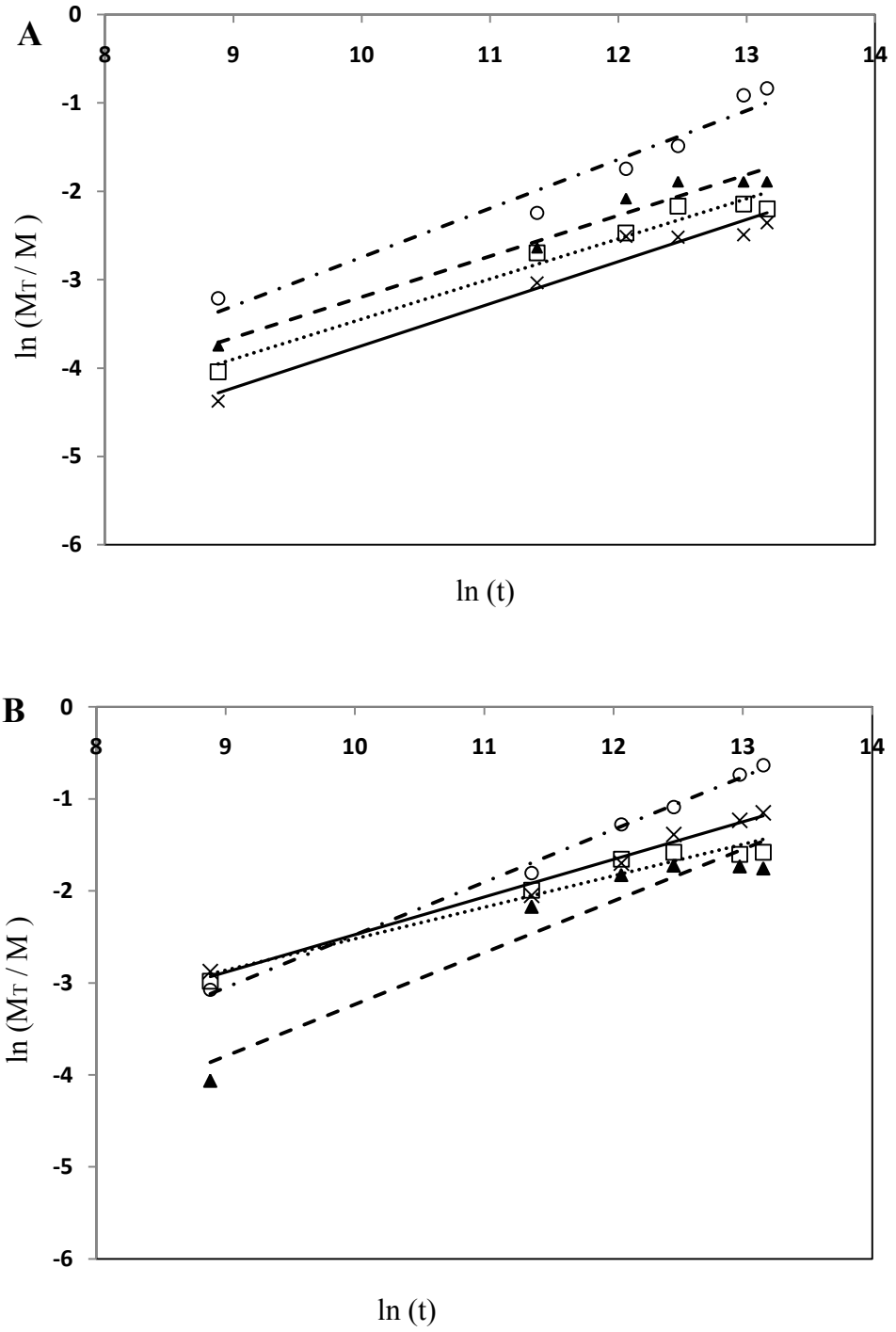


Figure 3: Curves of ln(MT/M) versus ln(t) for active films containing nisin (0.137 mg nisin cm-2). ○, HPMC active film; ▲, chitosan active film; ×, sodium caseinate active film; and □ PLA active film at 4°C (A) and 40°C (B).

The constant k is correlated to the macromolecular arrangement of the polymer matrix [19]. In the present study, Case I transport (Fickian) was observed for nisin diffusion in different biopolymers at both temperatures, as the predominant diffusion mechanism. Therefore, the diffusion exponent, n, is an important indicator of the mechanism of transport of a drug/active agent between the polymer and FSL.

The diffusion coefficients (D) were then calculated for each operating condition and reported after a statistical analysis (Table 1). The values of D determined ranged between $1.97 \pm 0.08 \times 10^{-13}$ and $97.8 \pm 3.2 \times 10^{-13} cm^2 s^{-1}$. In spite of the optimal solubility and bioactivity of nisin in different film forming solution, results show that diffusion coefficients obtained by active HPMC films were significantly higher than other biopolymers ($p < 0.001$). Contingent on the film structure and the affinity of the migrant active agent with the film polymers or with the desorption solution, diffusion coefficients might considerably differ [31]. As a matter of fact, nisin is a small polypeptide of 34 amino acids, with a molecular weight of about 3331 Dalton. Nisin polypeptide has overall positive charge [+4], with amphipathic properties [3]. Thus its relative affinity towards different polymer molecules influenced the nisin release into FSL. As it was clearly demonstrated at given temperature of 40°C the diffusion coefficients varied highly significantly among all polymer types. The pair-wise comparison indicated that polymer type effects the nisin diffusion as CTS and PLA revealed very lower D value as compared to $18.1 \pm 0.4 \times 10^{-13} cm^2 s^{-1}$ for SC, which itself was significantly inferior to $97.8 \pm 3.20 \times 10^{-13} cm^2 s^{-1}$ for HPMC (Table 1). HPMC is a disaccharide of D-glucose with methoxy and hydroxypropyl side chains to increase the hydrophilicity. HPMC carries no electrical charge [32], and due to stronger hydrophilic nature possess less structural stability. On the other hand, chitosan has positive charge under acidic conditions, which is due to protonation of its free amino groups. The implications of this are very important to biomedical applications as CTS maintains its structure in a neutral environment but solubilizes and degrade in an acidic environment [33]. As the FSL used in the present study had neutral pH, it helped CTS film to demonstrate higher stability with compact structure and thus lower D values. On the contrary, SC is made up of many hundreds of individual amino acids, each of which may have a positive or a negative charge, however at its isoelectric point (pH, 4.6), the net charge on SC is zero. At pH around neutral value, SC exhibits a net negative charge [34], which may possibly explain the higher retention of nisin due to opposite charge affinity between polymer and peptide. Similar interactions between PLA – nisin

is likely to occur as supports or particles that are generated from PLA are expected to have a neutral to slightly negative surface charge [35], a desirable characteristic for controlled release of cationic active agents. In a parallel manner, higher retention of nisin in the PLA matrix may be elucidated by the greater hydrophobicity of PLA films compared to HPMC and SC. These results are in accordance with the previous study, which had reported that nisin‟ tendency to aggregate, form large complexes, and to adhere to hydrophobic surfaces was attributable to its hydrophobicity [8].

Considering the influence of temperature, the experiments showed that D increased with temperature increased. In particular, the D values increased significantly for SC and HPMC, but temperature variation resulted in negligible change for diffusion coefficients of nisin related with PLA and CTS films. Temperature influence on diffusion coefficients had already been reported by several authors as activation energy required to induce diffusion of molecule is available. Thus higher nisin release was expected with increase in temperature, because molecular motion (Brownian motion) was increased by rising temperature.

3.3. Partition coefficient

In the present study, the partition coefficient (K) values were helpful to imply binding/interaction of nisin with the different film matrices; as low values of K, in the range of suggest that there is some affinity of the active agent with the film material [36]. It is reported earlier that the partition coefficient is directly correlated with the polarities of the diffusing substance and the biopolymer used as coating material. In case of similar chemical nature (polar), they will exhibit affinity and will interact with each other leading to a low partition coefficient and to a high retention of the migrating nisin in the polymer matrix. If the migrating active agent (nisin) is highly soluble in the film forming polymer than in the food (K « 1), it points out that at equilibrium just a little percentage of the migrating nisin may liberate [37, 38].

The values of K determined from experimental data revealed that nisin had good affinity with polymers than FSL as value of K< 1 for CTS, SC and PLA active films. Only HPMC expressed a value of K around 1 that shows some affinity for polymer and FSL (Table 3). As nisin has less affinity for HPMC than for other biopolymer films, it forms more free volume in HPMC network, which could result in weaker stability of film structure due to the intervention of

nisin molecules. For this reason, nisin particles without interaction with HPMC chains were released quickly, and that possibly explain reason for the high values of K observed for HPMC.

As explained earlier, nisin is a peptidic bacteriocin with amphipatic character; and may interact with non-polar sites of biodegradable polymers (Figure 1). However, the experimental values of D imply that the nisin did not interact strongly with HPMC. Nevertheless, the observed values of D are lower than what has been predicted earlier for nisin integrated in different film polymers [8, 39]. These results were expected as water-ethanol food simulating liquid exhibits lower water activity which subsequently results in lesser degradation or swelling phenomena of biopolymers.

Table 3: Partition coefficients (K) of nisin release from active films (HPMC, CTS, SC, PLA) at 4° and 40°C in water-ethanol simulant

Film type	Temperature (°C)	Partition coefficient (K)
HPMC	4°	0.79 ± 0.12^{a}
	40°	1.3 ± 0.50^{a}
CTS	4°	0.18 ± 0.01^{b}
	40°	0.21 ± 0.04^{b}
SC	4°	0.10 ± 0.05^{b}
	40°	0.47 ± 0.09^{b}
PLA	4°	0.13 ± 0.02^{b}
	40°	0.26 ± 0.05^{b}

$^{a \text{ to } b}$ means with different superscripts indicate significant difference ($p < 0.05$)

One important factor to consider while studying diffusion is the glass transition temperature (Tg) of biopolymers, Tg is the critical temperature at which the material changes its behavior from glassy/hard to rubbery/flexible. SC films are essentially plasticized to reduce its brittleness, however it renders the film with Tg around 40°C which is relatively lower as compared to other films [25, 40-42]. Due to rearrangement of biopolymer molecules and elastic/flexible characteristics at Tg, diffusion rates of nisin release from SC active films at higher temperature were elevated.

3.4. Microstructure study

Micrographs obtained from scanning electron microscope (SEM) with nisin incorporated HPMC, CTS, SC and PLA films revealed homogenous distribution and solubility of labeled nisin in film matrices (Figure 4).

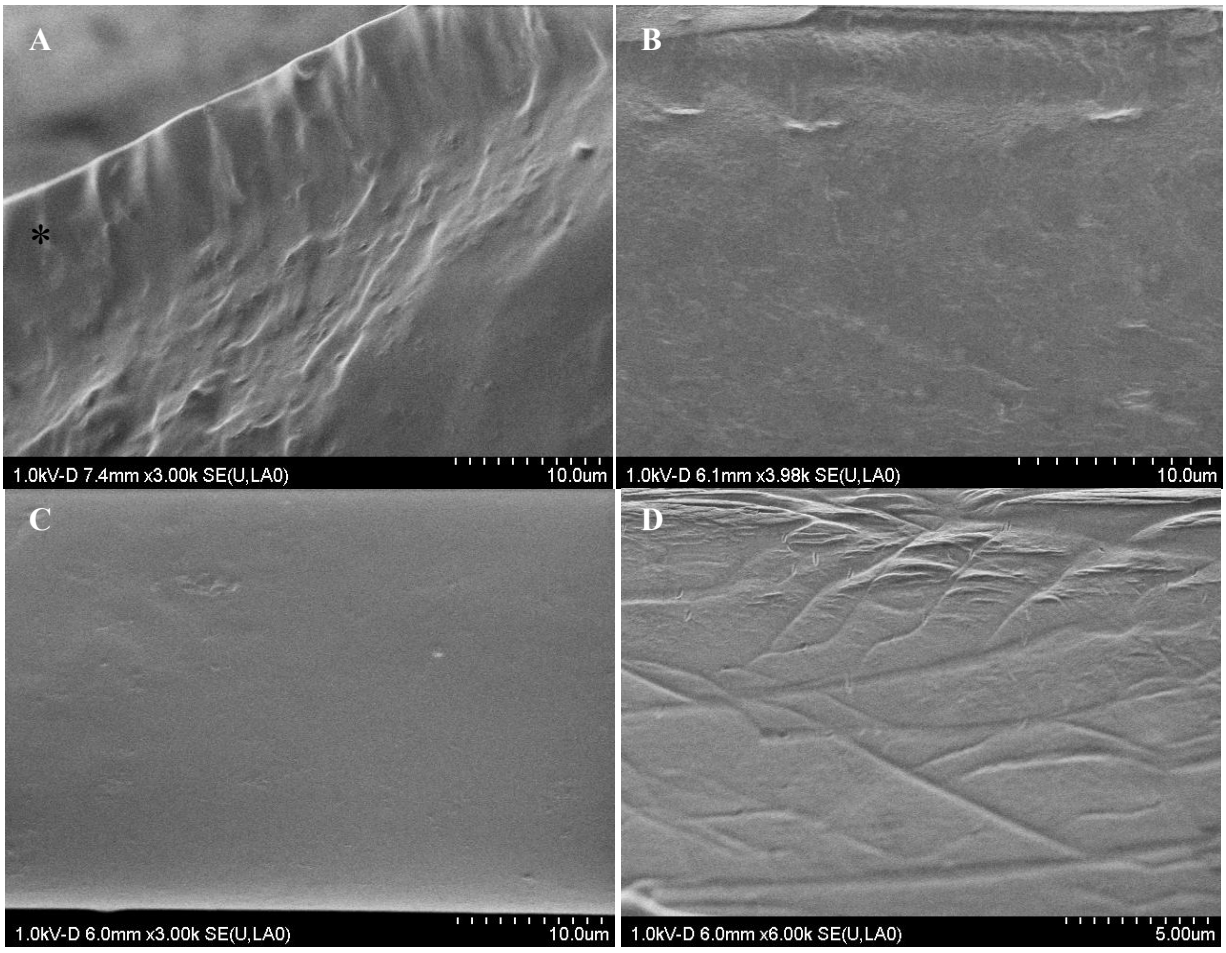


Figure 4: SEM micrographs of cryofractured nisin film of different biopolymeric origin (A to D: HPMC, CTS, SC and PLA respectively, * represents the film area in A)

At given nisin concentration (0.137 mg nisin cm^{-2} based on surface area of active films), micrographs suggested that the film forming solutions used to cast the investigated active films were homogeneous, at the concentrations tested in this study. In case of SC films, the films cross-section was highly smooth due to the plasticizing action of glycerol used essentially for overcoming brittleness of SC films. Most probably, the irregular lines pattern on PLA active

films cross-section is due to higher force and friction required to cut PLA film during cryo-fracturing with pre-chilled razor before mounting on SEM stub. It is worth noting that homogenous compact and cohesive structure without pores or cracks in films are in agreement with our objective, thus diffusion variations were solely influenced by biopolymer nature (affinity, charge), food simulating liquid (polarity, a_w, pH) and temperature (4°, 40°C).

3.5. Microbial growth control

Our previous work [26] revealed that HPMC-based active films with nisin as antimicrobial agent are efficient to control foodborne pathogenic *L. monocytogenes* strains when tested in a disc-diffusion assay. Since nisin is an antimicrobial agent with broad-spectrum efficacy against Gram-positive bacteria, the introduction of the nisin as antilisterial (*L. monocytogenes* CIP 82110^T) and antistaphylococcal (*Staphylococcus aureus* CIP 4.83) strains were tested in the HPMC, CTS, SC and PLA based active films. The results (Figure 5) revealed that *L. monocytogenes* CIP 82110^T and *Staphylococcus aureus* CIP 4.83 strains were inhibited by the incorporation of nisin into the films, as we expected, and the zones of inhibition were dependent of the film biopolymer used. A superior inhibitory effect against *L. monocytogenes* and *Staphylococcus aureus* was observed with HPMC, CTS and SC in association with nisin. However, antimicrobial activity only under the PLA-nisin films (1 cm^2) was observed against both spp. These results are in accordance with the nisin release rates from PLA based films in FSL discussed above, and hydrophobic yet biodegradable nature of PLA due to which its polymeric network resisted the water molecules to erode and widen the pores to control nisin liberation in surrounding medium. No significant difference existed in the zones of inhibition obtained with active HPMC or CTS films against bacterial strains tested, however, PLA based films significantly reduced the effective zone of inhibition due to lower desorption of nisin (Table 4). CTS had non-significant difference of nisin release kinetics as compared to PLA, but higher water activity of TSA inoculated medium initiated swelling of CTS biopolymer [43, 44]. This had resulted in lowering its structural integrity which correlates directly with package‟s capacity to retain active agent nisin. Similar explanation is valid for significantly higher inhibition zone (approx. double than the value obtained for PLA) by HPMC and SC, where nisin liberated and diffused well in agar to imply better inhibition zone [25, 26]. Comparison between active and control films without nisin revealed average control of pathogens only for inoculated solid

Table 4: The inhibition zone (cm) of the growth of *Staphylococcus aureus* CIP 4.83 (A) and *Listeria monocytogenes* CIP 82110^T by active films of different origin containing nisin (0.137 mg nisin cm^{-2} of film)

Biopolymers	*L. monocytogenes* CIP 82110^T		*S. aureus* CIP 4.83	
	Control	Active (nisin)	Control	Active (nisin)
HPMC	-	1.83 ± 0.06 ^{a}	-	1.5 ± 0.10 ^{a}
Chitosan	1.2 ± 0.2	2.0 ± 0.10 ^{a}	1.2 ± 0.1	1.6 ± 0.06 ^{a}
Sodium casseinate	-	1.73 ± 0.05 ^{b}	-	1.4 ± 0.10 ^{a}
Poly-lactic acid	-	1.07 ± 0.06 ^{c}	-	1.0 ± 0.10 ^{c}

HPMC= Hydroxypropyl methylcellulose; nisin= 0.137 mg nisin cm^{-2} of film
Tukey test: ^{c}, p<0.001; ^{b}, p<0.01; ^{a}, non-significant p>0.05

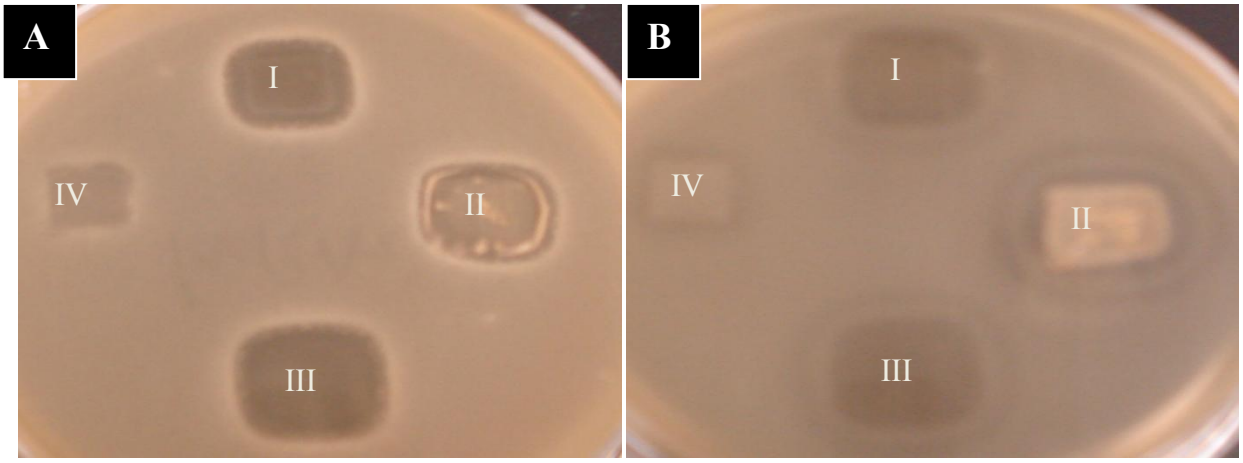


Figure 5: Growth inhibition of *Staphylococcus aureus* CIP 4.83 (A) and *L. monocytogenes* CIP 82110^T (B) inoculated in TSA-YE by nisin containing active films: I, HPMC film; II, chitosan film ; III, sodium caseinte film; IV, poly-lactic acid film at 37°C with over-night incubation.

growth medium in contact with CTS film. While the inhibitory effect was slightly improved for CTS-nisin as compared to other films, no significant increase of inhibitory zone suggested non-existence of synergistic interaction between CTS and nisin. Hence, it can be proposed that for foods with higher exposure to bacterial contamination at surface, PLA active films provide better nisin retention at elevated water activity. Whereas, foods having higher bacterial load in the food

depths, rest of the three biopolymers can be optimized to obtain effective control against foodborne pathogens.

4. Conclusion

The present study demonstrated the effectiveness of different biopolymers that may lead to the development of antimicrobial-nisin containing packaging materials. A rapid approach employing fluorescently labeled nisin can be successfully used to characterize the controlled release rates of bioactive peptides from active biodegradable films. HPMC, CTS, SC and PLA packaging films can act as a reservoir and progressively release nisin to sustain a constant anti-listerial and anti-staphylococcal inhibitory effect. Bacterial load is maximum at food surface, for this reason, food contact films that do not let antimicrobials to diffuse towards food or control their release are successful in preventing pathogen contamination or growth on the food surface but not as efficient in preventing growth inside the food as compared to those packaging films that allow faster migration to the food. Results illustrated that PLA and CTS films gave the highest percent retention of nisin in food simulating liquid. Whereas, higher temperature increase the diffusion rates of nisin from SC and HPMC due to biopolymer nature (Tg, solubility). Release tests run at 4° and 40°C with fluorescently labeled nisin revealed that the Fick‟s second law satisfactorily explains the release kinetic, with the nisin diffusion / partition coefficients practically dependent on biopolymer nature or temperature. A superior inhibitory effect against *L. monocytogenes* and *Staphylococcus aureus* was observed with HPMC, CTS and SC in association with nisin. However, inhibitory effect only under the area of PLA-nisin disc films was observed against both spp. due to hydrophobic nature of PLA and its higher nisin retention capability. These results imply that the investigated active films can be favorably used for prolonging the shelf life of packed foods, however in future, the ideal rate of antimicrobial release that offers the most effective inhibitory effect on pathogenic bacteria specific to a given food conditions (T°, pH, a_w, concentration…) may be achieved by blending two biopolymers with different nature, retention capacity and interaction with antimicrobial peptide to improve the food shelf-life and control over foodborne-illness outbreaks.

Acknowledgements

The authors are grateful to Higher Education Commission (HEC) Pakistan for financial support. We also thank Dr. Eefjan Breukink for his scientific support and Carole Jeandel for her technical assistance.

References

[1] M. Mastromatteo, M. Mastromatteo, A. Conte, M.A. Del Nobile, Advances in controlled release devices for food packaging applications. Trends in Food Science and Technology (2010) 1-8.

[2] L. Cao-Hoang, A. Chaine, L. Gragoire, Y. Wacha, Potential of nisin-incorporated sodium caseinate films to control Listeria in artificially contaminated cheese. Food Microbiology (2010) 1-5.

[3] E. Breukink, B. De Kruijff, The lantibiotic nisin, a special case or not? Biochimica et Biophysica Acta - Biomembranes 1462(1-2) (1999) 223-234.

[4] A. Carnet Ripoche, E. Chollet, E. Peyrol, I. Sebti, Evaluation of nisin diffusion in a polysaccharide gel: Influence of agarose and fatty content. Innovative Food Science and Emerging Technologies 7(1-2) (2006) 107-111.

[5] A. Hagiwara, N. Imai, H. Nakashima, Y. Toda, M. Kawabe, F. Furukawa, J. Delves-Broughton, K. Yasuhara, S.M. Hayashi, A 90-day oral toxicity study of nisin A, an anti-microbial peptide derived from Lactococcus lactis subsp. lactis, in F344 rats. Food and Chemical Toxicology 48(8-9) (2010) 2421-2428.

[6] W. Guiga, Y. Swesi, S. Galland, E. Peyrol, P. Degraeve, I. Sebti, Innovative multilayer antimicrobial films made with NisaplinÂ® or nisin and cellulosic ethers: Physico-chemical characterization, bioactivity and nisin desorption kinetics. Innovative Food Science and Emerging Technologies 11(2) (2010) 352-360.

[7] M. Imran, A.M. Revol-Junelles, A. Martyn, E.A. Tehrany, M. Jacquot, S. Desobry, Active food packaging evolution: Transformation from micro- to nanotechnology. Critical reviews in food science and nutrition 50(9) (2010) 1-24. Article in press.

[8] A. Teerakarn, D.E. Hirt, J.C. Acton, J.R. Rieck, P.L. Dawson, Nisin diffusion in protein films: Effects of film type and temperature. Journal of Food Science 67(8) (2002) 3019-3025.

[9] V. Coma, Bioactive packaging technologies for extended shelf life of meat-based products. Meat Science 78(1-2) (2008) 90-103.

[10] E. Chollet, Y. Swesi, P. Degraeve, I. Sebti, Monitoring nisin desorption from a multi-layer polyethylene-based film coated with nisin loaded HPMC film and diffusion in agarose gel by an immunoassay (ELISA) method and a numerical modeling. Innovative Food Science and Emerging Technologies 10(2) (2009) 208-214.

[11] L.R. Franssen, T.R. Rumsey, J.M. Krochta, Whey protein film composition effects on potassium sorbate and natamycin diffusion. Journal of Food Science 69(5) (2004).

[12] W. Guiga, S. Galland, E. Peyrol, P. Degraeve, A. Carnet-Pantiez, I. Sebti, Antimicrobial plastic film: Physico-chemical characterization and nisin desorption modeling. Innovative Food Science and Emerging Technologies 10(2) (2009) 203-207.

[13] I. Sebti, A. Ripoche Carnet, D. Blanc, R. Saurel, V. Coma, Controlled diffusion of an antimicrobial peptide from a biopolymer film. Chemical Engineering Research and Design 81(9) (2003) 1099-1104.

[14] D.S. Cha, K. Cooksey, M.S. Chinnan, H.J. Park, Release of nisin from various heat-pressed and cast films. LWT - Food Science and Technology 36(2) (2003) 209-213.

[15] P.L. Dawson, D.E. Hirt, J.R. Rieck, J.C. Acton, A. Sotthibandhu, Nisin release from films is affected by both protein type and film-forming method. Food Research International 36(9-10) (2003) 959-968.

[16] G.G. Buonocore, M.A. Del Nobile, A. Panizza, M.R. Corbo, L. Nicolais, A general approach to describe the antimicrobial agent release from highly swellable films intended for food packaging applications. Journal of Controlled Release 90(1) (2003) 97-107.

[17] M. Sameiro, T. Gonçalves, Fluorescent labeling of biomolecules with organic probes. Chemical Reviews 109(1) (2009) 190-212.

[18] M. Jamshidian, E.A. Tehrany, M. Imran, M. Jacquot, S. Desobry, Poly-Lactic Acid: Production, applications, nanocomposites, and release studies. Comprehensive Reviews in Food Science and Food Safety 9(5) (2010) 552-571.

[19] C.M.P. Yoshida, C.E.N. Bastos, T.T. Franco, Modeling of potassium sorbate diffusion through chitosan films. LWT - Food Science and Technology 43(4) (2010) 584-589.

[20] E. Mascheroni, V. Guillard, F. Nalin, L. Mora, L. Piergiovanni, Diffusivity of propolis compounds in Polylactic acid polymer for the development of anti-microbial packaging films. Journal of Food Engineering 98(3) (2010) 294-301.

[21] M.A. Del Nobile, A. Conte, A.L. Incoronato, O. Panza, Antimicrobial efficacy and release kinetics of thymol from zein films. Journal of Food Engineering 89(1) (2008) 57-63.

[22] S. Min, T.R. Rumsey, J.M. Krochta, Diffusion of the antimicrobial lysozyme from a whey protein coating on smoked salmon. Journal of Food Engineering 84(1) (2008) 39-47.

[23] E. Breukink, B. de Kruijff, Lipid II as a target for antibiotics. Nature Reviews Drug Discovery 5(4) (2006) 321-323.

[24] H. Müller, S. Grelier, P. Pardon, V. Coma, Antimicrobial and physicochemical properties of chitosan - HPMC-based films. Journal of Agricultural and Food Chemistry 52(21) (2004) 6585-6591.

[25] E. Kristo, K.P. Koutsoumanis, C.G. Biliaderis, Thermal, mechanical and water vapor barrier properties of sodium caseinate films containing antimicrobials and their inhibitory action on *Listeria monocytogenes*. Food Hydrocolloids 22(3) (2008) 373-386.

[26] M. Imran, S. El-Fahmy, A.M. Revol-Junelles, S. Desobry, Cellulose derivative based active coatings: Effects of nisin and plasticizer on physico-chemical and antimicrobial properties of hydroxypropyl methylcellulose films. Carbohydrate Polymers 81(2) (2010) 219-225.

[27] S. Liang, Q. Huang, L. Liu, K.I. Yam, Microstructure and molecular interaction in glycerol plasticized chitosan/poly(vinyl alcohol) blending films. Macromolecular Chemistry and Physics 210(10) (2009) 832-839.

[28] H.E. Hasper, N.E. Kramer, J.L. Smith, J.D. Hillman, C. Zachariah, O.P. Kuipers, B. De Kruijff, E. Breukink, An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. Science 313(5793) (2006) 1636-1637.

[29] J. Crank, The Mathematics of Diffusion. Oxford university press, London, UK (1975) 44-68.

[30] G. Rossi-Marquez, J.H. Han, B. Garcia-Almendarez, E. Castao-Tostado, C. Regalado-Gonzalez, Effect of temperature, pH and film thickness on nisin release from antimicrobial whey protein isolate edible films. Journal of the Science of Food and Agriculture 89(14) (2009) 2492-2497.

[31] I. Sebti, D. Blanc, A. Carnet-Ripoche, R.E. Saurel, V.E. Coma, Experimental study and modeling of nisin diffusion in agarose gels. Journal of Food Engineering 63(2) (2004) 185-190.

[32] R.M.M.A. Nuijts, In: Ocular toxicity of intraoperatively used drugs and solutions. Kugler Publications, New Amsterdam (1995) 19-20.

[33] S.A. Agnihotri, N.N. Mallikarjuna, T.M. Aminabhavi, Recent advances on chitosan-based micro- and nanoparticles in drug delivery. Journal of Controlled Release 100(1) (2004) 5-28.

[34] T. Kobori, A. Matsumoto, S. Sugiyama, pH-Dependent interaction between sodium caseinate and xanthan gum. Carbohydrate Polymers 75(4) (2009) 719-723.

[35] O.C. Farokhzad, S. Jon, A. Khademhosseini, T.N.T. Tran, D.A. LaVan, R. Langer, Nanoparticle-aptamer bioconjugates: A new approach for targeting prostate cancer cells. Cancer Research 64(21) (2004) 7668-7672.

[36] D. Chung, S.E. Papadakis, K.L. Yam, Release of propyl paraben from a polymer coating into water and food simulating solvents for antimicrobial packaging applications. Journal of Food Processing and Preservation 25(1) (2001) 71-87.

[37] E.A. Tehrany, S. Desobry, Partition coefficient of migrants in food simulants/polymers systems. Food Chemistry 101(4) (2007) 1714-1718.

[38] M.A.J.S. Van Boekel, Kinetic modeling of food quality: A critical review. Comprehensive Reviews in Food Science and Food Safety 7(1) (2008) 144-158.

[39] L. Bastarrachea, S. Dhawan, S.S. Sablani, J. Powers, Release kinetics of nisin from biodegradable poly(butylene adipate-co-terephthalate) films into water. Journal of Food Engineering (2010).

[40] L. Chen, C.Y. Tang, N.Y. Ning, C.Y. Wang, Q. Fu, Q. Zhang, Preparation and properties of chitosan/lignin composite films. Chinese Journal of Polymer Science (English Edition) 27(5) (2009) 739-746.

[41] A. Narladkar, E. Balnois, G. Vignaud, Y. Grohens, J.F. Bardeau, Morphology and glass transition of thin polylactic acid films. Polymer Engineering and Science 48(9) (2008) 1655-1660.

[42] N. Nyamweya, S.W. Hoag, Assessment of polymer-polymer interactions in blends of HPMC and film forming polymers by modulated temperature differential scanning calorimetry. Pharmaceutical Research 17(5) (2000) 625-631.

[43] H.K.S. Yadav, G.B. Joshi, M.N. Singh, H.G. Shivakumar, Naturally occurring chitosan and chitosan derivatives: A review. Current Drug Therapy 6(1) (2011) 2-11.

[44] M.P. Patel, R.R. Patel, J.K. Patel, Chitosan mediated targeted drug delivery system: A review. Journal of Pharmacy and Pharmaceutical Sciences 13(4) (2010) 536-557.

IV. Conclusion générale

« *There's plenty of room at the bottom* » est l'idée énoncée par Richard Freyman en décembre 1959 qui a initié le concept de Nanotechnologie. Cependant, ce n'est que très récemment que l'implantation de la nanotechnologie dans les produits alimentaires et l'emballage actif a été approuvé. L'émergence de la possibilité de maitriser la sécurité alimentaire *via* l'emballage actif est liée au concept « 3-BIOS » apparu avec la nanotechnologie, qui se réfère aux notions Bioactif - Biodégradable – Bio-nanocomposite. Le principal objectif du travail présenté dans cette thèse est de développer des films d'emballage biodégradables qui contiennent des peptides bio-actifs nano-vectorisés. Les connaissances des propriétés fonctionnelles et des interactions entre ces films nano-actifs et leurs composants, *i. e.* les nano-vecteurs délivrant les composés actifs, avec des aliments modèles et les microorganismes pathogènes alimentaires sont des pré-requis pour atteindre les objectifs définis.

-- Dans la première partie de ce projet, des films biodégradables à base d'HPMC, composé dérivé de la cellulose, incorporant une forme commerciale disponible de la nisine, la Nisaplin®, ont été élaborés et leurs capacités à remplir les fonctions attribués aux emballages évaluées. Il a été démontré que la présence de sels et de protéines dénaturés contenus dans la Nisaplin®, affecte fortement la transparence, l'épaisseur et le comportement de sorption d'eau des films actifs. Cependant, l'ajout d'agent plastifiant améliore de façon significative la flexibilité et la transparence, mais a un impact négatif sur la perméabilité et la résistance mécanique. Par ailleurs, les résultats montrent clairement que les films contenant Nisaplin® et 30% de glycérol possèdent des caractéristiques permettant la réalisation d'un film d'emballage bio-actif en raison de ses propriétés de transparence, d'homogénéité de la matrice, de structure stable possédant une bonne valeur mécanique, d'absorption de l'eau modérée et d'efficacité antibactérienne vis-à-vis de quelques souches de bactéries pathogènes rencontrées en industrie alimentaire.

-- La deuxième partie de ce travail est relatif à la réalisation d'un système nanométrique de libération de la nisine. La nisine a été encapsulée dans des nano-liposomes de différentes natures (lécithine d'origine marine, végétale ou pro-liposome) en utilisant une méthodologie rapide, innovante, applicable à échelle industrielle et non toxique qui utilise un système en continu de désintégration des cellules (Continuous Cell Disrupteur System - CCDS). Les lécithines de soja (SL) ont un plus fort taux d'oméga-6 et de lipides polaires comparativement aux lécithines marines et aux pro-liposomes qui contiennent uniquement des phospholipides saturés. Les liposomes préparés à partir de 5% de SL possèdent les meilleures efficacités d'encapsulation (EE

47%) et la meilleure stabilité physique. Tous les ingrédients utilisés sont non-toxiques (et peuvent également avoir des propriétés nutritionnelles comme les PUFA), ont un statut GRAS avec une notion de bioactif. Les études de microscopie électronique à transmission confirment la formation de nano-liposomes ainsi que les phénomènes de fusion et de formation de pores dans les liposomes de taille plus importante, qui sont indicateurs d'une libération de nisine. Les essais préliminaires réalisés en microplaque indiquent qu'un mélange nisine libre et encapsulée (1:1) possède une meilleure efficacité inhibitrice que la nisine seule ou la nisine nano-encapsulée seule.

-- Une fois les deux principaux composants, soit le film actif biodégradable et les systèmes de nano-transport de la nisine, obtenus et caractérisés, un emballage bio-actif biodégradable contenant les nanoparticules de nisine a été élaboré. Un des points majeurs limitant l'application des liposomes est leur tendance à « fuir » et perdre les composés encapsulés au cours du temps et la possibilité de déstabilisation des structures dans les aliments. Une approche novatrice de fusion des deux concepts de relargage contrôlé que sont la nano-vectorisation et l'immobilisation dans un bio-polymère, permet de formuler une nouvelle génération de films biodégradables, contenant soit le composé actif libre, soit le composé actif nano-encapsulé, soit les deux. Pour les films nano-actifs, les résultats indiquent clairement que les différentes propriétés physico-chimiques, incluant les propriétés barrière (oxygène et vapeur d'eau), la couleur et la transparence, restent similaires à celles des films d'HPMC natifs. Ces propriétés sont significativement améliorées quand la lécithine est utilisée directement, sans nano-restructuration. Les résultats des isothermes de sorption de l'eau ont montré une faible réduction de l'adsorption de l'eau quand la nisine se trouve sous forme de nano-émulsion dans la matrice d'HPMC. Les observations de la structure et de la morphologie du film par SEM et TEM permettent de visualiser ce nouveau concept de film d'emballage biodégradable contenant de la nisine nano-encapsulée qui permet d'augmenter la rétention de l'agent actif dans le polymère. Les résultats préliminaires des activités antibactériennes suggèrent que l'incorporation de la nisine (encapsulée et libre) dans des nano-émulsions peut être un moyen efficace de contrôle des bactéries pathogènes, sans altérer les propriétés physico-chimiques fondamentales d'un film d'HPMC.

-- Le marquage de biomolécules par des marqueurs fluorescents est apparu ces dernières années comme une méthodologie innovante à des fins analytiques en microbiologie alimentaire, médecine ou pharmacie en raison de sa précision, sa rapidité, ses limites de détection très faibles, son faible coût d'obtention, sa possibilité de détection *in vivo*…L'utilisation de nisine Z

marquée par un composé fluorescent est une nouvelle stratégie permettant de détecter et quantifier sa biodisponibilité dans un système alimentaire complexe, dans un film d'emballage (cinétique de relargage) et dans des systèmes de libération tels que des nanocapsules. Cette molécule marquée peut de plus être utilisée pour la compréhension des mécanismes membranaires d'action de la nisine vis-à-vis des bactéries pathogènes. La nisine Z fluorescente obtenue a un poids moléculaire déterminé par spectrométrie de masse de 3717,3 Da, confirmé par un état de charge des ions $[M+3H]^{3+}$ (m/z 1240,1) et $[M+2H]^{2+}$ (m/z 1859,4) de la nisine marquée. Cette masse résulte de la liaison entre le carboxyl de la nisine Z et le groupe aminé du fluorochrome. L'efficacité du marquage a été mesurée par des analyses TLC et les essais à l'acide bicinchominique ont révélé un taux de 10% de produit marqué. La comparaison des fuites de K^+ intracellulaires et des modifications de potentiel membranaire induit par la nisine et la nisine marquée, sur trois souches de *Listeria,* indique des différences d'activité non significatives. Ces résultats indiquent que le processus de marquage de la nisine n'a pas altéré son activité de façon significative. Pour illustrer une des applications potentielles de la nisine marquée, des études de microscopie confocale ont été réalisées sur des souches de *L. monocytogenes*, *L. ivanovii* et *L. grayi*. Ces études indiquent que des interactions entre la nisine Z et la membrane sont localisées au niveau des sites de division cellulaire chez les trois souches étudiées.

-- Prédire la diffusion de la nisine du film d'emballage vers l'aliment est nécessaire pour s'assurer de son efficacité antibactérienne. Une méthode rapide utilisant la nisine marquée par le composé fluorescent a été utilisée avec succès pour caractériser le taux de relargage de composé actif à partir de film biodégradables. Les films HPMC, CTS, SC et PLA peuvent agir comme réservoir et libérer progressivement la nisine, afin d'obtenir un effet inhibiteur anti-staphylocoques et anti-*Listeria*. Le taux de bactéries est maximal au niveau de la surface de l'aliment. Le film en contact avec l'aliment qui ne permet pas à l'agent antibactérien de diffuser vers l'aliment ou qui ne permet pas un contrôle de sa libération, exercera une action inhibitrice efficace en surface uniquement. Ce film sera moins efficace par rapport à un film permettant un relargage contrôlé et une migration plus rapide du composé actif dans l'aliment. Les résultats indiquent que les films de PLA et de CTS ont les plus hauts pourcentages de rétention de la nisine dans un aliment simulé liquide. Des températures plus élevées augmentent les taux de diffusion de la nisine à partir des films SC et des films d'HPMC en raison de la nature du bio-

polymère (Tg, solubilité). Les tests de relargage effectués à 4 °C et 40 °C montrent que la deuxième loi de Fick explique de façon satisfaisante les cinétiques de relargage, avec un coefficient de diffusion/partition pratiquement dépendant de la nature du bio-polymère et de la température. Un effet inhibiteur supérieur vis-à-vis de *L. monocytogenes* et *Staphylococcus aureus* a été observé avec l'HPMC, le CTS et le CS en association avec la nisine. Ces résultats indiquent que le choix du bio-polymère est important, et doit permettre la biodisponibilité de l'antibactérien requise en surface et en profondeur de l'aliment pour un effet antibactérien significatif.

"There's plenty of room at the bottom", a famous idea proffered by Richard Feynman in December 1959 initiated the conception of Nanotechnology. However, it is very recent that implementation of nanotechnology in food products and active packaging has been approved due to its enabling nature. The emerging revolution concerning food safety through packaging relies on 3-BIOs blend with nanotechnology, which refers to Bioactive, Biodegradable and Bio-nanocomposite. The principal objective of the present study was to develop the biodegradable packaging films containing bioactive peptides nano-vectorized. Knowledge about the functional properties and interactions of innovative nano-active films, and their sub-component i.e. nano-delivery systems carrying active agents, with model foods and food borne pathogens is pre-requisite to attain the objectives of the present thesis work.

-- In the first part of this project, biodegradable films based on cellulose derivative HPMC embedded with commercially available food bio-preservative „nisin" were fabricated and their suitability to fulfill basic packaging functions was studied. It was demonstrated that along-with nisin the presence of salt and denatured protein in Nisaplin® greatly affected the transparency, thickness and water sorption behaviour of active films. However, the presence of plasticizer substantially improved the stretch-ability and transparency but adversely altered the permeability and tensile strength. Furthermore, the results clearly demonstrated that formulation containing HPMC, nisin and 30% glycerol is a promising bioactive film due to its transparent and homogenous matrix, stable structure with good stretch-ability, moderate water sorption and good antimicrobial efficiency against a spectrum of food pathgens.

-- Second part of the project comprises the fabrication of nano-delivery systems for antimicrobial peptide nisin (only approved bacteriocin by WHO and FDA). Nisin was encapsulated in different nanoliposomes (Marine-, Soy-, Pro-liposomes) using an innovative, rapid, industrial scale and nontoxic continuous cell disruption system (CCDS) method of microfluidic format. Soy-lecithin (SL) had higher omega-6 and polar lipids as compared to marine-lecithin while proliposomes contained only saturated phospholipids. Soy liposomes prepared from SL 5 % provided best possible EE (47%) and physical stability. All of the ingredients used in the present study are non-toxic (even nutritive as PUFA), GRAS (nisin) with bio-additive notion. The microscopical study confirmed successful formation of nanoliposomes as well as the fusion and pore formation phenomenon in larger liposomes that indicated slow

release of lantibiotic nisin. The initial microplate antimicrobial assay had revealed that blend of free and encapsulated nisin (1:1) had indicated better control of *Listeria monocytogenes* as compared to 100% encapsulated nisin or free nisin.

-- Once the successful completion of two major sub-components i.e. biodegradable active films and nano-carrier systems containing nisin was achieved, formerly unknown properties attributed to engineered nanoparticles were introduced in the active coatings domain. One of the major limitations of liposome application is the tendency to leak and lose encapsulated components over time, moreover, gradual coalescence and destability may occur in food system. Thus innovative approach of fusion of two concepts of controlled release i.e. nisin nanoencapsulation and biopolymer immobilizing to formulate the next generation biodegradable films embedded with either active agent, nano-encapsulated active agent or both of them had resolved liposome delivery limitations. For nano-active films, the results clearly showed that different physic-chemical properties including barrier (oxygen and water vapor permeability), color and transparency remained similar to native HPMC films and significantly improved than using lecithin directly without nano-scale restructuring. The results of water sorption isotherms have shown a slight improvement in resistance to water adsorption when nisin was embedded as nano-emulsion in HPMC matrix. The observation of the structure and morphology of the film by SEM and TEM established the new concept of biodegradable packaging containing nano-encapsulated nisin that can help to improve the active agent retention in polymeric network. Preliminary antimicrobial results suggest that incorporation of nisin in nano-emulsion form (encapsulated and free) can possibly be an effective approach to control pathogen without compromising the basic physico-chemical attributes of composite HPMC films.

-- The labeling of biomolecules by fluorescent markers has emerged as an innovative methodology for bio-analytical purposes in food microbiology, medicine and pharmaceutics due to the advantages of precision, rapidity, wide detection limits, no extraction cost, in vivo recognition… The optimization, purification and characterization of nisin Z fluorescent labeling will not only form the basis of new strategies to detect and quantify the nisin bioavailability in food complex systems, food packaging (release kinetics) and nano-delivery systems (peptide encapsulation) but also facilitate us to understand the mechanism of action against food-borne pathogens. The fluorescently labeled nisin Z prepared had a molecular weight of 3717.3 Da by

mass spectroscopy, confirmed by charge state of $[M+3H]^{3+}$ (m/z 1240.1) and $[M+2H]^{2+}$ (m/z 1859.4) ions of labeled peptide as a result of bond between carboxyl and amine groups of nisin Z and AAA-flu respectively. The efficiency of labeling process was revealed by TLC analyses while bicinchoninic acid assay demonstrated 10 % yield of labeled product as compared to initial nisin concentration in reaction mix. Comparison of intracellular K^+ leakage and transmembrane electrical potential ($\Delta\Psi$) against three listerial strains between pure and labeled nisin demonstrated non-significant difference, which implies that nisin had endured the labeling process without any loss to its activity. As one of the applications of fluorescein labeled nisin, confocal microscopic study against *L. monocytogenes, L.innocua* and *L. ivanovii* successfully demonstrated the peptide-lipid II interaction at the cell-division sites as possible mechanism of action against this food borne pathogen.

-- Predicting nisin diffusion from packaging to the model food system is necessary to assess its antimicrobial activity. A rapid approach employing fluorescently labeled nisin can be successfully used to characterize the controlled release rates of bioactive peptides from active biodegradable films. HPMC, CTS, SC and PLA packaging films can act as a reservoir and progressively release nisin to sustain a constant anti-listerial and anti-staphylococcal inhibitory effect. Bacterial load is maximum at food surface, for this reason, food contact films that do not let antimicrobials to diffuse towards food or control their release are successful in preventing pathogen contamination or growth on the food surface but not as efficient in preventing growth inside the food as compared to those packaging films that allow faster migration to the food. Results illustrated that PLA and CTS films gave the highest percent retention of nisin in food simulating liquid. Whereas, higher temperature increase the diffusion rates of nisin from SC and HPMC due to biopolymer nature (Tg, solubility). Release tests run at 4° and 40°C with fluorescently labeled nisin revealed that the Fick‟s second law satisfactorily explains the release kinetic, with the nisin diffusion / partition coefficients practically dependent on biopolymer nature or temperature. A superior inhibitory effect against *L. monocytogenes* and *Staphylococcus aureus* was observed with HPMC, CTS and SC in association with nisin. These results imply that choice of biopolymer is significant in providing requisite bioavailability of antimicrobial compounds at exterior surface and inside food system.

Perspectives

Les connaissances relatives aux propriétés fonctionnelles du film, aux interactions internes à l'emballage ou entre l'emballage et l'aliment et aux mécanismes mis en jeux entre le nanosystème de relargage et les systèmes biologiques (bactéries pathogènes responsables des intoxications alimentaires) sont fondamentales pour une meilleure compréhension du fonctionnement de ces systèmes complexes. Les travaux développés dans cette thèse ouvrent de perspectives dans ces domaines :

-- Un film composite à base d'HPMC, de nisine et d'agent plastifiant a démontré des propriétés physico-chimiques et des capacités antibactériennes prometteuses. *Dans le futur, la durée de vie des aliments peut être améliorée en utilisant la théorie des barrières ou « hurdle technology », qui repose sur l'action synergique entre plusieurs composés actifs, ou l'association de différents bio-polymères ;*

-- L'encapsulation de la nisine dans des systèmes de taille nanométrique en utilisant le CCDS est une méthode rapide, efficace, non toxique et applicable à une échelle industrielle. *D'autres composés actifs (antioxydants, micronutriments, antibactériens, ...) peuvent être véhiculés par des nano-vecteurs pour améliorer leur biodisponibilité ;*

-- Les films biodégradables nanoactifs sont prometteurs comparativement aux films natifs. *Différents nano-systèmes libérateurs de composés actifs peuvent être utilisés simultanément ou dans différents bio-polymères pour améliorer les propriétés fonctionnelles fondamentales des films d'emballage et les taux de diffusion des composés actifs* ;

-- Le marquage de biomolécules à l'aide de marqueurs fluorescents présente des atouts de précision, rapidité, limites de détection basses, coûts d'obtention faibles et possibilité d'utilisation *in vivo… D'autres composés actifs ou bio-polymères peuvent être marqués de façon à pouvoir expliquer des mécanismes d'interactions non encore caractérisés et ceci afin de résoudre des problèmes de contamination des aliments* ;

-- Le choix des bio-polymères est important pour obtenir la biodisponibilité désirée du composé actif. *Des films multicouches et des biopolymères en mélange ou nanocomposites peuvent remplir différemment les fonctions demandées pour permettre la conservation du produit*

-- *La compréhension des interactions/migrations entre les composés actifs et les nano-systèmes de libération est importante pour maîtriser la capacité de libération de la nisine et ainsi améliorer la sécurité alimentaire ;*

-- *Les mécanismes d'action des « nano-billes » de nisine vis-à-vis des systèmes biologiques restent encore à élucider….*

Ces différents travaux permettront dans le futur de mieux maitriser le développement d"emballages actifs garants de la sécurité des aliments.

Perspectives:

Information about the bioactive film functional properties, intra-packaging and package-food interaction, and mechanism of nano-delivery systems with biological systems (pathogens responsible for food borne illness outbreaks) is important as far as food safety is concerned.

-- Composite films of HPMC, nisin and plasticizer provided promising physico-chemical and antimicrobial results. *Therefore, in future food shelf-life can be improved by Hurdle technology (synergistic action of multiple active agents) or blend of different biopolymers.*

-- Encapsulation of nisin in nano-delivery systems by CCDS is rapid, efficient, non-toxic and industrial scale technique. *Thus, other vital active agents (antioxidants, antimicrobials, micronutrients…) can be transformed in nano-carriers to improve their bioavailability.*

-- Nano-active biodegradable films are promising as compared to native films. *So, different nano-delivery systems possibly will be used simultaneously or in different biopolymers to improve fundamental film attributes and active agent diffusion rates*

-- The labeling of biomolecules with fluorescent markers has advantages of precision, rapidity, wide detection limits, no extraction cost, and *in vivo* recognition. *Thus other active agents or biopolymer constituents may well be labeled to detect, quantify or understand the unknown interactions to solve food spoilage problems.*

-- Choice of biopolymer is significant in providing requisite bioavailability of antimicrobial compounds. *Multi-layer films, biopolymer blending or nano-composites use may distinctively fulfill the conservation needs of desired food product.*

-- *The interaction/migration study between active agents and nano-delivery systems might be of significant importance concerning the superior delivery of nisin to improve food safety.*

-- *Nisin nano-bullets' mechanism of action against biological systems is yet a question, requiring answers…*

IV. Annexes

Comprehensive REVIEWS in Food Science and Food Safety

Poly-Lactic Acid: Production, Applications, Nanocomposites, and Release Studies

Majid Jamshidian, Elmira Arab Tehrany, Muhammad Imran, Muriel Jacquot, and Stéphane Desobry

Abstract: Environmental, economic, and safety challenges have provoked packaging scientists and producers to partially substitute petrochemical-based polymers with biodegradable ones. The general purpose of this review is to introduce poly-lactic acid (PLA), a compostable, biodegradable thermoplastic made from renewable sources. PLA properties and modifications via different methods, like using modifiers, blending, copolymerizing, and physical treatments, are mentioned; these are rarely discussed together in other reviews. Industrial processing methods for producing different PLA films, wrappings, laminates, containers (bottles and cups), are presented. The capabilities of PLA for being a strong active packaging material in different areas requiring antimicrobial and antioxidant characteristics are discussed. Consequently, applications of nanomaterials in combination with PLA structures for creating new PLA nanocomposites with greater abilities are also covered. These approaches may modify PLA weaknesses for some food packaging applications. Nanotechnology approaches are being broadened in food science, especially in packaging material science with high performances and low concentrations and prices, so this category of nano-research is estimated to be revolutionary in food packaging science in the near future. The linkage of a 100% bio-originated material and nanomaterials opens new windows for becoming independent, primarily, of petrochemical-based polymers and, secondarily, for answering environmental and health concerns will undoubtedly be growing with time.

Introduction

Today, polymers and materials used for food packaging consist of a variety of petrochemical-based polymers, metals, glass, paper, and board, or combinations hereof. The durability and degradability of packaging materials are 2 contradictory subjects; the 1st is desirable for packaging stability and protection for its contents during shelf life and the 2nd for its rapid degradation in the environment (Bohlaman 2005).

Advantages of petrochemical-based polymers, which encouraged industries to use them are: (a) low cost and high-speed production; (b) high mechanical performance; (c) good barrier properties; and (d) good heat sealability. On the other hand, several disadvantages include: (a) declining oil and gas resources; (b) increasing oil and gas prices during recent decades; (c) environmental concerns for their degradation or incineration and global warming; (d) uneconomical costs and cross-contaminations in their recycling; and (e) consumer toxicity risks about their monomers or oligomers migrating to edible materials (Amass and others 1998; Chandra and Rustgi 1998; Mohanty and others 2000; Siracusa and others 2008).

MS 20100340 Submitted 3/29/2010, Accepted 6/10/2010. *Authors are with École nationale supérieure d'agronomie et des industries alimentaires, Institut National Polytechnique de Lorraine, 2 avenue de la Forêt de Haye, 54501 Vandoeuvre, France. Direct inquiries to author Jamshidian (E-mail: majid.jamshidian@ensaia.inpl-nancy.fr).*

Mechanical recycling (segregated plastics, mixed plastics), biological recycling (sewage, compost, soil), and energy recovery (incineration, pyrolysis) are 3 alternative ways for plastics waste management, with each having some advantages and disadvantages as to economical, processing, and technological aspects (Scott 2000).

The above-mentioned concerns are negligible for biopolymers concerning the biodegradation process that takes place in nature. Biodegradation is defined as the degradation of a polymer in natural environments that includes changes in chemical structure, loss of mechanical and structural properties, and finally, changing into other compounds like water, carbon dioxide, minerals, and intermediate products like biomass and humic materials. The natural environments contain chemical, biological, and physical forces with impinging factors like temperature, humidity, pH, O_2 presence, and so on, which determine the rate and products of the biodegradation process (Zee 2005).

Biopolymers are produced from natural resources and crude oil. Four categories of biopolymers are recognized: (a) extracted directly from natural raw materials, such as polysaccharides like starch and cellulose; proteins like gelatin, casein, and silk; and marine prokaryotes; (b) produced by chemical synthesis from bio-derived monomers such as poly-lactic acid (PLA), also known as poly(lactic acid) in the literature; (c) produced by microorganisms or genetically modified bacteria such as polyhydroxyalkanoates (PHA), polyhydroxybutyrate (PHB), hydroxyl-valerate (PHV), bacterial cellulose, xanthan, and pullan; and (d) produced from crude oil like aliphatic and aromatic polyesters, polyvinyl alcohol, and modified

© 2010 Institute of Food Technologists®
doi 10.1111/j.1541-4337.2010.00126.x

polyolefins, which are sensitive to temperature and light (Chandra and Rustgi 1998; Clarinval and Halleux 2005).

PLA or poly-lactide was discovered in 1932 by Carothers (at DuPont). He was only able to produce a low molecular weight PLA by heating lactic acid under vacuum while removing the condensed water. The problem at that time was to increase the molecular weight of the products; and, finally, by ring-opening polymerization of the lactide, high-molecular weight PLA was synthesized. PLA was 1st used in combination with polyglycolic acid (PGA) as suture material and sold under the name Vicryl in the U.S.A. in 1974 (Mehta and others 2005).

In comparison to other biopolymers, the production of PLA has numerous advantages including: (a) production of the lactide monomer from lactic acid, which is produced by fermentation of a renewable agricultural source corn; (b) fixation of significant quantities of carbon dioxide via corn (maize) production by the corn plant; (c) significant energy savings; (d) the ability to recycle back to lactic acid by hydrolysis or alcoholysis; (e) the capability of producing hybrid paper-plastic packaging that is compostable; (f) reduction of landfill volumes; (g) improvement of the agricultural economy; and (h) the all-important ability to tailor physical properties through material modifications (Dorgan and others 2000).

Briefly, PLA is based on agricultural (crop growing), biological (fermentation), and chemical (polymerization) sciences and technologies. It is classified as generally recognized as safe (GRAS) by the United State Food and Drug Administration (FDA) and is safe for all food packaging applications (Conn and others 1995; FDA 2002).

Production steps, general properties, applications, processing technologies, modifications, and biodegradability of PLA are presented in this review. Consequently, migration and release studies of active compounds and PLA abilities making it a potential active food packaging are also discussed; finally, recent different types of nanocomposites used for improving PLA applications are reviewed.

PLA Production

Lactic acid (2-hydroxy propionic acid), the single monomer of PLA, is produced via fermentation or chemical synthesis. Its 2 optically active configurations, the L(+) and D(−) stereoisomers are produced by bacterial (homofermentative and heterofermentative) fermentation of carbohydrates. Industrial lactic acid production utilizes the lactic fermentation process rather than synthesis because the synthetic routes have many major limitations, including limited capacity due to the dependency on a by-product of another process, inability to only make the desirable L-lactic acid stereoisomer, and high manufacturing costs (Datta and Henry 2006).

The homofermentative method is preferably used for industrial production because its pathways lead to greater yields of lactic acid and to lower levels of by-products. The general process consists of using species of the *Lactobacillus* genus such as *Lactobacillus delbrueckii, L. amylophilus, L. bulgaricus, and L. leichmanii*, a pH range of 5.4 to 6.4, a temperature range of 38 to 42 °C, and a low oxygen concentration. Generally, pure L-lactic acid is used for PLA production (Mehta and others 2005).

PLA has a variable molecular weight and only its high molecular weight polymer is used in the packaging industry. Three ways are possible for the polymerization of lactic acid; (a) direct condensation polymerization; (b) direct polycondensation in an azeotropic solution (an azeotrope is a mixture of 2 or more chemical liquids in such a ratio that its composition cannot be changed by simple distillation. This occurs because, when an azeotrope is boiled, the resulting vapor has the same ratio of constituents as the original mixture); and (c) polymerization through lactide formation. The 1st method is based on esterification of monomers by the aid of some solvents and exudated water is removed using progressive vacuum and high temperatures. Obtaining high molecular weight polyesters with good mechanical properties via this method is not easy, although precondensates may be of interest for the preparation of biodegradable glues or lacquers, since the –OH and -COOH end groups allow cross-linking with suitable inorganic or organic multivalent additives (Hartmann 1998).

Producing high molecular weight PLA polymers by direct polycondensation in an azeotropic solution and also application of some catalysts is more practicable. The azeotropic solution helps to decrease the distillation pressures and facilitates PLA separation from the solvent by application of molecular sieves. The variety and content of catalysts, solvent volume percentages, and the reaction time on the preparation of PLA have been studied. The results identified by using improved experimental equipment, the proper complex catalyst, and solvent volume ratio, in order to obtain a molecular weight of PLA of 6.6×10^4 (Li and others 2006).

Polymerization through lactide formation is being industrially accomplished for high molecular weight PLA production. Lactide is a cyclic dimer formed by removing water under mild conditions and without solvent. L-lactide, meso (L,D) lactide, and D-lactide are products of L-lactic acid and D-lactic acid. The terms poly lactide and poly (L-lactide) have been used in many references instead of PLA.

Lactide purification is accomplished by vacuum-distillation of high temperatures. After the vacuum-distillation of L-lactide, high molecular weight PLA with a controlled optical and crystal purity is formed by ring-opening polymerization. Ring-opening polymerization of lactide can be carried out in melt or solution by cationic, anionic, and coordination mechanisms, depending on the initiator utilized. The most considered active initiator for the L-lactide ring-opening polymerization is stannous octoate (bis 2-ethyl hexanoate, $SnOct_2$), which causes a low degree of racemization at high temperature. It has a low toxicity and is accepted by FDA (Puaux and others 2007).

A kinetics study for ring-opening polymerization of L-lactide with stannous octoate has been done and a correlated mathematical modeling developed for that (Mehta and others 2007).

The choice of initiator system, co-initiator as chain control agent, catalyst concentration, monomer-to-initiator ratio, and polymerization temperature and time significantly affect the polymer properties. Properties such as molecular weight, degree of crystallinity, and residual monomer content, in turn affect the physico-mechanical properties of polylactide and its copolymers (Vink and others 2004).

Figure 1 shows PLA production steps by ring-opening polymerization using stannous octoate as an initiator.

New ideas for decreasing PLA final price and making production processes more eco-friendly, in comparison to earlier production process, include usage of crop residue (stems, straw, husks, and leaves) from corn or, potentially, other crops, and use of unfermentable residues as a heat source, as well as substituting some part of electricity energy by wind power energy. These approaches decrease the consumption of fossil fuels and corn starch as raw materials and also diminish polluting air, water, and waste emissions to the environment (Vink and others 2003).

One of the most positive points of PLA production in comparison with the other hydrocarbon-based polymers is the decrease of CO_2 emission. Carbon dioxide is believed to be the most

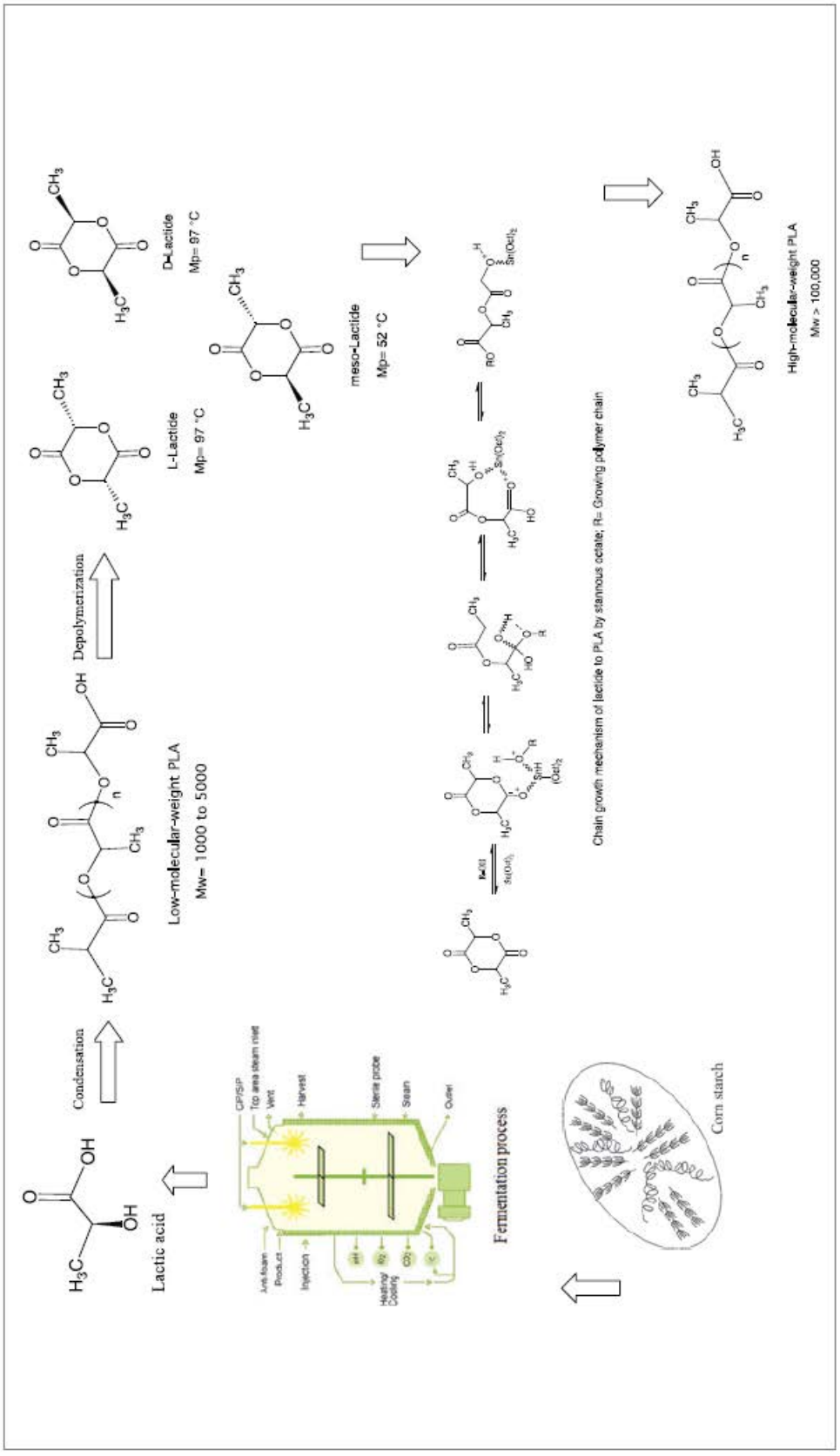


Figure 1–Current production steps for PLA.

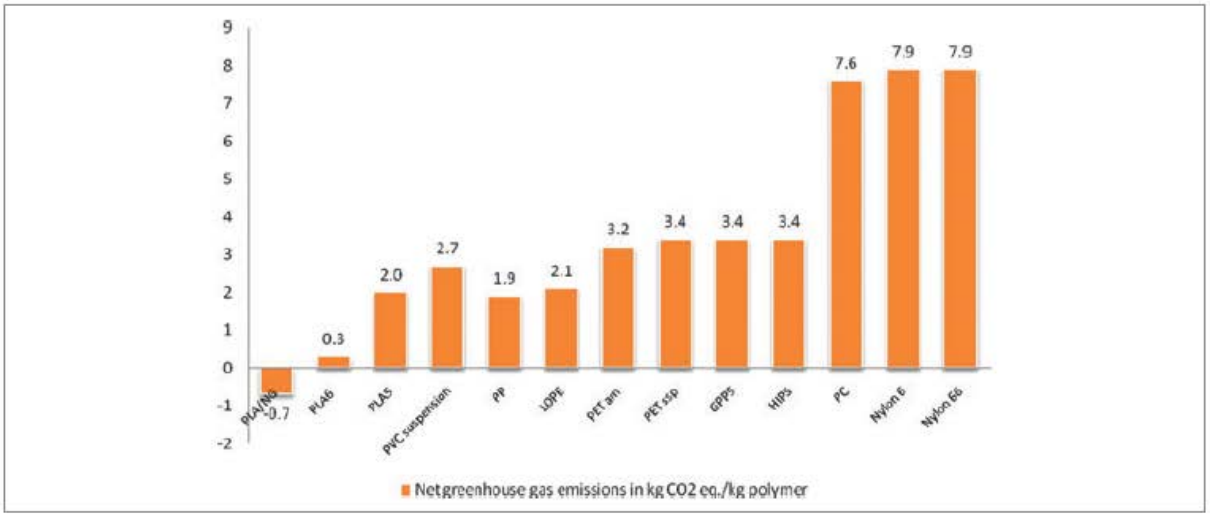


Figure 2–Net greenhouse gas emission of commercial PLAs and other polymers. PLA/NG = NatureWorks® PLA next generation, PLA5 = NatureWorks® PLA in 2005, PLA6 = NatureWorks® in 2006, HIPS = high impact poly(styrene), PC = poly(carbonate), GPPS = general purpose poly(styrene), PET am = PET amorph, PET ssp = PET solid sate polycondensed.

important contributor to global climate change and its warming. Because, carbon dioxide is absorbed from air when corn is grown, use of PLA has the potential to emit fewer greenhouse gases compared to competitive hydrocarbon-based polymers. "Net" or "residual" emissions are calculated as total emissions from the cradle to the factory gate minus carbon dioxide uptake that occurs during corn production. This amount is negative for present PLA production. It means the total CO_2 consumption from the cradle to factory is more than its emission to the environment (Bogaert and Coszach 2000). Vink and others (2003) concluded if all produced PLA articles enter into composting process which emits CO_2 in atmosphere; Nevertheless, their net CO_2 emission is less than for other polymers. Vink and others (2007) showed the net greenhouse gas emissions of NatureWorks® PLA polymers decreased from 2 kg CO_2 eq./kg polymer in 2003 to 0.3 kg in 2006. The authors also estimated the −0.7 kg of CO_2 for next PLA generation using wind energy in the near future. By this opportunity, PLA can even become a greenhouse gas sink by the implementation of a new process technology combined with the use of green power to drive the production processes (Figure 2).

NatureWorks® LLC, the present leader in PLA technology, has a 50 to 50 joint venture between Cargill incorporated and Dow Chemical Co. and was formed in November 1997. In 2002, they started the world's first full-scale PLA plant in Blair, Nebraska, U.S.A., capable of producing 140,000 metric tons per year. NatureWorks® entered into a joint venture between Cargill and Teijin Limited of Japan in December 2007 (www.natureworksllc.com).

Other major companies involved in PLA manufacturing are Toyobo, Dai Nippon Printing Co., Mitsui Chemicals, Shimadzu, NEC, Toyota (Japan), PURAC Biomaterials, Hycail (The Netherlands), Galactic (Belgium), Cereplast (U.S.A.), FkuR, Biomer, Stanelco, Inventa-Fischer (Germany), and Snamprogetti (China) (Wolf 2005; Platt 2006).

Sources for lactic acid fermentation

NatureWorks® exclusively uses corn starch as raw material for lactic acid production via lactic fermentation. Many studies have been conducted to find other sources of carbohydrates for lactic acid production. The use of a specific carbohydrate feedstock depends on its price, availability, and purity. Some agricultural by-products, which are potential substrates for lactic acid production include, cassava starch, lignocellulose/hemicellulose hydrolysates, cottonseed hulls, Jerusalem artichokes, corn cobs, corn stalks, beet molasses, wheat bran, rye flour, sweet sorghum, sugarcane press mud, cassava, barley starch, cellulose, carrot processing waste, molasses spent wash, corn fiber hydrolysates, and potato starch (Reddy and others 2008).

Other sources of carbohydrate for lactic acid production include kitchen wastes (Kim and others 2003; Zhang and others 2008), fish meal wastes (Huang and others 2008), and paper sludge (Budhavaram and Fan 2007). By using kitchen wastes, concerns about waste management in crowded cities could be automatically eased. Additionally, some parts of carbohydrates from wastes will return to the production cycle of lactic acid and, as a result, decrease a large amount of corn consumption. By using other carbohydrate sources rather than corn, the criticisms and debates about utilizing a food source as packaging material will be defused (Zhang and others 2008).

PLA Processing Technologies for Food Applications

The methods of manufacture for biopolymers are all established polymer-manufacturing techniques, but the control and application of these methods must be varied to cope with certain factors associated with exploiting the advantages of biopolymers. The manufacturing routes all show certain fundamental similarities, with the major differences depending on whether a thermoset or thermoplastic biopolymer is to be processed.

The conditions in biopolymer processes such as injection molding are least damaging to polymer melts, and most problematic in continuous processes like extrusion, particularly in processes where the extrudate is stretched, such as film blowing. The limiting factors for processing conditions for biopolymers are the same as for petrochemical-based ones: degradation at the upper limits of temperature and shear, and lack of homogeneity at the lower limits. However, these limits are somewhat more tightly drawn at the upper limits for biopolymers. The results of exceeding these upper limits are degradation of the polymer, resulting in molding

Table 1–Processing possibilities of typical commercial biodegradable polymers (Clarinval 2002; Clarinval and Halleux 2005, NatureWorks® datasheets).

	Injection molding	Extrusion	Extrusion blow molding	Cast film extrusion	Blow molding	Fiber spinning	Thermoforming
Starch	×	×	×	×			
Cellulose	×	×			×		
PHB	×	×	×	×	×		×
PHB-PHV	×	×	×	×	×	×	×
PLA	×	×		×	×	×	×
PBS	×	×					
PCL	×	×	×		×	×	×
PBST	×	×		×			×
PBAT		×	×	×			
PTMAT		×	×	×		×	
PVA	×	×		×		×	×
PP,PE + additives	×	×	×	×	×	×	×
Starch + PVA	×	×		×	×	×	
Starch + cellulose acetate	×	×	×		×		×

PHB = Poly(3-hydroxybutyrate); PHV = Poly(hydroxyl valerate); PBS = Poly(butylenes succinate); PCL = Poly(ε-caprolactone); PBST = Poly(butylene succinate terephthalate); PBAT = Poly(butylene adipate terephthalate);
PTMAT = Poly(tetramethylene adipate terephthalate); PVA = Poly(vinyl alcohol).

Table 2–Commercial Ingeo™ PLA resins adapted by NatureWorks® data sheets.

Product code	Applications	Usages
2002D	Extrusion, thermoforming	Dairy containers, food serviceware, transparent food containers, blister packaging, cold drink cups
3001D 3051D	Injection molding for applications with heat deflection temperatures lower than 55 °C (130 °F)	Cutlery, cups, plates, and saucers, and outdoor novelties
3251D	Injection molding, having higher melt flow capability than other PLA resins for easier molding of thin-walled parts	Injection molding applications, both clear and opaque, requiring high gloss, UV resistance, and stiffness
4032D	Biaxially oriented films with use temperatures up to 150 °C (300 °F), barrier to flavor and grease, and oil resistance	Laminations, printed films with higher curing temperatures, other packaging applications
4042D	Biaxially oriented films with use temperatures up to 130 °C (265 °F), barrier to flavor and grease, and superior oil resistance	Candy twist-wrap, salad, and vegetable bags, window envelope film, lidding film, label film, other packaging applications
4060D	Heat sealant with a seal initiation temperature of 80 °C	Can be coextruded with other PLA resin to form a sealant layer for biaxially-oriented PLA film
7000D	Injection stretch blow molding, for 1:2 stage operations	Fresh dairy, edible oils, fresh water
7032D	Injection stretch blow molding, for 1:2 stage operation	Fruit juices, sports drinks, jams, and jellies

defects such as weld lines, discoloration, or a strong odor in the final product (Johnson and others 2003).

Processing possibilities of typical commercial biodegradable polymers are presented in Table 1.

Commercial PLA resins are packaged in crystalline and amorphous pellet forms. Crystalline and amorphous pellets look significantly different. Semicrystalline pellets are opaque and amorphous pellets are transparent. Different types of PLA resins with different application ranges are being produced and each customer should specify packaging demands and match them with PLA data sheets. In Table 2, available commercial PLA resins for food packaging applications are characterized.

The processing technologies for producing different packaging applications with PLA resins are mentioned here.

Drying

As PLA is sensitive to high-relative humidity and temperature conditions, and for minimizing the risk of its molecular degradation, it is necessary to be dried less than 0.01% w/w. This value is expressed as 0.025% w/w or below in NatureWorks® data sheets. PLA resins normally are packaged with moisture content below 0.04% w/w in moisture-resistant foil liners to maintain that moisture level, and so the drying process is essential. Drying conditions are dependent on temperature, time, air flow rate, and dew point. Amorphous pellets must be dried below the T_g (43 to 55 °C) to prevent the resin pellets from sticking together, which can bridge and plug the dryer (Lim and others 2008).

For crystalline types, the recommended temperatures and times range between 80 to 100 °C and 4 to 2 h, respectively. Typical drying conditions are 4 h at 80 °C (175 °F) or to a dew point of −40 °C (−40 °F), with an airflow rate greater than 0.032 m^3/min per kg (0.5 cfm/lb) of resin throughout (NatureWorks® PLA processing guide for biaxially oriented film 2005b). PLA is a hygroscopic thermoplastic and readily absorbs moisture from the atmosphere; so its resins should not be exposed to atmospheric conditions after drying and the packages should be kept sealed until ready to use and promptly be dried and resealed if not entirely used.

Extrusion

The 1st major step in the conversion of plastic resin into films, sheets, containers and so on, is to change the pellets from solid to liquid or molten phase in an extruder.

Extrusion is a common way for melting thermoplasts and it is the 1st step for extrusion coating, cast film extrusion, blown film extrusion, and other polymer processes.

Screw extruders are typically used in the polymer industry. They consist of an electrically heated metal barrel, a hopper for feeding the resin, a motor for rotating a screw, and a die where the polymer melt exists. So, the combination of thermal energy generated by a heater and frictional heat due to friction between the plastic and the screw and barrel provide sufficient heat to melt the pellets.

The L/D ratio, which is the ratio of flight length of the screw to its outer diameter, determines the shear and residence time of the melt. Screws with a large L/D ratio provide greater shear heating, better mixing, and longer melt residence time in the extruder. Another important screw parameter is the compression ratio, which is the ratio of the flight depth in the feed section to the flight depth in the metering section. The greater the compression ratio a screw possesses, the greater the shear heating it provides (Giles and others 2005).

Recommended extrusion conditions for PLA pellets include general purpose screws with L/D ratios from 24:1 to 30:1 and compression ratio of 2.5:1 to 3:1, melt temperature of 200 to 220 °C, and also smooth barrels (NatureWorks® PLA 4042 data sheet 2006a).

Injection molding

Injection molding involves melting a thermoplastic by extrusion, injecting the polymer melt into a mold, cooling the part, and finally ejecting the part. Most polymers can be injection molded so long as they can flow and fill the mold cavity easily. The commonly used polymers are Poly(ethylene terephthalate) PET, Poly(styrene) (PS), Poly(propylene) (PP), high-density poly(ethylene) (HDPE), Low-density poly(ethylene) (LDPE), nylon, and Poly(vinyl chloride) PVC.

An injection molding machine is similar to an extruder and the main difference between 2 machines is in their screw operation. In an extruder, the screw rotates continuously providing output of continuous and long product (pipe, rod, sheet), but the screw of an injection molding machine, which is called reciprocating screw, does not only rotate but also moves forward and backward according to the steps of the molding cycle. The mold is equipped with a cooling system providing controlled cooling and solidification of the material. Injection molding may be used to manufacture a wide variety of parts such as bottle caps, food trays, containers, and preforms for blow molding (Rosato and others 2000).

Injection mold-grade PLA is injection molded on most conventional equipment, but there could be some torque limitations if the screw design has a high compression ratio. Compression ratios of 2.5 to 3 should be adequate and the recommended melting temperature is 200 to 205 °C. Since PLA has a lower glass transition temperature (about 58 °C) than PS or PET, it might take a little longer time to set up in the mold (NatureWorks® PLA injection molding guide for 3051D 2006b).

Physical aging occurs when a polymer is in a nonequilibrium state and is caused by molecular relaxations that are biased in the direction required to drive the material closer to equilibrium. This phenomenon is very common and is encountered in thermoplastics moldings that have been cooled rapidly from an elevated temperature during the shaping operation such as injection molding process (White 2006). Physical aging significantly affects the physical properties of the amorphous phase in glassy or partly glassy polymers. The effect of aging takes place around T_g and can be noticed by shrinkage of specific volume, decreases in specific enthalpy and entropy, and a decrease in molecular mobility. These effects are associated with decrease of free volume, which controls the mobility of large segments of the polymer chains and affects the mechanical properties of polymers such as shrinkage, stiffness, brittleness, and decrease in damping (Ke and Sun 2003b; Acioli-Moura and Sun 2008).

Injected molded PLA articles are relatively brittle, which caused by rapid physical aging of polymer since ambient temperature is approximately 25 °C below the T_g. Cai and others (1996) studied physical aging behavior of PLA in different times and temperatures by DSC. The results confirmed the augmentation of endothermic peak at T_g by increasing the aging time, which is related to the increase of the excess enthalpy of relaxation. The rate of physical aging was very fast initially and decreased as time increased. They also showed that the aging temperature of 37 °C had the maximum enthalpy of relaxation, but by increasing the temperature above 60 °C the enthalpy of relaxation was greatly reduced. However, storage conditions for injected molded PLA articles that are especially intended for further processing (like preforms) should be carefully controlled.

Shear-controlled orientation in injection molding (SCORIM) is a nonconventional injection molding technique that allows for the enhancement of the mechanical properties of semicrystalline polymers and has additional degrees of freedom over conventional injection molding. SCORIM technique manipulates the structure development of a solidifying polymer melt through an in-mold shearing action, thereby tailoring the morphology and hence controls the mechanical properties of polymers (Grossman 1995). Ghosh and others (2008) investigated the effect of operative parameters of SCORIM on Poly(L-lactic acid) (PLLA). They showed some modifications in energy at break and maximum stress of all the SCORIM processed PLLA. The overall increments in maximum stress and energy at break were 134% and 641%, respectively.

Blow molding

Blow molding is a process of blowing up a hot thermoplastic tube (called parison or preform, a term derived from the glass industry) with compressed air to conform to the shape of a chilled mold and releasing the finished product form the mold. The most widely used materials for blow molding are LDPE, HDPE, PP, PVC, and PET (Lee and others 2008).

There are 3 common types of blow molding: extrusion blow molding, injection blow molding, and injection stretch blow molding (ISBM).

Extrusion blow molding. Extrusion blow molding begins with extruding a polymer melt into a parison. The chilled mold is then closed, followed by blowing air through a blow pin to inflate the parison to conform to the shape to the mold cavity. After cooling the plastic, the mold is opened, and the part is ejected. Containers produced by extrusion blow molding must meet minimum stiffness requirements to undergo filling on automated lines. They must avoid, or limit, unsightly bulging under weight of their contents, both alone or when stacked. They must also withstand normal impacts of handling, transport, and accidental dropping. Such impact must be absorbed by the container walls, weld lines (pinch-off and handle areas), and screw cap closure threads, often under extremes of temperature. Basic polymers for extrusion blow molding are HDPE, PP, and PVC. These polymers are sometimes coextruded with Ethylene vinyl alcohol (EVOH) or nylon to provide a better gas barrier (Lee 2006).

PLA containers are not produced by this method, because of a lack of the required physical and mechanical properties; and PLA resins for this application have not been yet produced.

Injection blow molding. Injection blow molding is a 2-step process for making plastic containers. This method produces a molded parison called a preform. This method is preferred over extrusion blow molding for making small parts that require high-production volumes and closer quality dimensions. Injection blow molding consists of injecting a thermoplastic material into a cavity and around a core rod producing a hollow test tube like shape (preform). The molded preform still on the core rod is transferred to the blow mold. The mold is clamped around the preform and air is blown into it to shape to the cavity. The preform is injected onto a support pin or core, which forms a neck with threads to their required dimensions. The preform is then blown against the cavity wall to its final shape (Lee 2006; Lee and others 2008).

The use of preform allows the manufacture of bottles with more precise detail in the neck and finish (threaded) area than extrusion blow molding.

Injection blow molding requires lower degree of melt strength than extrusion blow molding and its tooling costs are higher. Common polymers for this method are PS, LDPE, Linear low-density poly(ethylene) (LLDPE), HDPE, PP, PVC, and PET (Lee and others 2008).

This process is typically limited to the production of relatively small bottles and PLA pellets are rarely recommended to be processed by this method.

Injection stretch blow molding. Injection stretch blow molding (ISBM) is an extension of injection blow molding with 2 modifications: (a) the preform is significantly shorter than the bottle and (b) a stretch rod is used to stretch the preform in the axial direction. This process became known in the blow molding industry with the introduction of plastic or PET soft drink bottles.

While all blow molding processes involve blowing air to stretch the parison or preform in some fashion, ISBM is designed to achieve and retain biaxial orientation to significantly improve gas barrier properties, impact strength, transparency, surface gloss, and stiffness. Biaxial orientation is achieved by elongating the preform with the stretch rod and blowing air to stretch the preform in a direction perpendicular to the axis of the preform, while precisely controlling a temperature warm enough to allow rapid inflation and molecular orientation, but cool enough to retard relaxation of its molecular structure once oriented (Rosato and others 2000)

PET bottles for carbonated soft drinks are the most common food packaging applications of this process. The combination of stretching by rod and blowing air at high pressure (about 4 MPa) induces biaxial molecular orientation, thereby making the bottles a better barrier to carbon dioxide and stronger to withstand the internal pressure (Rosato and others 2000; Lee and others 2008).

ISBM-grade PLA resins are accessible and they are generally used for bottles for different foods like fresh dairy liquids, fruit juices, sport drinks, edible oils, and so on.

ISBM-grade PLA resin is typically run at lower processing temperatures than bottle-grade PET and the blow molding conditions include: preform temperature at 80 to 100 °C, stretch rod speed 0.8 to 1.2 m/s, and blow mold temperature at 100 to 120 °C. In fact, the heating of the preforms is critical in getting a container with good clarity and material distribution. Normal preform temperatures for running on a 2-step process have been between 80 and 100 °C. This temperature may be lower or higher depending on the preform design, bottle design, and reheating equipment that is being used (NatureWorks® PLA ISBM bottle guide 2005a).

Preform design is critical in getting a container with good clarity and physical properties. Designing a preform for use as a PLA container is specific to the blow mold equipment, bottle design, and mold tooling.

Cast film extrusion

The cast film process involves extruding a molten polymer through a slit die and drawing it around 2 or more highly polished high-speed rolls, typically chrome-plated and water-cooled. In less than 1 revolution, the chill roll solidifies the product as it draws it down to the correct thickness. Cast film is used in packaging, food wrap, substrate for coating, protective film, agricultural film for weed control, general purpose polyethylene film as a protective barrier to prevent scratching of parts during shipment, and many other applications (Giles and others 2005).

Due to rapid cooling by the chilled rolls, cast films typically have a low degree of crystallinity and transparent appearance. Besides providing good optical properties, cast film extrusion has the advantages of high production rate, good control of film thickness and uniformity, and little or no additive is required for processing.

Similar to PP, PET, and PS films, the physical properties of PLA films can be enhanced through orientation. Uniaxial orientation of PLA is achieved with conventional machine direction orientation (MDO) rolls. Since PLA tends to neck in (neck in happens by contacting the melt film with the 1st point of the die; the hot film shrinks on its way down so its width from the die to the chill roll is reduced. At the same time, beading or thickening of the edges occurs) during drawing, nipped rolls are usually required. It is possible to improve both the thermal resistance and impact resistance of PLA films or sheets by drawing, orientation, and crystallization to the same level of strength and stiffness as oriented polypropylene (OPP) or PET, while maintaining its high transparency. An oriented film is obtained by stretching it to 2 to 10 times its original length at 60 to 80 °C, and further annealing it at temperatures between the stretching temperature and melting point. An oriented film may be either processed for dry lamination, printing, and heat seal or other applications including various types of packaging (Kawashima and others 2002).

Thermoforming

Thermoforming is a generic term encompassing many techniques for producing useful plastic articles from flat sheets. Thermoforming is a process that deals with the pressing or drawing of pliable plastic into final shape by vacuum or air pressure. A wide range of thermoplastics, including PP, LDPE, LLDPE, HDPE, PET, PS, and nylon, may be thermoformed. Food packaging is the largest application for thermoformed containers, trays, cups, and tubs. Typical thermoforming steps are clamping, heating, shaping, cooling, and trimming (Throne 1996)

PLA sheet can be thermoformed with vacuum, compressed air/vacuum, or only compressed air assistance. The radiant heater of the thermoforming line for PLA must be adjusted to very low temperatures. Preheating is not absolutely necessary; however it has the general advantage that the sheet is homogeneously preheated.

PLA sheet is quite brittle at room temperature and requires some special handling and storage considerations. There is a greater risk of cracking and breaking during transporting compared with Oriented poly(Styrene) (OPS) or PET. Neither the sheet nor the finished product can be stored at temperatures above 40 °C or greater than 50% relative humidity. These conditions minimize moisture uptake and consequently sheet blocking, and resistance to unwinding. Exposure to high temperatures or humidity, even for a short period, can cause the material to deform and eventually break down. Sheet and formed products must be transported in cooled trucks and stored in a climate-controlled warehouse. The toughness of PLA increases with orientation, and therefore, thermoformed articles are less brittle than PLA sheet, particularly in the regions that have been highly stretched during the forming operations rather than flanges and lips. So, flange or lip areas that receive less orientation tend to be more brittle than the rest of the thermoformed part (NatureWorks® processing guide for thermoforming articles 2005c; Patey 2010).

PLA is frequently thermoformed using forming ovens, molds, and trim tools designed for PET or polystyrene. Because of higher shrinkage of PP than PLA, the molds and trim tools designed for PP are less optimally used for PLA.

PLA has a lower softening temperature than PET or PS. Typically oven settings are about 55 °C (100 °F) or lower than PS and about 40 °C (75 °F) or lower than PET oven settings. The sheet should be about 90 to 110 °C (190 to 230 °F) entering the mold. Aluminum molds are recommended for thermoforming PLA. PLA thermal properties indicate that the cooling time in the mold will be greater for PLA than either PS or PET (NatureWorks® processing guide for thermoforming articles 2005c).

Patey (2010) discussed some essential factors for PLA thermoforming process. According to his suggestions, optimizing a

conventional polymer thermoforming line for PLA just needs some minor modifications in tools and equipments.

PLA crystallinity diminishes its shrinkage after thermoforming process. Uradnisheck (2009) showed the shrinkage of PLA thermoformed articles is minimized by longer dwell times and crystallinity of polymer. He concluded that the crystallinity in a thermoformed article dwelling in the heated mold raised to a higher level due to supplemental crystallinity generated in the preheat step and forming or stretching step.

PLA Thermal Stability

PLA is thermally unstable and exhibits rapid loss of molecular weight as the result of thermal treatment at processing temperatures. The ester linkages of PLA tend to degrade during thermal processing or under hydrolytic conditions. PLA undergoes thermal degradation at temperatures lower than the melting point of the polymer, but the degradation rate rapidly increases above the melting point. It has been postulated that thermal degradation mainly occurs by random main-chain scissions. Several reactions such as hydrolysis, depolymerization, oxidative degradation, and inter- and intramolecular trans-esterification reactions to mononmer and oligomeric esters, are suggested to be involved in the degradation process during thermal treatments (Taubner and Shishoo 2001; Södergård and Stolt 2002).

Taubner and Shishoo (2001) studied 3 parameters on thermal degradation of PLA during extrusion processing, including processing temperature (210 and 240 °C), residence time in the melt (1.75 and 7 min), and the inherent moisture content of polymer. Their results confirmed higher polymer degradation by increasing processing temperature and time. In a temperature of 210 °C, the loss in M_n (Number-average molecular weights) was less dependent on the residence time in the melt compared to when processed at a temperature of 240 °C. The presence of moisture in the material affected the loss in M_n to a great extent when processing was done at 210 °C. The rate of degradation at 240 °C and 7 min was so high that they concluded the moisture content in the polymer probably does not contribute further to the degradation process.

Different factors like particle size and shape of polymer, temperature, moisture, crystallinity, % D-isomer, residual lactic acid concentration, molecular weight, molecular weight distribution, water diffusion, and metal impurities from the catalyst will affect the polymer degradation rate. Yu and others (2003) have developed a mathematical model to describe the molecular weight and polydispersity index (Q) in PLA thermal degradations. They claimed model ability to predict changes of the molecular weight and poly dispersity index in the PLLA thermal degradation. Their model was based on the random chain scission mechanism, effects of temperature, and time on the molecular weight and polydispersity index.

PLA Properties

PLA has unique properties like good appearance, high mechanical strength, and low toxicity; and good barrier properties have broadened its applications. Numerous researchers have studied the different properties of PLA alone and in combination with other polymers as blend or copolymer; and here some of them will be introduced.

Auras and others (2003) studied mechanical, physical, and barrier properties of 2 PLA films by the names of 4030-D, which was made with nominally 98% L-lactide, and 4040-D, which was made with nominally 94% L-lactide resins. Finally, the data from these 2 PLA film samples were compared to those of polystyrene (PS) and polyethylene terephthalate (PET). PLA films showed good tensile strength with higher values than PS but lower than PET. Both 4030-D and 4040-D had lower T_m (melting point) and T_g (glass transition temperature) than PET and PS, which makes PLA better for heat-sealing and thermal processing. In terms of barrier properties of PLA, the permeability coefficients of CO_2 and O_2 were lower than those of PS and comparable to those of PET.

For tensile modulus and flexural modulus, PLA has the highest value in comparison to PS, PP, and HDPE. For notched izod impact (izod impact strength testing is an American society for testing and materials [ASTM] standard method [D256 – 06ae1] of determining impact strength. A notched sample is generally used to determine impact strength. An arm held at a specific height is released. The arm hits the sample and breaks it. From the energy absorbed by the sample, its impact strength is determined), PLA has the lowest one between PS, PP, and HDPE. The elongation at break is low and nearly 4% that is just higher than that of PS (Dorgan and others 2000).

Low glass transition temperature of PLA limits its usages in thermally processed packages. Because of its deformation and its low melting temperature, it is better to use it for heat-sealing and thermoforming applications.

Five major properties of typical biodegradable polymers are compared with LDPE, PS, and PET in Table 3. It is approximately possible to predict the application fields of a polymer by these properties and barrier properties.

The other important property of polymers is their rate of crystallinity. Crystallinity is the indication of amount of crystalline region in the polymer with respect to amorphous content. Crystallinity influences many polymer properties including hardness, modulus, tensile strength, stiffness, crease point, and melting point. So, while selecting a polymer for a required application its crystallinity plays the foremost role.

PLA crystals can grow in 3 structural positions called α, β, and γ forms. They are characterized by different helix conformations and cell symmetries, which develop upon different thermal and/or mechanical treatments. The α form grows upon melt or cold crystallization, the β form develops upon mechanical stretching of the more stable α form, and the γ form, which only recently has been reported to develop on hexamethylbenzene substrate (Di Lorenzo 2005).

Di Lorenzo (2005) measured crystallization rates of PLA over a wide temperature range, using both isothermal and nonisothermal methods. He determined that the crystallization rate of PLA at temperatures between 100 and 118 °C is very high. He concluded that the high crystallization rate of PLA below 120 °C has to be ascribed to the high rate of radial growth of the spherulites (spherical semicrystalline regions inside nonbranched linear polymers).

By modification of the chain architecture through the introduction of branching, different melt flow properties will be obtained. Thermal and rheological properties of 2 commercial types of PLA, linear and branched, were investigated by Dorgan and others (2000). The crystallization kinetic of the branched polymer was faster than that of the linear analog. Longer relaxation times in the terminal region of the branched material introduced it as a higher zero shear rate viscosity. They concluded that by utilizing the structure modifications through polymer branching the ability of using PLA in many processing operations will be extended.

Optical properties of PLA are important in dyeing operations for textiles and in various packaging applications where clarity is desirable. Hutchinson and others (2006) determined the optical

Table 3–Comparison of typical biodegradable polymer properties with LDPE, PS, and PET adapted from Clarinval and Halleux (2005).

	T_g (°C)	Tm (°C)	Tensile strength (MPa)	Tensile modulus (Mpa)	Elongation at break (%)
LDPE	−100	98 to 115	8 to 20	300 to 500	100 to 1000
PCL	−60	59 to 64	4 to 28	390 to 470	700 to 1000
Starch	–	110 to 115	35 to 80	600 to 850	580 to 820
PBAT	−30	110 to 115	34 to 40	–	500 to 800
PTMAT	−30	108 to 110	22	100	700
PS	70 to 115	100	34 to 50	2300 to 3300	1.2 to 2.5
Cellulose	–	–	55 to 120	3000 to 5000	18 to 55
PLA	40 to 70	130 to 180	48 to 53	3500	30 to 240
PHB	0	140 to 180	25 to 40	3500	5 to 8
PHA	−30 to 10	70 to 170	18 to 24	700 to 1800	3 to 25
PHB-PHV	0 to 30	100 to 190	25 to 30	600 to 1000	7 to 15
PVA	58 to 85	180 to 230	28 to 46	380 to 530	–
Cellulose acetate	–	115	10	460	13 to 15
PET	73 to 80	245 to 265	48 to 72	200 to 4100	30 to 300
PGA	35 to 40	225 to 230	890	7000 to 8400	30
PEA	−20	125 to 190	25	180 to 220	400

PGA = Poly(glutamic acid); PEA = Poly(ester amide).

properties of PLA with different amounts of stereoisomer proportions by ellipsometric measurements. They developed an equation for index of refraction of PLA with a wide range of stereoisomer proportions (L-content) within the range of wavelengths from 300 to 1300 nm by using Cauchy coefficients.

There are many PLA resins for different applications with different properties; the general characteristics of a commercial amorphous PLA, injection mold grade and having a 96:4 L:D ratio content, are summarized in Table 4.

PLA barrier properties

One of the most important factors in food packaging polymers is their barrier or permeability performance against transfer of gases, water vapor, and aroma molecules. Gas permeation properties of PLA (L:D ratio 96:4) have been studied by Lehermeier and others (2001) and these values have been reported: at 30 °C, N_2 permeation in PLA was 1.3 (10^{-10} cm^3cm/cm^2scm Hg), and the activation energy was 11.2 kJ/mol. For oxygen, the corresponding values were 3.3 (10^{-10} cm^3cm/cm^2scm Hg) and 11.1 kJ/mol. The values for carbon dioxide permeation were 1.2 (10^{-10} cm^3cm/cm^2scm Hg) and 6.1 kJ/mol. For methane, a value of 1.0 (10^{-10} cm^3cm/cm^2scm Hg) and an activation energy of 13.0 kJ/mol were found.

The authors concluded that polymer chain branching and small changes in L:D stereochemical content have no effect on permeation properties, but film crystallinity profoundly impacted of the permeation of mentioned gases. For example, due to higher crystallinity of biaxially oriented PLA film, CH_4 permeation is 4.5 times lower than that of the other films. The permeation properties of PLA for all gases studied were very similar to polystyrene.

In research done by Bao and others (2006), different results for pure gas permeation of PLA were obtained, which disagreed with those of previous work. They used a time-lag method for the determination of PLA permeation to pure gases and also determined diffusivity and solubility of N_2, CO_2, and O_2 in PLA film. For example, at 30 °C, N_2 permeability, diffusivity, and solubility in PLA (98.7% L, 1.3% D) were 0.05 (10^{-10} cm^3cm/cm^2scm Hg), $2.4 \times 10^{-8} cm^2/s$, and $2.2 \times 10^{-4} cm^3/cm^3$(polymer) cm Hg, respectively. The measured activation energy of N_2 permeation was 34.6 kJ/mol.

Sorption of nitrogen, oxygen, carbon dioxide, and water in PLA has also been studied at 293.2, 303.2, and 313.2 °K (Oliveira and others 2004).

Shogren (1997) reported that the water vapor transmission rate of crystalline and amorphous PLA in 6, 25, and 49 °C as 27, 82, and 333 g/m^2 per day for the crystalline form and 54, 172, and 1100 g/m^2 per day for the amorphous form, respectively. He reported activation energies of 5 and − 0.1 kJ/mol for amorphous and crystalline PLA, respectively.

Siparsky and others (1997) used a "solution-diffusion" model to determine the water vapor permeability parameters of different PLA films, PLA copolymers with caprolactone, and blends with polyethylene glycol. These parameters included the solubility coefficient S, which is a measure of the equilibrium water concentration available for hydrolysis and the diffusion coefficient D, which characterizes the rate of water vapor diffusion into the film under specific conditions. They calculated the permeability coefficient by the equation of P = SD. They studied S and D for PLA films by different percent of L and D Lactide. They found the degree of crystalline had little influence on the measured permeability parameters.

In a more detailed research done by Tsuji and others (2006), the effects of D-Lactide content, degree of crystallinity (% Xc), and molecular weight of PLA films on water vapor transfer rate (WVTR) were studied. They observed the WVTR of PLA films decreased monotonically with increasing Xc from 0% to 20%, while leveled off for Xc exceeding 30%; so they suggested this change due to the higher resistance of restricted amorphous regions to water vapor permeation compared with that of the free amorphous regions. They also concluded that changes in M_n of PLA films in the range of 9×10^4 to 5×10^5 g/mol and D-lactide unit content of PLA films in the range of 0% to 50% have insignificant effects on their WVTR values.

Some aforementioned PLA permeability parameters are summarized in Table 5.

Orientation changes the barrier properties. In a study done by Auras and others (2005), oriented PLA (OPLA) was investigated with PET and oriented polystyrene (OPS) with regard to physical, mechanical, and barrier properties. They concluded, in terms of water vapor barrier, that PET gave the best performance, followed by OPS and OPLA. In the case of oxygen barrier properties, PET showed the lowest oxygen permeability coefficients, followed by OPLA and OPS that showed very poor oxygen barrier performance.

According to these results, the barrier properties of PLA are remarkable and better than those of OPS. As a consequence, PLA

Table 4–General characteristics of commercial amorphous poly L-lactid acid film, injection mold grade, 96:4 L:D, produced by NatureWorks® Co.

Characteristics	Unit	Amount	Reference
Physical:			
Molecular weight	g/mol	66000	Garlotta (2001)
Specific gravity	–	1.27	
Solid density	g/cm^3	1.2515	
Melt density	g/cm^3	1.0727	
Glass transition temperature	°C	55	Mehta and others (2005)
Melting temperature	°C	165	
Specific heat (Cp)	J/Kg °C		
190 °C		2060	
100 °C		1955	
55 °C		1590	
Thermal conductivity	W/m °C		
190 °C		0.195	www.natureworkslic.com (technical data sheet)
109 °C		0.197	
48 °C		0.111	
Optical:			
UV light transmission:			Auras and others (2004)
190 to 220 nm		<5%	
225 to 250 nm		85%	
>300 nm		95%	
Visible light transmission		95%	
Color			
L*		90.64 ± 0.21	
a*		−0.99 ± 0.01	
b*		−0.50 ± 0.04	
Mechanical:			
Tensile strength	MPa	59	
Elongation at break	%	7.0	
Elastic modulus	MPa	3500	
Shear modulus	MPa	1287	
Poissons ratio	–	0.3600	www.natureworks.com (technical data sheet)
Yield strength	MPa	70	
Flexural strength	MPa	106	
Unnotched izod impact	J/m	195	
Notch izod impact	J/m	26	
Rockwell hardness	HR	88	
Heat deflection temp.	°C	55	
Vicat penetration	°C	59	
Ultimate tensile strength	MPa	73	
Percent of elongation	%	11.3	
Young's modulus	MPa	1280	
Rehological:			
Cross WLF Viscosity Model:			
n		0.2500	
Tau	Pa	1.00861e + 005	www.natureworkslic.com (technical data sheet)
D1	Pa-s	3.31719e + 009	
D2	K	373.15	
D3	K/P	0	
A1		20.194	
A2	K	51.600	

is suitable for packaging a wide range of foods that are mentioned in the section of PLA applications.

Studies on Migration from PLA

Lactic acid is the lone monomer in the PLA structure and so, migrated agents are lactic acid monomers, dimmers, and oligomers. Conn and others (1995) investigated the safety of PLA as a food contact polymer under different conditions and studied the migration of most probable species from PLA. They concluded: (1) Very limited migration can be expected from PLA into foods that it contacts during the intended conditions of use. (2) The small amount of any material that might migrate from PLA into food will be lactic acid, or its dimers (lactoyl lactic acid and lactide) and oligomers that will be subsequently hydrolyzed in aqueous systems to lactic acid. Based on these findings, they concluded that PLA is safe and GRAS for its intended uses in fabricating articles intended for use in contact with food. The authors also mentioned that the projected intake of lactic acid from PLA is approximately 700 times less than the estimated daily lactic acid intake of a breast-fed infant.

Table 5–PLA permeability parameters.

		L:D, 96:4 (30 °C) (Lehermeier and others 2001)	L:D, 98.7:1.3 (30 °C) (Bao and others 2006)
CO_2	10^{-10} cm^3 cm/cm^{2}scm Hg	1.2	1.1
O_2		3.3	0.26
N_2		1.3	0.05

		Water vapor permeation property		
(Shogren 1997)		6 °C	25 °C	49 °C
Crystalline, 66% crystallinty	g/m^2/day	27	82	333
Amorphous		54	172	1100
(Siparsky and others 1997)		**20 °C**	**40 °C**	**50 °C**
L:D (100:0), 39% crystallinity	cm^{3}cm/cm^{2}sPa	1.6	1	2
L:D (100:0), Amorphous		1.9	0.8	2.1
L:D (95:5)		1.4	2.2	2.1
L:D (50:50)		2.2	8.7	6.1

Mutsuga and others (2008) determined the PLA migration products for 4 different PLA sheets, which are used in lunch boxes in Japan. They applied 3 food simulants such as water, 4% acetic acid, and 20% ethanol at temperatures of 40, 60, and 95 °C for different periods of time. They concluded that the rate of migration is augmented by high temperatures. The total migrated levels, including lactic acid, lactide, and oligomers, at 40 °C after 180 d were 0.28 to 15 μg.cm^{-2} and 0.73 to 2840 μg.cm^{-2} for 60 °C after 10 d. The migration test at 95 °C for 30, 60, and 120 min mimicked the use of lunch boxes in a microwave oven at 100 °C or above, and the total migrated levels for 120 min were 2.04 to 49.63 μg.cm^{-2}.

So, for a PLA much of the concerns about migrations of potential dangerous materials, which exist for petrochemical-based polymers are resolved. These results are only for pure PLA polymer and more studies are needed for its blends and copolymers, also for all the compounds that are applied or added for improving physical, mechanical, and barrier properties of PLA.

PLA Applications

PLA has potential for use in a wide range of applications; Table 6 shows an overview of NatureWorks™ PLA and Ingeo™ fibers, PLA opportunities, and examples of commercially available products. As be seen, PLA food packaging applications are ideal for fresh products and those whose quality is not damaged by PLA oxygen permeability.

PLA is a growing alternative as a "green" food packaging polymer. New applications have been claimed in the field of fresh products, where thermoformed PLA containers are used in retail markets for fruits, vegetables, and salads. The market capacity of these products packaged in PLA is unlimited.

The major PLA application today is in packaging (nearly 70%); the estimation for 2020 shows the increase of other applications especially in fibers and fabrics (Table 7).

Table 6–Business segments for products based on Ingeo™ plastic and Ingeo™ fibers PLA (NatureWorks®) adapted from Vink and others (2004).

Business segment	Commercially available applications
1- Ingeo plastic applications	
Rigid thermoforms	Clear fresh fruit and vegetable clamshells
	Deli meat trays
	Opaque dairy (yogurt) containers
	Bakery, fresh herb, and candy containers
	Consumer displays and electronics packaging
	Disposable articles and cold drink cups
Biaxially oriented films	Candy twist and flow wrap
	Envelope and display carton windows
	Lamination film
	Product (gift basket) overwrap
	Lidding stock
	Die cut labels
	Floral wrap
	Tapes
	Shrink sleeves
	Stand-up pouches
	Cake mix, cereal, and bread bags
Bottles	Short shelf-life milk
	Edible oils
	Bottled water
2- Ingeo fiber applications	
Apparel	Casual (sports-), active, and underwear fashion item
Nonwovens	Wipes, hygiene products, diapers, shoe liners, automotive head and door liners, and paper reinforcement
Furnishings	Blankets and panel, upholstery, and decorative fabrics
Industrial carpets	Agricultural and geotextiles[a]
	Residential/institutional broadloom and carpet tiles
Fiberfill	Pillows, comforters, mattresses, Duvets, and furniture

[a] Geotextiles are permeable fabrics which, when used in association with soil, have the ability to separate, filter, reinforce, protect, or drain. Usually geotextiles are placed at the tension surface to strengthen the soil.

Table 7–Main applications of PLA in 2003 and the estimation for 2020 (Wolf 2005).

	Percent of total production (2003)		Estimated percent of total production (2020)	
Sector	Cargill Dow	Hycail	Cargill Dow	Hycail
Packaging	70	70	20	55
Building				
Agriculture	1	12		6
Transportation			20	2
Furniture				
Electric appliance and electronics	1	1	10	10
Houseware		12		6
Other (fibers and fabrics)	28	3 to 5	50	21
Other (analytics)				
Total	100	100	100	100

Table 8–PLA functional properties for packaging (Kawashima and others 2002).

Functional property	Packaging improvement	Comment
Dead fold, twist, and crimp[a]	Improved folding and sealing	OPLA has excellent dead fold and twist retention
High gloss, and clarity	Package aesthetics	Comparable with PET and cellophane, 3 times more than nylon and PP, 10 times more than LDPE
Barrier properties	Grease and oil resistance	Good resistant to oils and terpens
Renewable resource	Made from CO_2 and H_2O	
Flavor and aroma properties	Reduced taste/odor issues	
Low temperature heat seal	Stronger seals at lower temperatures	PLA can provide an "easy-open" package
High tensile and modulus	Wet paper strength, ability to down guage coating	
Low coefficient of friction, polarity	Printability	Excellent printability, metallizable, antifogging ability
GRAS status	Food contact approved	

[a] The ability to hold a crease or fold, or the ability to retain a twist that is imparted in order to close the edges of the film around a small object.

Table 9–Some commercialized PLA products (Platt 2006; www.natureworksllc.com).

Product	Company name[a]
Packaging	
Films and trays for biscuits, fruit, vegetables, and meat	Treophan, Natura, IPER, Sainsburys, Sulzer, Ecoproducts, RPC
Yogurt cup	Cristallina/Cargill Dow
Rigid transparent packaging of batteries with removable printed film on back side	Panasonic
Trays and bowls for fast food	McDonalds
Envelope with transparent window, paper bag for bread with transparent window	Mitsui, Ecocard
Agriculture and horticulture	
Mulching films	Novamont, Cargill Dow
Long life consumer good	
Apparel (T-shirt, socks)	FILA/Cargill Dow, Kanebo Gosen
Blanket	Ingeo
Casing of walkman	Sony
CD (compact disk)	Sanyo Marvic Media/Lacea
Computer keys	Fujitsu
Small component of laptop housing	Fujitsu/Lacea
Sapre wheel cover	Toyota

[a] List is not exhaustive.

In the field of packaging, 2 specific areas have received close attention, namely high-value films and rigid-thermoformed containers. PLA brings a new combination of attributes to packaging, including stiffness, clarity, deadfold and twist retention, low-temperature heat sealability, as well as an interesting combination of barrier properties including flavor, and aroma barrier characteristics. The functional properties and benefits of PLA in these areas are presented in Table 8.

Commercialized PLA products demonstrate this fact that PLA is not being used solely because of its degradability, nor because it is made from renewable resources; it is being used because it functions very well and provides excellent properties at a competitive price. There are many commercialized PLA products in today's market and their variety and consumption are increasing rapidly (Table 9). The reader can find the partners and consumers of PLA from the NatureWorks® Co. website (www.natureworksllc.com).

PLA is also used in biomedical applications, with various uses as internal body components mainly in the of restricted load for example, interference screws in ankle, knee, and hand; tacks and pins for ligament attachment; rods and pins in bone, plates and screws for craniomaxillofacial bone fixation (Lim and others 2003); and also for surgical sutures, implants, and drug delivery systems (Furukawa and others 2005; Mills and others 2006).

PLA as an Active Packaging Material

Release studies from PLA

Active packaging is defined as an intelligent or smart system that involves interactions between package or package components and food or internal gas atmosphere and complies with consumer demands for high quality, fresh-like, and safe products (Labuza and Breene 1989).

Active packaging is an innovative approach to change the condition of the packaging to extend shelf-life or improve safety or sensory properties while maintaining the quality of the food. Traditional packaging concepts are limited in their ability to prolong shelf-life of food products. The most important active packaging concepts are O_2 and ethylene scavenging, CO_2 scavengers and emitters, moisture regulators, antimicrobial packaging concepts, antioxidant release, release or adsorption of taste, and aroma molecules (Vermeiren and others 1999; Lopez-Rubio and others 2004; Kerry and others 2006).

As a GRAS and biodegradable material, and also because of its biosorbability and biocompatible properties in the human body, PLA and its copolymers (especially poly-glycolic acid) is attractive to pharmaceutical and medical scientists as a carrier for releasing various drugs and agents like bupivacaine (Sokolsky-Papkov and others 2009), rapamycin (Miao and others 2008), melittin (Cun and others 2008), 5-fluorouracil (Liu and others 2008), amoxicillin (Xu and Czernuszka 2008), human nerve growth factor (rhNGF) (Gu and others 2007), and gentamicin (Schnieders and others 2006) and many others.

In food domains, little research has been done studying the ability of PLA as an active packaging material. PLA is a relatively new polymer and needs time to become an acceptable and an effective active packaging in the market.

Antioxidants have been added to food packaging material for the intentional purpose of migration into food, because prooxidant effects are often seen to a high extent and could be reduced by antioxidants. Van Aardt and others (2007) studied the release of antioxidants from loaded poly (lactide-co-glycolide) (PLGA) (50:50) films, with 2% α-tocopherol, and a combination of 1% butylated hydroxytoluene (BHT) and 1% butylated hydroxyanisole (BHA), into water, oil (food simulant: Miglyol 812), and milk products at 4 and 25 °C in the presence and absence of light. They concluded that in water medium PLGA (50:50) showed hydrolytic degradation of the polymer and release of BHT into water. In Miglyol 812, no degradation or antioxidant release took place, even after 8 wk at 25 °C. Milk fat was stabilized to some extent when light-exposed dry whole milk and dry buttermilk were exposed to antioxidant-loaded PLGA (50:50). The authors also suggested potential use of degradable polymers as a unique active packaging option for sustained delivery of antioxidants, which could be a benefit to the dairy industry by limiting the oxidation of high-fat dairy products, such as ice cream mixes.

PLA and antimicrobial packaging trends

The innovative strength of PLA antimicrobial packaging has a direct impact on consumer health by creating safer and more wholesome packaged foods. Active packaging realizes certain extraordinary and vital functions other than providing an inert barrier between product and external conditions.

Active substances that are important and considered for novel bioactive packaging include antimicrobials, vitamins, phytochemicals, prebiotics, marine oils, and immobilized enzymes (Lopez-Rubio and others 2006).

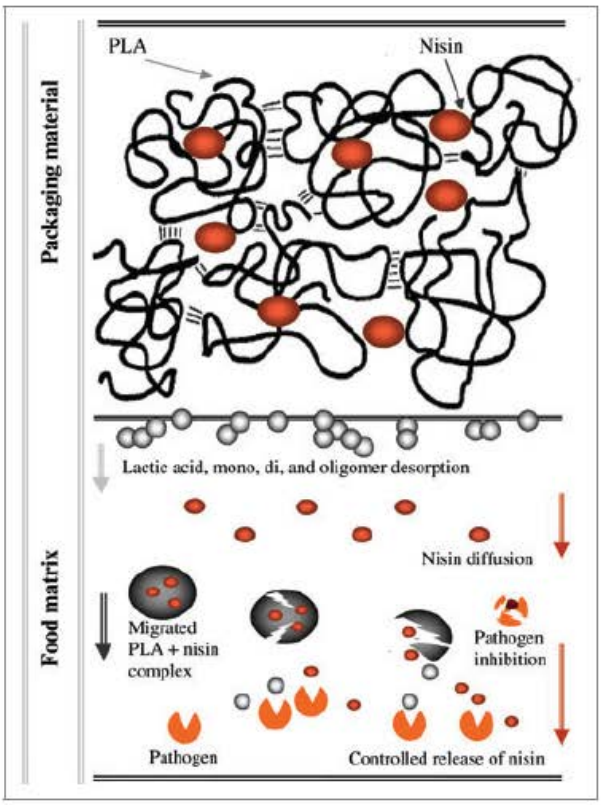


Figure 3–Schematic representation of PLA film with nisin as an active agent incorporated and release thereof.

A whole range of active additives, including silver-substituted zeolite, organic acids and their salts, bacteriocins such as nisin and pediocin, enzymes such as lysozyme, a chelator like ethylenediaminetetraacetic acid (EDTA), lactoferrin, and plant extracts have already been successfully incorporated in antimicrobial active packaging (Joerger 2007).

The most widely used bacteriocin in active food packaging is nisin due to its GRAS status (FDA 2001). Successful introduction of a new active packaging requires careful attention to the interactions in the active agent, packaging, and food triangle.

Notion of controlled release. In particular for active packaging, the major complexity emerges for migration/diffusion as either slow release of package component itself or as an active agent being incorporated. In both contexts, the evaluation of materials compliance with regulations includes migration monitoring for package component (monomer) and additives (active agent). A schematic representation is showed in Figure 3 for release of an active agent (nisin) alone and in conjunction with packaging material. Recently, the predictive mathematical modeling for active agent-controlled release and its various approaches were excellently reviewed by Poças and others (2008).

On the contrary, to study an additive's release from a package, active agent desorption from the multilayer biodegradable film and diffusion in agarose gels were monitored. The data attained after 2 or 6 d of contact between antimicrobial films and agarose gels were employed to find out nisin mass transfer by numerical modeling following Fick's 2nd law. The values were in the range from 0.87×10^{-3} m/s to 4.30×10^{-3} m/s and 6.5×10^{-11} m^2/s to 3.3×10^{-10} m^2/s, for nisin apparent desorption and diffusion

coefficients, respectively. The diffusion process was governed by interactions between nisin, package, and food matrix simulant (Chollet and others 2009).

Mode of incorporation. The customized direct incorporation of active agents may result in a loss of activity due to interactions with food components, thus showing from a diminution of active concentration and dilution into bulk foods (Kim and others 2002; Coma 2008). The incorporation of antimicrobial agents into PLA packaging material slows down their release and helps to maintain high concentrations of the active compounds against pathogenic bacteria like *Listeria monocytogenes* (Jin and others 2009).

In the last decade, the above-mentioned slow release approach has been used for PLA. In this regard, major antimicrobial agents include bacteriocins, predominantly nisin (Ariyapitipun and others 1999; Jin and others 2009), lactic acid (Ariyapitipun and others 1999), lysozyme (Del Nobile and others 2009), and chitosan (Torres-Giner and others 2008).

Novel PLA active packaging potential approaches

Although the above-mentioned PLA systems reduced resistant bacterial strain development and guaranteed a higher level of microbial protection for certain food products, their casting and preparation was complicated and inactivation of the active proteins was observed. Active agent modification by attachment to a polymer did not yield biologically active derivatives. Up till now, a literature study reveals that relatively low attention has been given to micro-encapsulated active agents in foods. Active agent-loaded polymeric micro-/nanoparticles give the impression of being promising formulations to achieve long-lasting antimicrobial activity (Salmaso and others 2004; Sanchez-Garcia and others 2007).

Thus, this particular controlled release concept can be enlarged to the applications of other active agents like antioxidants for oil-rich foods and antisticking/antifogging agents for cheese slices and fresh fruits, respectively.

PLA Modifications

The special characteristics of PLA can make it a good fit for some applications but may also require modifications for some others. For example, the oxygen and moisture permeability of PLA is much higher than for most other plastics, such as PE, PP, and even PET. However, the applications of PLA are limited by several factors such as low glass transition temperature, weak thermal stability, and low toughness and ductility (Harada and others 2007).

For extending PLA applications, the properties like impact strength or flexibility, stiffness, barrier properties, thermal stability, and production costs must be improved. Generally, modifiers have been studied to improve stiffness at elevated temperatures, reduce cost, or increase the degradation rate of PLA.

Some efforts of PLA modifications in the field of packaging are presented in Table 10.

A large number of investigations have been performed on the blending of PLA with various polymers, for example, thermoplastic starch, poly (ethylene oxide), poly (ethylene glycol), poly (ε-caprolactone), poly (vinyl acetate), poly (hydroxy butyrate), cellulose acetate, poly (butylene succinate), and poly (hexamethylene succinate). Low molecular weight compounds have also been used as plasticizers for PLA, for example, oligomeric lactic acid, glycerol, triacetine, and low molecular weight citrates (Ljungberg and others 2005).

The choice of polymers or plasticizers to be used as modifiers for PLA is limited by the requirements of the application. For packaging and hygiene applications, only nontoxic substances approved for food contact and personal care can be considered as plasticizing agents. The plasticizer should be miscible with PLA, thus creating a homogeneous blend. The plasticizer should not be too volatile, because this would cause evaporation to occur at the high temperature used during processing. Furthermore, the plasticizer should not be prone to migration into the materials in contact with the plasticized PLA. It would also cause the blended materials to regain the brittleness of pure PLA (Ren and others 2006).

There is a tendency for plasticizers to migrate to the surface of a polymer. A possible way to prevent this migration would be to increase the molecular weight of the plasticizers.

However, increasing the molecular weight too much would eventually decrease the solubility causing phase separation and formation of a 2-phase system.

The final properties of these blends depend on the chemical structure of the original components, the mixing ratio of the constituent polymers, the interaction between the components, and the processing steps to which they are then subjected.

Amorphous PLA exhibits lower modulus above the glass transition temperature and poor heat resistance, which limits the wide application of PLA in the general plastic use. Thus, how to improve the crystallization behavior or enhance the degree of crystallinity (% Xc) of PLA becomes the main problem that must be solved. Decreasing the cooling rate of PLA from melt and providing an annealing process for PLA articles is believed to be the most efficient way to enhance the (% Xc) of PLA. It has been reported that the smaller the cooling rate, the higher the (% Xc). Annealing endows PLA chain segments enough activation energy and promotes the crystallization through the reorganization process. Especially, in a certain condition, the annealing process also induces the polymorphic transition in PLA (Li and others 2009).

Other kinds of modifications, such as surface modifications, are being applied in biomedical uses for improving polymer release properties (Janorkar and Hirt 2004; Koo and Jang 2008).

Nanotechnology and PLA Food Packaging

Nanotechnology and its applications in food science have recently been studied by several researchers. The use of nanoparticles, such as micelles, liposomes, nanoemulsions, biopolymeric nanoparticles, and cubosomes, as well as the development of nanosensors aimed at ensuring food safety, are some novel nano-food applications.

Nanoparticles can be used as bioactive compounds in functional foods. Bioactive compounds that can be found naturally in certain foods have physiological benefits and might help to reduce the risk of certain diseases, including cancer. Omega-3 and omega-6 fatty acids, probiotics, prebiotics, vitamins, and minerals have found their applications in food nanotechnology as bioactive compounds (Sozer and Kokini 2009).

Nanotechnology is also applicable in food packaging in the form of elementary components of food packaging. This approach includes improving packaging performances like its gas, moisture, ultraviolet, and volatile barriers, increasing mechanical strength, decreasing weight, and increasing the heat resistance and flame retardancy of the packaging material. Nanoadditives, intelligent packaging (using nanosensors), delivery and controlled release of neutraceuticals, antibacterial agents, self-cleaning packaging, and systems to monitor product conditions during transportation are other novel nano-approaches in food packaging (Ray and Bousmina 2005; Sozer and Kokini 2009).

Table 10–Summary of PLA modifications for packaging applications.

Type of modification	Treatment or added material	Effect	Reference
Modifier	Citrate esters	Lowering the Tg and improving the elongation at break	Labrecque and others (1997)
	Triacetine or tributyl citrate	Decrease in Tg and increase in crystallinity	Ljungberg and Wesslén (2002)
	Oligomeric malonate esteramides	Decrease in Tg and improvement of the strain at break	Ljungberg and others (2005)
	4,4-Methylene diphenyl diisocyanate	Tg value increased to 64 °C, tensile strength increased from 4.9 to 5.8 MPa good nucleating agent for PLA crystallization	Li and Yang (2006)
	Polyglycerol esters	Improving the elongation at break	Uyama and others (2006)
	Polyethylene glycol and acetyl triethyl citrate	Decrease in Tg and increase in crystallization rate	Li and Huneault (2007)
	Talc	Increase the ductility at more than 10%	Li and Huneault (2007)
	Bifunctional cyclic ester	Enhance PLA toughness	Jing and Hillmyer (2008)
	Poly(1,3-butylene adipate)	Decrease in storage modulus and glass transition temperature but increase in elongation at break	Wang and others (2008)
	Polycarbodiimide	Improve the thermal stability at 210 °C for up to 30 min	Yang and others (2008)
Blending with:	Polyvinyl acetate	Increase in tensile strength and percent elongation	Gajria and others (1996)
	Poly ethylene oxide (PEO)	Elongation at break of more than 500%	Nijenhuis and others (1996)
	Poly ε-caprolactone (PCL)	High improvement in mechanical properties	Tsuji and Ikada (1996)
	Poly ethylene glycol (PEG)	Enhance the crystallinity of PLA and biodegradability	Sheth and others (1997)
	Starch with different plasticizers	Lowering the price, decreasing Tg, and increasing crystallinity and biodegradability	Ke and Sun (2001); Jacobsen and Fritz (1996); Ke and others (2003)
	Polyvinyl alcohol and starch	Increase in tensile strength	Ke and Sun (2003a)
	Ethylene vinyl alcohol (EVOH)	Improvement of mechanical, thermal, and biodegradability properties	Lee and others (2005)
	Polycarbonate	Improvement of mechanical properties and biodegradation rate	Wang and others (2007)
	Poly ethylene glycidyl methacrylate (PEGMA)	Production of super-tough PLA materials	Oyama (2009)
Copolymerization of PLA and:	DL-mandelic acid	Increasing Tg and improving mechanical properties	Kylmä and others (1997)
	ε-Caprolactone	Improving the decomposition temperatures and crystallinity	Park and others (1998)
	Polyvinyl chloride	Improving strength and toughness	Lu and others (2008)
	Acrylonitrile–butadiene–styrene	Improved impact strength and elongation at break with a slight loss in modulus and tensile strength	Li and Shimizu (2009)
Physical treatment	Vacuum compression-molding and solid-state extrusion techniques	Flexural strength and flexural modulus were improved up to 221 MPa and 8.4 GPa, respectively	Lim and others (2001)
	Orientation	Significant improvement in tensile and impact properties	Grijpma and others (2002)
	Annealing	Increasing the toughness	Park and others (2004)
	Aging	Increasing the Tg	Quan and others (2004)
	Drawing	Improvement in tensile and fracture properties	Todo (2007)

Addition of different fillers to polymers for improving their performances like their strength and stiffness, barrier properties, resistance to fire and ignition, and also decreasing their price has always been a common objective in packaging technology. Traditionally, mineral fillers such as clay, silica, and talc are incorporated in film preparations in the range of 10% to 50% by weight to reduce film cost or to improve its performance in some way. However, mechanical strength of such films, in general, decreases when fillers are present. Recently, nanocomposites have received significant attention as an alternative to conventional filled polymers (Rhim 2007).

Nanocomposites

Nanocomposites are a new class of composites that are particle-filled polymers for which at least 1 dimension of the dispersed particles is in the nanometer range. Three types of nanocomposites include isodimensional nanoparticles (with 3 nano dimensions), nanotubes or whiskers (with 2 nano dimensions), and polymer-layered crystal nanocomposites (with 1 nano dimension) (Alexandre and Dubois 2000).

Although several nanoparticles have been recognized as possible additives to enhance polymer performance, the packaging industry has focused its attention mainly on layered inorganic solids like clays and silicates, due to their availability, low cost, significant enhancements, and relative simple processability (Azeredo 2009). These nanocomposites exhibit markedly improved mechanical, thermal, optical, and physicochemical properties when compared with the pure polymer or conventional (microscale) composites. The layered silicates commonly used in nanocomposites consist of 2-dimensional layers, which are 1-nm thick and several microns long depending on the particular silicate (Alexandre and Dubois 2000).

The commonly used layered silicates for the preparation of polymer-layered silicate (PLS) nanocomposites are montmorillonite (MMT), hectorite, and saponite (Sinha Ray and Okamoto 2003).

The matrix of polymer/clay nanocomposites consists mainly of synthetic polymers including thermosets such as epoxy, thermoplastics like poly (methyl methacrylate), nonpolar polymers like polyethylene and polypropylene, polar polymers like nylon, and conductive polymers like polyaniline. In addition, biodegradable polymers such as PLA and polycaprolactone (PCL) have also been tested for the manufacture of nanocomposites with layered silicate (Rhim 2007).

PLA nanocomposites

The combination of PLA and montmorillonite-layered silicate may result in a nanocomposite with good barrier properties that is suitable for film packaging material. The modulus of PLA would be increased by the addition of montmorillonite. However, the incorporation of the montmorillonite clay into PLA could decrease the toughness of the PLA composites. There are various technical approaches to achieve a balance of good strength and toughness for PLA nanocomposites. The addition of poly ethylene glycol could act as a good plasticizer in a PLA/clay systems (Shibata and others 2006).

A comprehensive review is provided by Sinha Ray and Okamoto (2003) for the preparation, characterization, materials properties, crystallization behavior, melt rheology, and foam processing of pure polylactide (PLA) and PLA/layered silicate nanocomposites. They concluded this new family of composite materials frequently exhibits remarkable improvements in its material properties when compared with those of virgin PLA. Improved properties can include a high storage modulus both in the solid and melt states, increased tensile and flexural properties, decreased gas permeability, increased heat distortion temperature, and increased rate of biodegradability of pure PLA.

In a complementary review, Sinha Ray and Bousmina (2005) presented recent developments on the above-mentioned properties for many biodegradable polymers' nanocomposites. They described 2 types of biodegradable polymers: (a) originating from renewable sources like PLA, poly (3-hydroxybutyrate), thermoplastic starch (TPS), plant-based polymers, cellulose, gelatin, or chitosan; and (b) originating from petroleum sources like poly (butylene succinate), aliphatic polyesters, poly(ε-caprolactone), or poly (vinyl alcohol).

Recent research on PLA nanocomposites

The potential applications of PLA-based nanocomposites are in food packaging, medical applications, and tissue cultures. Some research conducted on PLA nanocomposites in the field of food packaging after the year 2005 is presented here.

Biodegradability of polymers through photodegradation has been studied by using TiO_2 nanoparticles as photocatalysts that decompose various organic chemicals like aldehyde, toluene, and polymers such as PE, PP, PVC, and PS.

In a study done by Nakayama and Hayashi (2007), TiO_2 nanoparticles were prepared and the surface of TiO_2 was modified using propionic acid and n-hexylamine, with the modified TiO_2 uniformly dispersed into PLA matrixes without aggregation. They studied the PLA-TiO_2 nanocomposite's photodegradation under UV light and concluded photodegradability of nanocomposites can be efficiently promoted.

Melt intercalation is a method where the blending of polymer and silicate layers is followed by molding to form a polymer-layered silicate nanocomposite. In general, for intercalation, polymers and layered hosts are annealed above the softening point of the polymer. Chow and Lok (2009) used this method for studying the effect of maleic anhydride-grafted ethylene propylene rubber (EPMgMA) on the thermal properties of PLA/organo-montmorillonite nanocomposites. They concluded that the addition of OMMT (Organo-montmorillonite) and EPMgMA did not influence much the T_g and T_m (melting temperature) of PLA nanocomposites. The degree of crystallinity of PLA increased slightly in the presence of OMMT; it had been supposed that OMMT could act as a nucleating agent to increase the crystallinity of PLA. In contrast, the addition of EPMgMA may restrict the crystallization process and crystal formation of PLA, which subsequently reduces the degree of crystallinity of PLA/OMMT nanocomposites. Finally, they claimed that the thermal stability of PLA/OMMT was greatly enhanced by the addition of EPMgMA.

Kim and others (2009) studied the effect of bacterial cellulose on the transparency of PLA/bacterial nanocomposites, since bacterial cellulose had shown good potential as reinforcement or preparing optically transparent materials due to its structure, which consists of ribbon-shaped fibrils with diameters in the range from 10 to 50 nm. They found that light transmission of the PLA/bacterial cellulose nanocomposite was quite high due to the size effect of the nanofibrillar bacterial cellulose. Additionally, the tensile strength and Young's modulus of the PLA/bacterial cellulose nanocomposite were increased by 203% and 146%, respectively, compared with those of the PLA.

Carbon nanotubes (CNTs) have been the subject of much attention because of their outstanding performance including excellent mechanical, electrical, and thermal properties. The most promising area of nanocomposite research involves the reinforcement of polymers using CNTs as reinforcing filler (Kim and others 2007).

Li and others (2009) introduced functionalized multiwalled carbon nanotubes (f-MWCNTs) into PLA to investigate the effect of such filler on the crystallization behavior of PLA. They concluded that the addition of f-MWCNTs accelerates the crystallization of PLA dramatically and induces formation of homogeneous and very small spherulites. The results of polarized optical microscopy showed that the average spherulite diameter is about 200 μm, but for nanocomposites it was very difficult to differentiate the spherulites one by one.

Numerous studies have also been done on PLA nanocomposites in medical science regarding drug delivery systems, tissue engineering, and bone fixation (Jo and others 2004; Sakata and others 2006; Chen and others 2007).

PLA Degradability, Biodegradability, and Recyclability

Almost all the conventional plastics such as PE, PP, PS, and PVC are resistant to microbial attack; on the contrary aliphatic polyesters like PLA are readily degraded by microorganisms present in the environment. According to ASTM D6400–04, a biodegradable plastic is "a plastic that degrades because of the action of naturally occurring microorganisms such as bacteria, fungi, and algae," and a compostable plastic is "a plastic that undergoes degradation by biological processes during composting to yield carbon dioxide, water, inorganic compounds, and biomass at a rate consistent with other known compostable materials and leaves no visually distinguishable or toxic residues.

PLA degradation was studied in animal and human bodies for medical applications like implants, surgical sutures, and drug delivery materials (Vainionpaa and others 1989). In these environments, PLA is initially degraded by hydrolysis and the soluble oligomers formed are metabolized by cells. PLA degradation upon disposal in the environment is more challenging because PLA is largely resistant to attack by microorganisms in soil or sewage under

ambient conditions. The polymer must 1st be hydrolyzed at elevated temperatures (about 58 °C) to reduce the molecular weight before biodegradation can commence. No degradation was observed on PLA sheets after 6 wk in soil, thus PLA will not degrade in typical garden compost (Brandrup and others 1999; Ohkita and Lee 2006). Urayama and others (2002) reported that the molecular weight of PLA films with different optical purity of the lactate units (100% L and 70% L) decreased by 20% and 75%, respectively, after 20 mo in soil.

Kale and others (2007) studied the degradation of PLA bottles in a real composting condition (compost containing cow manure, wood shavings, and waste feed) at 65 °C for 30 d. They observed major fragmentation, which produces decomposition of the polymer chain into shorter oligomer chains and monomers since the 4th day, and on the15th day, the bottles were already in pieces and mostly consisted of parts from cap threads, and neck (bottle parts having higher thickness) and finally on the 30th day the bottles were completely degraded.

Microbial and enzymatic degradation of PLA have recently been studied by many researchers because these types of degradations usually do not need the high temperatures to be accomplished. Williams (1981) 1st reported the degradation for PLLA by proteinase K from *Tritirachium.album.*, afterward many studies were done for finding different enzymes corresponding PLA degradation. Reported enzymes that enable to degrade PLA in different scale include, alkaline protease (Oda and others 2000), serine proteases such as subtilisin, trypsin, elastase, and α-chymotrypsin (Lim and others 2005), Cutinase-like enzyme (Masaki and others 2005). Lipase could hydrolyze low molecular weight PLLA and some copolymers such as PDLLA (poly D,L-lactic acid) and, poly(D-lactid-co-glycolide) but not PDLA (poly D-lactic acid) and high molecular weight PLLA (Fukuzaki and others 1989). Pranamuda and others (2001) found an enzyme from *Amycolatopsis* sp. cultures and named it PLLA depolymerase. The optimum pH and temperature for this enzyme were 6.0 and 37 to 45 °C, respectively. PLLA depolymerase can also hydrolyze casein, silk fibroin, succinyl-*p*-nitroanilide, but not PHB and PCL. The enzymatic degradation of aliphatic polyesters by hydrolysis is a 2-step process. The 1st step is adsorption of the enzyme on the surface of the substrate through surface-binding and the 2nd step is hydrolysis of the ester bond (Tokiwa and Calabia 2006).

Pranamuda and others (2001) 1st isolated a PLA-degrading microorganism of *Amycolatopsis* strain from soil environment, which was capable of degrading 60% of the PLA film after 14 d. Suyama and others (1998) reported that PLA-degrading microorganisms are not widely distributed in the natural environment and, thus, PLA is less susceptible to microbial attack in the natural environment than other synthetic aliphatic polyesters like PHB, PCL, and Poly(butylenes succinate) (PBS). Several PLA-degrading microorganisms, their enzymes, and substrate specificities are reported in Table 11. Upon disposal in the environment, PLA is hydrolyzed into low molecular weight oligomers and then mineralized into CO_2 and H_2O by the microorganisms present in the environment.

Microbial degradation of PLA should be studied for packaging of foods containing microorganisms including lactic acid bacteria, and fungi for their probable abilities of PLA degradation. Torres and others (1996) reported the ability of assimilation of lactic acid and racemic oligomer products of PLA for 2 strains of *Fusarium moniliforme* (widely distributed in soil) and on strain of *Penicillium roqueforti* (the main fungus in blue cheese, and can be isolated from soil).

Recycling diverts material from alternative waste streams such as land filling or incineration, as well as conserves natural resources and energy. PET and HDPE make up a large percentage of the plastic bottles that get recycled. Sorting PLA in recycling facilities is difficult due to low volumes and in many cases, the PLA container looks like PET. Because of this, the possibility of mixing the different materials together exists. As a result, there is concern in the recycling community that PLA bottles, at high enough levels, would contaminate the PET recycle stream due to chemical and thermal property differences. The National Association for PET Container Resources (NAPCOR) recently announced its concern for potential contamination of the PET recycling stream associated with PLA bottles. This trade association for the PET plastic industry in the U.S. and Canada cited its concerns involving cost of separation, increased contamination, yield loss, and impact on recycled PET (RPET) quality and processing (www.napcor.com).

Consequently, NatureWorks® and Primo Water Corp. conducted a commercial scale bottle recycling evaluation to demonstrate that automated systems being used today in the recycling industry are capable of separating PLA bottles from PET bottles with good accuracy and efficiency (93%). In this evaluation, near-infrared equipment was used since it is a common sorting technology in large recycling operations and can accurately identify many different types of polymers (NatureWorks® 2009).

Recycled bottles crushed, chopped into flakes, and pressed into bales. They enter to final recycling step and are changed to PLA monomers; L-lactic acid or L-lactide. There are 2 methods for PLA recycling, primarily hydrolysis or solvolysis to L-lactic acid or L-lactic acid-based compounds and, 2nd, depolymerization to the cyclic dimer, L-lactide. Both methods have problems with low yield of monomers in a short period and require the removal of catalysts and additives used for hydrolysis, solvolysis, or depolymerization (Tsuji and others 2003).

High-temperature hydrolysis, normally above the melting point, is an effective way to hydrolyze PLA rapidly to L-lactic acid without the aid of catalysts. The highest maximum yield of L-lactic acid (about 90%) in a high temperature and high pressure water was attained at 250 °C for 10 to 20 min (Tsuji and others 2001).

Conclusion

In previous years, the most negative point of PLA was its price in comparison with petrochemical-based polymers. Today, by using other sources of dextrose, optimizing lactic acid production processes and its costs, substituting electricity energy by wind and solar energy for PLA production, optimizing PLA production processes, and increasing PLA demands, reduction of its price can be attained. The present PLA price is much lower than in previous years, but it is not fixed and it even will be considerably lower in the future because, according to expert forecasts, beyond 2010 the global demand for biodegradable plastics will continue to increase by 30% each year and PLA will take a large part of this market because of its valuable properties (Bastioli 2005).

The linkage of a 100% bio-originated material and nanomaterials opens new windows for becoming independent from petrochemical-based polymers and also free of environmental and health concerns.

Substituting PET with PLA in food packages, which require high-barrier properties, is not feasible unless some modifications are applied to develop its permeability. Also, the brittleness of PLA may also limit its applications where toughness and impact resistance are critical. However, with the help of nanotechnology

Table 11–PLA-degrading microorganism, their enzymes substrate specificities, and detection methods used in degradation tests.

Microorganism	Enzyme	Substrate specificity	Detection method for PLA degradation
Amycolatopsis sp. strain HT 32	Protease	L-PLA	Film-weight loss; monomer production (lactic acid)
Amycolatopsis sp. strain 3118	Protease	L-PLA	Film-weight loss; monomer production
Amycolatopsis sp. strain KT-s-9	Protease	Silk fibroin, L-PLA	Clear-zone method
Amycolatopsis sp. strain 41	Protease	L-PLA, silk powder, casein, Suc-(Ala)3-pNA	Film-weight loss; monomer production
Amycolatopsis sp. strain K104–1	Protease	L-PLA, casein, fibrin	Turbidity method
Lentzea waywayandensis (formerly Saccharothrix waywayandensis)	Protease	L-PLA	Film-weight loss; monomer production
Kibdelosporangium aridum	Protease	L-PLA	Film-weight loss; monomer production
Tritirachium album ATCC 22563	Protease	L-PLA, silk fibroin, elastin	Film-weight loss; monomer production
*Brevibacillus (formerly Bacillus brevis)**	Protease	L-PLA	Change in molecular weight and viscosity
*Bacillus stearothermophilus**	Protease	D-PLA	Change in molecular weight and viscosity
*Geobacillus thermocatenulatus**	Protease	L-PLA	Change in molecular weight and viscosity
*Bacillus sinithii strain PL 21**	Lipase (Esterase)	L-PLA, pNP-fatty acid esters	Change in molecular weight
Paenibacillus amylolyticus strain TB-13	Lipase	DL-PLA, PBS, PBSA, PES, PCL, triolein, tributyrin	Turbidity method
Cryptococcus sp. strain S-2	Lipase (Curtinase)	L-PLA, PBS, PCL, PHB	Turbidity method

Adapted from Tokiwa and Calabia (2006).

and providing safe PLA nanocomposites, many of its weakness compared to petrochemical-based polymer will be resolved.

According to its safety, biodegradability, and ability for being improved in a tailor-made fashion, the authors predict the substituting of many petrochemical-based polymers by PLA for almost all pharmaceutical and direct food contact packaging materials in the near future.

Nomenclature

ASTM = American society for testing and materials;
EVOH = Ethylene vinyl alcohol;
GPPS = General purpose poly(styrene);
HDPE = High-density poly(ethylene);
HIPS = High-impact poly(styrene);
LDPE = Low-density poly(ethylene);
LLDPE = Linear low-density poly(ethylene);
MMT = Montmorillonite;
MWCNT = Multiwalled-carbon nanotube;
OMMT = Organo-montmorillonite;
OPLA = Oriented poly(lactic acid);
OPP = Oriented poly(propylene);
OPS = Oriented poly(Styrene);
PBAT = Poly(butylene adipate terephthalate);
PBS = Poly(butylenes succinate);
PBST = Poly(butylene succinate terephthalate);
PC = Poly(carbonate);
PCL = Poly(ε-caprolactone);
PEA = Poly(ester amide);
PEG = Poly(ethylene glycol);
PEGMA = Poly(ethylene-glycidyl methacrylate);
PEO = Poly(ethylene oxide);
PET = Poly(ethylene terephthalate);
PGA = Poly(glutamic acid);
PHB = Poly(3-hydroxybutyrate);
PHV = Poly(hydroxyl valerate);
PLLA = Poly(L-lactic acid);
PP = Poly(propylene);
PS = Poly(styrene);
PTMAT = Poly(tetramethylene adipate terephthalate);
PVA = Poly(vinyl alcohol);
PVC = Poly(vinyl chloride);
TPS = Thermoplastic starch.

References

Acioli-Moura R, Sun XS. 2008. Thermal degradation and physical aging of poly(lactic acid) and its blends with starch. Polym Eng Sci 48:829–36.

Alexandre M, Dubois P. 2000. Polymer-layered silicate nanocomposites: preparation, properties and uses of a new class of materials. Mater Sci Eng R: Rep 28:1–63.

Amass W, Amass A, Tighe B. 1998. A review of biodegradable polymers: uses, current developments in the synthesis and characterization of biodegradable polyesters, blends of biodegradable polymers and recent advances in biodegradation studies. Polym Int 47:89–144.

Ariyapitipun T, Mustapha A, Clarke AD. 1999. Microbial shelf life determination of vacuum-packaged fresh beef treated with polylactic acid, lactic acid, and nisin solutions. J Food Prot 62:913–20.

Auras R, Harte B, Selke S, Hernandez R. 2003. Mechanical, physical, and barrier properties of poly(lactide) films. J Plastic Film Sheet 19:123–35.

Auras R, Harte B, Selke S. 2004. An overview of polylactides as packaging materials. Macromol Biosci 4:835–64.

Auras RA, Singh SP, Singh JJ. 2005. Evaluation of oriented poly(lactide) polymers vs. existing PET and oriented PS for fresh food service containers. Packag Technol Sci 18:207–16.

Azeredo HMCD. 2009. Nanocomposites for food packaging applications. Food Res Int 42:1240–53.

Bao L, Dorgan JR, Knauss D, Hait S, Oliveira NS, Maruccho IM. 2006. Gas permeation properties of poly(lactic acid) revisited. J Membr Sci 285:166–72.

Bastioli C. 2005. Handbook of biodegradable polymers. 1st ed. Shropshire, U.K.: Rapra Technology Limited. 5 p.

Bogaert JC, Coszach P. 2000. Poly(lactic acids): a potential solution to plastic waste dilemma. Macromol Symp 153:287–303.

Bohlaman GM. 2005. General characteristics, processability, industrial applications and market evolution of biodegradable polymers. In: Bastioli C, editor. Handbook of biodegradable polymers. 1st ed. Shropshire, U.K.: Rapra Technology Limited. p 183–218.

Brandrup J, Immergut EH, Grulke EA. 1999. Polymer handbook. 4th ed. New York: John Wiley and Sons. 163 p.

Budhavaram NK, Fan Z. 2007. Lactic acid production from paper sludge using thermophilic bacteria. AIChE Annual Meeting.

Cai H, Dave V, Gross RA, McCarthy SP. 1996. Effects of physical aging, crystallinity, and orientation on the enzymatic degradation of poly(lactic acid). J Polym Sci, Part B: Polym Phys 34:2701–8.

Chandra R, Rustgi R. 1998. Biodegradable polymers. Prog Polym Sci 23:1273–335.

Chen C, Lv G, Pan C, Song M, Wu C, Guo D, Wang X, Chen B, Gu Z. 2007. Poly(lactic acid) (PLA)-based nanocomposites—A novel way of drug-releasing. Biomed Mater 2:L1–4.

Chollet E, Swesi Y, Degraeve P, Sebti I. 2009. Monitoring nisin desorption from a multi-layer polyethylene-based film coated with nisin-loaded HPMC film and diffusion in agarose gel by an immunoassay (ELISA) method and a numerical modeling. Innov Food Sci Emerg Technol 10:208–14.

Chow WS, Lok SK. 2009. Thermal properties of poly(lactic acid)/organo-montmorillonite nanocomposites. J Therm Anal Calorim 95:627–32.

Clarinval AM. 2002. Classification and comparison of thermal and mechanical properties of commercialized polymers. International Congress & Trade Show, The Industrial Applications of Bioplastics, 2002 February 3–5; York, UK.

Clarinval AM, Halleux J. 2005. Classification of biodegradable polymers. In: Smith R, editor. Biodegradable polymers for industrial applications. 1st ed. Boca Raton, FL, USA: CRC Press. p 3–31.

Coma V. 2008. Bioactive packaging technologies for extended shelf life of meat-based products. Meat Sci 78:90–103.

Conn RE, Kolstad JJ, Borzelleca JF, Dixler DS, Filer LJ, LaDu BN, Pariza MW. 1995. Safety assessment of polylactide (PLA) for use as a food-contact polymer. Food Chem Toxicol 33:273–83.

Cun D, Cui F, Yang L, Yang M, Yu Y, Yang R. 2008. Characterization and release mechanism of melittin-entrapped poly (lactic acid-co-glycolic acid) microspheres. J Drug Deliv Sci Technol 18:267–72.

Datta R, Henry M. 2006. Lactic acid: recent advances in products, processes and technologies: a review. J Chem Technol Biotechnol 81:1119–129.

Del Nobile MA, Conte A, Buonocore GG, Incoronato AL, Massaro A, Panza O. 2009. Active packaging by extrusion processing of recyclable and biodegradable polymers. J Food Eng 93:1–6.

Di Lorenzo ML. 2005. Crystallization behavior of poly(l-lactic acid). Eur Polym J 41:569–75.

Dorgan JR, Lehermeier H, Mang M. 2000. Thermal and rheological properties of commercial-grade poly(lactic acids)s. J Polym Environ 8:1–9.

FDA. 2001. FDA/CFSAN/OPA: Agency response letter: GRAS Notice No. GRN 000065.

FDA. 2002. Inventory of Effective Food Contact Substance (FCS) Notifications No. 178. http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=fcsListing&id=178.

Fukuzaki H, Yoshida M, Asano M, Kumakura M. 1989. Synthesis of copoly(D,L-Lactic acid) with relatively low molecular weight and in vitro degradation. Eur Polym J 25:1019–26.

Furukawa T, Sato H, Murakami R, Zhang J, Duan YX, Noda I, Ochiai S, Ozaki Y. 2005. Structure, dispersibility, and crystallinity of poly(hydroxybutyrate)/poly(L-lactic acid) blends studied by FT-IR microspectroscopy and differential scanning calorimetry. Macromol 38:6445–54.

Gajria AM, Davé V, Gross RA, McCarthy SP. 1996. Miscibility and biodegradability of blends of poly(lactic acid) and poly(vinyl acetate). Polymer 37:437–44.

Garlotta D. 2001. A literature review of poly(lactic acid). J Polym Environ 9:63–84.

Ghosh S, Viana JC, Reis RL, Mano JF. 2008. Oriented morphology and enhanced mechanical properties of poly(l-lactic acid) from shear controlled orientation in injection molding. Mater Sci Eng A 490:81–9.

Giles FH, Wagner JR, Mount EM. 2005. Extrusion, the definitive processing guide and handbook. 1st ed. New York: William Andrew Publishing. 547 p.

Grijpma DW, Altpeter H, Bevis MJ, Feijen J. 2002. Improvement of the mechanical properties of poly(D,L-lactide) by orientation. Polym Int 51:845–51.

Grossman EM. 1995. Annual Technical Conference—ANTEC 95, Conference Proceedings. SCORIM- principles, capabilities and applications. Society of plastic engineers 1995 p 461–76.

Gu H, Song C, Long D, Mei L, Sun H. 2007. Controlled release of recombinant human nerve growth factor (rhNGF) from poly[(lactic acid)-co-(glycolic acid)] microspheres for the treatment of neurodegenerative disorders. Polym Int 56:1272–80.

Harada M, Ohya T, Iida K, Hayashi H, Hirano K, Fukuda H. 2007. Increased impact strength of biodegradable poly(lactic acid)/poly(butylene succinate) blend composites by using isocyanate as a reactive processing agent. J Appl Polym Sci 106:1813–20.

Hartmann MH. 1998. High-molecular-weight polylactic acid polymers. In: Kaplan DL, editor. Biopolymers from renewable resources. Berlin: Springer. p 367–411.

Huang L, Sheng J, Chen J, Li N. 2008. 2nd International Conference on Bioinformatics and Biomedical Engineering, iCBBE 2008. Direct fermentation of fishmeal wastewater and starch wastewater to lactic acid by Rhizopus oryzae. 2008.

Hutchinson MH, Dorgan JR, Knauss DM, Hait SB. 2006. Optical properties of polylactides. J Polym Environ 14:119–24.

Jacobsen S, Fritz HG. 1996. Filling of poly(lactic acid) with native starch. Polym Eng Sci 36:2799–804.

Janorkar AV, Hirt DE. 2004. Annual Technical Conference—ANTEC, Conference Proceedings. Surface modification of poly(lactic acid) films via grafting hydrophilic polymers. 2004.

Jin T, Liu L, Zhang H, Hicks K. 2009. Antimicrobial activity of nisin incorporated in pectin and polylactic acid composite films against Listeria monocytogenes. Int J Food Sci Technol 44:322–9.

Jing F, Hillmyer MA. 2008. A bifunctional monomer derived from lactide for toughening polylactide. J Am Chem Soc 130:13826–7.

Jo YS, Kim MC, Kim DK, Kim CJ, Jeong YK, Kim KJ, Muhammed M. 2004. Mathematical modelling on the controlled-release of indomethacin-encapsulated poly(lactic acid-co-ethylene oxide) nanospheres. Nanotechnology 15:1186–94.

Joerger RD. 2007. Antimicrobial films for food applications: a quantitative analysis of their effectiveness. Packag Technol Sci 20:231–73.

Johnson RM, Mwaikambo LY, Tucker N. 2003. Biopolymers. Rapra Rev Rep 43:1–26.

Kale G, Auras R, Singh SP, Narayan R. 2007. Biodegradability of polylactide bottles in real and simulated composting conditions. Polym Test 26:1049–61.

Kawashima N, Ogawa S, Obuchi S, Matsuo M, Yagi T. 2002. Poly lactic acid "LACEA." In: Doi Y, Steinbuchel A, editors. Biopolymers polyesters III applications and commercial products. Weinheim: Wiley–VCH Verlag GmbH. p 251–74.

Ke T, Sun X. 2001. Thermal and mechanical properties of poly(lactic acid) and starch blends with various plasticizers. Trans Am Soc Agric Eng 44:945–53.

Ke T, Sun XS. 2003a. Starch, poly(lactic acid), and poly(vinyl alcohol) blends. J Polym Environ 11:7–14.

Ke T, Sun XS. 2003b. Thermal and mechanical properties of poly(lactic acid)/starch/methylenediphenyl diisocyanate blending with triethyl citrate. J Appl Polym Sci 88:2947–55.

Ke T, Sun SX, Seib P. 2003. Blending of poly(lactic acid) and starches containing varying amylose content. J Appl Polym Sci 89:3639–46.

Kerry JP, O'Grady MN, Hogan SA. 2006. Past, current and potential utilisation of active and intelligent packaging systems for meat and muscle-based products: a review. Meat Sci 74:113–30.

Kim YM, An DS, Park HJ, Park JM, Lee DS. 2002. Properties of nisin-incorporated polymer coatings as antimicrobial packaging materials. Packag Technol Sci 15:247–54.

Kim KI, Kim WK, Seo DK, Yoo IS, Kim EK, Yoon HH. 2003. Production of lactic acid from food wastes. Appl Biochem Biotechnol 107:637–48.

Kim JY, Park HS, Kim SH. 2007. Multiwall-carbon-nanotube-reinforced poly(ethylene terephthalate) nanocomposites by melt compounding. J Appl Polym Sci 103:1450–7.

Kim Y, Jung R, Kim HS, Jin HJ. 2009. Transparent nanocomposites prepared by incorporating microbial nanofibrils into poly(l-lactic acid). Curr Appl Phys 9:S69–71.

Koo GH, Jang J. 2008. Surface modification of poly(lactic acid) by UV/ozone irradiation. Fibers Polym 9:674–8.

Kylmä J, Härkönen M, Seppälä JV. 1997. The modification of lactic acid-based poly(ester-urethane) by copolymerization. J Appl Polym Sci 63:1865–72.

Labrecque LV, Kumar RA, Davé V, Gross RA, McCarthy SP. 1997. Citrate esters as plasticizers for poly(lactic acid). J Appl Polym Sci 66:1507–13.

Labuza TP, Breene W. 1989. Application of 'active packaging' technologies for the improvement of shelf-life and nutritional quality of fresh and extended shelf-life foods. Bibl Nutr Dieta 43:252–9.

Lee NC. 2006. The extrusion blow moulding system. In: Practical guide to blow moulding. Shawbury, U.K.: Rapra Technology Limited. p 81–98.

Lee CM, Kim ES, Yoon JS. 2005. Reactive blending of poly(L-lactic acid) with poly(ethylene-co-vinyl alcohol). J Appl Polym Sci 98:886–90.

Lee DS, Yam KL, Piergiovanni L. 2008. Food packaging science and technology. 1st ed. New York: Taylor and Francis 631 p.

Lehermeier HJ, Dorgan JR, Way JD. 2001. Gas permeation properties of poly(lactic acid). J Membr Sci 190:243–51.

Li H, Huneault MA. 2007. Effect of nucleation and plasticization on the crystallization of poly(lactic acid). Polymer 48:6855–66.

Li Y, Shimizu H. 2009. Improvement in toughness of poly(l-lactide) (PLLA) through reactive blending with acrylonitrile-butadiene-styrene copolymer (ABS): Morphology and properties. Eur Polym J 45:738–46.

Li BH, Yang MC. 2006. Improvement of thermal and mechanical properties of poly(L-lactic acid) with 4,4-methylene diphenyl diisocyanate. Polym Adv Technol 17:439–43.

Li L, Tang SC, Wang QH, Pan YK, Wang TL. 2006. Preparation of poly (lactic acid) by direct polycondensation in azeotropic solution. J East China Univ Sci Technol 32:672–5.

Li Y, Wang Y, Liu L, Han L, Xiang F, Zhou Z. 2009. Crystallization improvement of poly(L-lactide) induced by functionalized multiwalled carbon nanotubes. J Polym Sci Part A: Polym Chem 47:326–39.

Lim JY, Kim SH, Lim S, Kim YH. 2001. Improvement of flexural strengths of poly(L-lactic acid) by solid-state extrusion. Macromol Chem Phys 202:2447–53.

Lim JY, Kim SH, Lim S, Kim YH. 2003. Improvement of flexural strengths of poly(L-lactic acid) by solid-state extrusion, 2: extrusion through rectangular die. Macromol Mater Eng 288:50–7.

Lim HA, Raku T, Tokiwa Y. 2005. Hydrolysis of polyesters by serine proteases. Biotechnol Lett 27:459–64.

Lim LT, Auras R, Rubino M. 2008. Processing technologies for poly(lactic acid). Prog Polym Sci 33:820–52.

Liu FT, He R, Zhao YD, Gao F, Zhang YX, Cui DX. 2008. Modified biodegradable poly(D, L-lactic-co-glycolic acid) film implants for sustained release of 5-fluorouracil. Shanghai Jiaotong Daxue Xuebao/J Shanghai Jiaotong Univ 42:822–6,30.

Ljungberg N, Wesslén B. 2002. The effects of plasticizers on the dynamic mechanical and thermal properties of poly(lactic acid). J Appl Polym Sci 86:1227–34.

Ljungberg N, Colombini D, Wessleén B. 2005. Plasticization of poly(lactic acid) with oligomeric malonate esteramides: dynamic mechanical and thermal film properties. J Appl Polym Sci 96:992–1002.

Lopez-Rubio A, Almenar E, Hernandez-Munoz P, Lagaron JM, Catala R, Gavara R. 2004. Overview of active polymer-based packaging technologies for food applications. Food Rev Int 20:357–87.

Lopez-Rubio A, Gavara R, Lagaron JM. 2006. Bioactive packaging: turning foods into healthier foods through biomaterials. Trends Food Sci Technol 17:567–75.

Lu D, Zhang X, Zhou T, Ren Z, Wang S, Lei Z. 2008. Biodegradable poly (lactic acid) copolymers. Prog Chem 20:339–50.

Masaki K, Kamini NR, Ikeda H, Iefuji H. 2005. Cutinase-like enzyme from the yeast *Cryptococcus* sp. strain S-2 hydrolyses polylactic acid and other biodegradable plastics. Appl Environ Microbiol 7:7548–50.

Mehta R, Kumar V, Bhunia H, Upadhyay SN. 2005. Synthesis of poly(lactic acid): a review. J Macromol Sci Polym Rev 45:325–49.

Mehta R, Kumar V, Upadhyay SN. 2007. Mathematical modeling of the poly(lactic acid) ring-opening polymerization using stannous octoate as a catalyst. Polym Plast Technol Eng 46:933–7.

Miao LF, Yang J, Huang CL, Song CX, Zeng YJ, Chen LF, Zhu WL. 2008. Rapamycin-loaded poly (lactic-co-glycolic) acid nanoparticles for intraarterial local drug delivery: preparation, characterization, and in vitro/in vivo release. Acta Acad Med Sinicae 30:491–7.

Mills CA, Navarro M, Engel E, Martinez E, Ginebra MP, Planell J, Errachid A, Samitier J. 2006. Transparent micro- and nanopatterned poly(lactic acid) for biomedical applications. J Biomed Mater Res 76:781–7.

Mohanty AK, Misra M, Hinrichsen G. 2000. Biofibres, biodegradable polymers and biocomposites: an overview. Macromol Mater Eng 276–277:1–24.

Mutsuga M, Kawamura Y, Tanamoto K. 2008. Migration of lactic acid, lactide and oligomers from polylactide food-contact materials. Food Addit Contam Part A, Chem, Anal, Control, Expo Risk Assess 25:1283–90.

Nakayama N, Hayashi T. 2007. Preparation and characterization of poly(l-lactic acid)/TiO_2 nanoparticle nanocomposite films with high transparency and efficient photodegradability. Polym Degrad Stab 92:1255–64.

NatureWorks. 2005a. PLA ISBM bottle guide. Minnetonka, Minn.: NatureWorks LLC.

NatureWorks. 2005b. PLA processing guide for biaxially oriented film. Minnetonka, Minn.: NatureWorks LLC.

NatureWorks. 2005c. Processing guide for thermoforming articles. Minnetonka, Minn.: NatureWorks LLC.

NatureWorks. 2006a. PLA 2002D, 3001D, 3051D, 3251D, 4032D, 4042D, 4060D, 7000D, 7032D data sheets. Minnetonka, Minn.: NatureWorks LLC.

NatureWorks. 2006b. PLA injection molding guide for 3051D. Minnetonka, Minn.: NatureWorks LLC.

Natureworks. 2009. Using near-infrared sorting to recycle PLA bottles. Minnetonka, Minn.: Natureworks LLC.

Nijenhuis AJ, Colstee E, Grijpma DW, Pennings AJ. 1996. High-molecular-weight poly(L-lactide) and poly(ethylene oxide) blends: thermal characterization and physical properties. Polymer 37:5849–57.

Oda Y, Yonetsu A, Urakami T, Tonomura K. 2000. Degradation of polylactide by commercial proteases. J Polym Environ 8:29–32.

Ohkita T, Lee SH. 2006. Thermal degradation and biodegradability of poly(lacticacid)/cornstarch biocomposites. J Appl Polym Sci 100:3009–17.

Oliveira NS, Oliveira J, Gomes T, Ferreira A, Dorgan J, Marrucho IM. 2004. Gas sorption in poly(lactic acid) and packaging materials. Fluid Phase Equilib 222–223:317–24.

Oyama HT. 2009. Super-tough poly(lactic acid) materials: reactive blending with ethylene copolymer. Polymer 50:747–51.

Park S, Chang Y, Cho JH, Noh I, Kim C, Kim SH, Kim YH. 1998. Synthesis and thermal properties of copolymers of L-lactic acid and ε-caprolactone. Polymer 22:1–5.

Park SD, Todo M, Arakawa K. 2004. Effect of annealing on fracture mechanism of biodegradable poly(lactic acid). Key Eng Mater 261–263:105–10.

Patey W. 2010. Thermoforming PLA: how to do it right. Plastics Technol 56:30–1.

Platt K. 2006. The global biodegradable polymers market. In: Biodegradable polymers. Shawbury, UK: Smithers Rapra Technology Limited. p 31–48.

Poças MF, Oliveira JC, Oliveira FAR, Hogg T. 2008. A critical survey of predictive mathematical models for migration from packaging. Crit Rev Food Sci Nutr 48:913–28.

Pranamuda H, Tsuchii A, Tokiwa Y. 2001. Poly(L-lactide)-degrading enzyme produced by *Amycolatopsis* sp. Macromol Biosci 1:25–9.

Puaux JP, Banu I, Nagy I, Bozga G. 2007. A study of L-lactide ring-opening polymerization kinetics. Macromol Symp 259:318–26.

Quan D, Liao K, Zhao J. 2004. Effects of physical aging on glass transition behavior of poly(lactic acid)s. Acta Polym Sinica 5:726–30.

Ray SS, Bousmina M. 2005. Biodegradable polymers and their layered silicate nanocomposites: in greening the 21st century materials world. Prog Mater Sci 50:962–1079.

Reddy G, Altaf M, Naveena BJ, Venkateshwar M, Kumar EV. 2008. Amylolytic bacterial lactic acid fermentation—A review. Biotechnol Adv 26:22–34.

Ren Z, Dong L, Yang Y. 2006. Dynamic mechanical and thermal properties of plasticized poly(lactic acid). J Appl Polym Sci 101:1583–90.

Rhim JW. 2007. Potential use of biopolymer-based nanocomposite films in food packaging applications. Food Sci Biotechnol 16:691–709.

Rosato DV, Rosato DV, Rosato MG. 2000. Injection molding handbook. 3rd ed. Boston: Kluwer Academic Publishers 1488 p.

Sakata S, Kei T, Uchida K, Kaetsu I. 2006. Nano-particle of hydrophobic poly lactic acid for DDS. Polym Preprints Japan 55:2074.

Salmaso S, Elvassore N, Bertucco A, Lante A, Caliceti P. 2004. Nisin-loaded poly-L-lactide nano-particles produced by CO_2 anti-solvent precipitation for sustained antimicrobial activity. Int J Pharm 287:163–73.

Sanchez-Garcia MD, Gimenez E, Lagaron JM. 2007. Novel PET nanocomposites of interest in food packaging applications and comparative barrier performance with biopolyester nanocomposites. J Plastic Film Sheet 23:133–48.

Schnieders J, Gbureck U, Thull R, Kissel T. 2006. Controlled release of gentamicin from calcium phosphate-poly(lactic acid-co-glycolic acid) composite bone cement. Biomaterials 27:4239–49.

Scott G. 2000. 'Green' polymers. Polym Degrad Stab 68:1–7.

Sheth M, Kumar RA, Davé V, Gross RA, McCarthy SP. 1997. Biodegradable polymer blends of poly(lactic acid) and poly(ethylene glycol). J Appl Polym Sci 66:1495–505.

Shibata M, Someya Y, Orihara M, Miyoshi M. 2006. Thermal and mechanical properties of plasticized poly(L-lactide) nanocomposites with organo-modified montmorillonites. J Appl Polym Sci 99:2594–602.

Shogren R. 1997. Water vapor permeability of biodegradable polymers. J Environ Polym Degrad 5:91–5.

Sinha Ray S, Okamoto M. 2003. Polymer/layered silicate nanocomposites: a review from preparation to processing. Prog Polym Sci 28:1539–641.

Siparsky GL, Voorhees KJ, Dorgan JR, Schilling K. 1997. Water transport in polylactic acid (PLA), PLA/polycaprolactone copolymers, and PLA/polyethylene glycol blends. J Environ Polym Degrad 5:125–36.

Siracusa V, Rocculi P, Romani S, Rosa MD. 2008. Biodegradable polymers for food packaging: a review. Trends Food Sci Technol 19:634–43.

Södergård A, Stolt M. 2002. Properties of lactic acid based polymers and their correlation with composition. Prog Polym Sci (Oxford) 27:1123–63.

Sokolsky-Papkov M, Golovanevski L, Domb AJ, Weiniger CF. 2009. Prolonged local anesthetic action through slow release from poly(lactic acid co castor oil). Pharm Res 26:32–9.

Sozer N, Kokini JL. 2009. Nanotechnology and its applications in the food sector. Trends Biotechnol 27:82–9.

Suyama T, Tokiwa Y, Ouichanpagdee P, Kanagawa T, Kamagata Y. 1998. Phylogenetic affiliation of soil bacteria that degrade aliphatic polyesters available commercially as biodegradable plastics. Appl Environ Microbiol 64:5008–11.

Taubner V, Shishoo R. 2001. Influence of processing parameters on the degradation of poly(L-lactide) during extrusion. J Appl Polym Sci 79:2128–35.

Throne JL. 1996. Technology of thermoforming. 1st ed. New York: Hanser Publishers. 922 p.

Todo M. 2007. Effect of unidirectional drawing process on fracture behavior of poly(l-lactide). J Mater Sci 42:1393–6.

Tokiwa Y, Calabia BP. 2006. Biodegradability and biodegradation of poly(lactide). Appl Microbiol Biotechnol 72:244–51.

Torres A, Li SM, Roussos S, Vert M. 1996. Screening of microorganisms for biodegradation of poly(lactic acid) and lactic acid-containing polymers. Appl Environ Microbiol 62:2393–7.

Torres-Giner S, Ocio MJ, Lagaron JM. 2008. Development of active antimicrobial fiber-based chitosan polysaccharide nanostructures using electrospinning. Eng Life Sci 8:303–14.

Tsuji H, Ikada Y. 1996. Blends of aliphatic polyesters. I. Physical properties and morphologies of solution-cast blends from poly(DL-lactide) and poly(ε-caprolactone). J Appl Polym Sci 60:2367–75.

Tsuji H, Nakahara K, Ikarashi K. 2001. Poly(L-lactide), high-temperature hydrolysis of poly(L-lactide) films with different crystallinities and crystalline thicknesses in phosphate-buffered solution. Macromol Mater Eng 286:398–406.

Tsuji H, Daimon H, Fujie K. 2003. A new strategy for recycling and preparation of poly(L-lactic acid): hydrolysis in the melt. Biomacromol 4:835–40.

Tsuji H, Okino R, Daimon H, Fujie K. 2006. Water vapor permeability of poly(lactide)s: effects of molecular characteristics and crystallinity. J Appl Polym Sci 99:2245–52.

Uradnisheck J. 2009. Annual Technical Conference—ANTEC 2009, Conference Proceedings. Improved dimensional stability of thermoformed polylactic acid articles. 2009 June 22–26; Chicago IL, USA, p 1612–5.

Urayama H, Kanamori T, Kimura Y. 2002. Properties and biodegradability of polymer blends of poly(l-lactide)s with different optical purity of the lactate units. Macromol Mater Eng 287:116–21.

Uyama H, Ueda H, Doi M, Takase Y, Okubo T. 2006. Plasticization of poly (lactic acid) by bio-based resin modifiers. Polym Preprints Japan 55:5595.

Vainionpaa S, Rokkanen P, Tormall P.1989. Surgical application of biodegradable polymers in human tissues. Prog Polym Sci 14:679–716.

Van Aardt M, Duncan SE, Marcy JE, Long TE, O'Keefe SF, Sims SR. 2007. Release of antioxidants from poly(lactide-co-glycolide) films into dry milk products and food simulating liquids. Int J Food Sci Technol 42:1327–37.

Vermeiren L, Devlieghere F, Van Beest M, De Kruijf N, Debevere J. 1999. Developments in the active packaging of foods. Trends Food Sci Technol 10:77–86.

Vink ETH, Rabago KR, Glassner DA, Gruber PR. 2003. Applications of life cycle assessment to NatureWorks® polylactide (PLA) production. Polym Degrad Stab 80:403–19.

Vink ETH, Rajbago KR, Glassner DA, Springs B, O'Connor RP, Kolstad J, Gruber PR. 2004. The sustainability of NatureWorks polylactide polymers and ingeo polylactide fibers: an update of the future. Initiated by the 1st International Conference on Bio-based Polymers (ICBP 2003), November 2003, Saitama, Japan. Macromol Biosci 4:551–64.

Vink ETH, Glassner DA, Kolstad JJ, Wooley RJ, O'Connor RP. 2007. The eco-profiles for current and near-future NatureWorks® polylactide (PLA) production. Ind Biotechnol 3:58–81.

Wang S, Tao J, Guo T, Fu T, Yuan X, Zheng J, Song C. 2007. Thermal characteristics, mechanical properties and biodegradability of polycarbonates/poly(lactic acid) (PPC/PLA) blends. Lizi Jiaohuan Yu Xifu/Ion Exch Adsorp 23:1–9.

Wang N, Zhang X, Yu J, Fang J. 2008. Study of the properties of plasticised poly(lactic acid) with poly(1,3-butylene adipate). Polym Polym Compos 16:597–604.

White JR. 2006. Polymer ageing: physics, chemistry or engineering? Time to reflect. C.R Chimie 9:1396–408.

Williams DF. 1981. Enzymatic hydrolysis of polylactic acid. Eng Med 10:5–7.

Wolf O. 2005. Techno-economic feasibility of large-scale production of bio-based polymers in Europe. Institute for Prospective Technological Studies, Spain: European Communities. p 50–64.

Xu Q, Czernuszka JT. 2008. Controlled release of amoxicillin from hydroxyapatite-coated poly(lactic-co-glycolic acid) microspheres. J Control Release 127:146–53.

Yang L, Chen X, Jing X. 2008. Stabilization of poly(lactic acid) by polycarbodiimide. Polym Degrad Stab 93:1923–9.

Yu H, Huang N, Wang C, Tang Z. 2003. Modeling of poly(L-lactide) thermal degradation: theoretical prediction of molecular weight and polydispersity index. J Appl Polym Sci 88:2557–62.

Zee MV. 2005. Biodegradability of polymers–Mechanisms and evaluation methods. In: Bastioli C, editor. Handbook of biodegradable polymer. 1st ed. Shropshire, U.K.: Rapra Technology Limited. p 1–22.

Zhang B, He PJ, Ye NF, Shao LM. 2008. Enhanced isomer purity of lactic acid from the nonsterile fermentation of kitchen wastes. Bioresour Technol 99:855–62.

Critical Reviews in Food Science and Nutrition, 50:799–821 (2010)
Copyright © Taylor and Francis Group, LLC
ISSN: 1040-8398 print / 1549-7852 online
DOI: 10.1080/10408398.2010.503694

Taylor & Francis
Taylor & Francis Group

Active Food Packaging Evolution: Transformation from Micro- to Nanotechnology

MUHAMMAD IMRAN, ANNE-MARIE REVOL-JUNELLES, AGNIESZKA MARTYN, ELMIRA ARAB TEHRANY, MURIEL JACQUOT, MICHEL LINDER, and STÉPHANE DESOBRY

Laboratoire d'Ingénierie des Biomolécules, ENSAIA–INPL, Nancy Université, 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France

Predicting which attributes consumers are willing to pay extra for has become straightforward in recent years. The demands for the prime necessity of food of natural quality, elevated safety, minimally processed, ready-to-eat, and longer shelf-life have turned out to be matters of paramount importance. The increased awareness of environmental conservation and the escalating rate of foodborne illnesses have driven the food industry to implement a more innovative solution, i.e. bioactive packaging. Owing to nanotechnology application in eco-favorable coatings and encapsulation systems, the probabilities of enhancing food quality, safety, stability, and efficiency have been augmented. In this review article, the collective results highlight the food nanotechnology potentials with special focus on its application in active packaging, novel nano- and microencapsulation techniques, regulatory issues, and socio-ethical scepticism between nano-technophiles and nano-technophobes. No one has yet indicated the comparison of data concerning food nano- versus micro-technology; therefore noteworthy results of recent investigations are interpreted in the context of bioactive packaging. The next technological revolution in the domain of food science and nutrition would be the 3-BIOS concept enabling a controlled release of active agents through bioactive, biodegradable, and bionanocomposite combined strategy.

Keywords nanoencapsulation, biodegradable, liposome, antimicrobial, regulatory issues, controlled release

INTRODUCTION

The word "necessity" has been transfigured into a more diabolical notion "Fear of death," and thus Fear of death is the mother of invention in the twenty-first century. This fear factor is assessed by the Centers for Disease Control and Prevention (CDC); Foodborne diseases cause approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States each year (Mead et al., 1999). More than 200 known diseases are transmitted through food. Acute gastroenteritis affects 250 to 350 million people in the United States annually and an estimated 25–30% of these cases are thought to be foodborne disease. Approximately one person out of four may experience some form of foodborne illnesses each year (McCabe-Sellers et al., 2004). Such incidences of foodborne illness are mounting in developing countries as well as in the developed world (Greig et al., 2007). The Foodborne Diseases Active Surveillance Network (Food Net) states that comparing 2007 with 2004–2006, the estimated incidence of infections caused by *Campylobacter, Listeria, Salmonella, Shigella, Vibrio,* and *Yersinia* did not decline significantly, and the incidence of *Cryptosporidium* infections increased by 44% (Vugia et al., 2008). At the same time these illness-outbreaks create an enormous social and economic burden due to food recalls. As a result of several food-related incidents and reported outbreaks worldwide, consumer confidence has begun to oscillate (Jevsnik et al., 2008; Sofos, 2008).

The post-process contamination caused by product mishandling and faulty packaging is responsible for about two-thirds of all microbiologically related class I recalls in the United States, with most of these recalls originating from contamination of ready-to-eat (RTE) food products (Cagri et al., 2004; Gounadaki et al., 2007). Post-processing protection using "active packaging and coatings" has been proposed as an

Address correspondence to: Prof. Stéphane Desobry. Laboratoire d'Ingénierie des Biomolécules, ENSAIA–INPL, Nancy Université, 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France. Tel:+33 (0) 3 83 59 58 80; Fax: +33 (0) 3 83 59 57 72. E-mail: Stephane.Desobry@ensaia.inpl-nancy.fr

Carbohydrate Polymers 81 (2010) 219–225

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Cellulose derivative based active coatings: Effects of nisin and plasticizer on physico-chemical and antimicrobial properties of hydroxypropyl methylcellulose films

Muhammad Imran, Soumaya El-Fahmy, Anne-Marie Revol-Junelles, Stéphane Desobry*

Nancy Université, ENSAIA–INPL, Laboratoire d'Ingénierie des Biomolécules (LIBio), 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy Cedex, France

ARTICLE INFO

Article history:
Received 4 December 2009
Received in revised form 3 February 2010
Accepted 9 February 2010
Available online 4 March 2010

Keywords:
Carbohydrate edible coating
HPMC
Polypeptide nisin
Biodegradable packaging
Listeria

ABSTRACT

Bioactive composite coatings based on hydroxypropyl methylcellulose (HPMC), broad-spectrum food preservative nisin (Nisaplin®), and hydrophilic plasticizer glycerol were evaluated for mechanical, barrier (O_2, H_2O), transparency and microbiological effectiveness. Incorporation of Nisaplin® into cellulose derivative, i.e. HPMC-based films strongly increased the film thickness due to salt crystallization while glycerol had normalized it by homogenous dispersibility. The tensile strength of composite films decreased, however ultimate elongation was increased significantly. The dynamic vapour sorption experimental data fitted by different models had shown lesser values of respective energy constants for composite films. The transparency and water permeability of HPMC films were negatively affected by the additives as an effect individual but conversely as combined effect for film transparency. Film bioactivity demonstrated efficacy against *Listeria* > *Enterococcus* > *Staphylococcus* > *Bacillus* spp. These cellulose derivative based active films may thus be a key approach towards eradicating post-process contamination of healthy foods.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Post-process contamination caused by product mishandling and faulty packaging is responsible for about two-thirds of all microbiologically related class I recalls in the USA with most of these recalls originating from contamination of ready-to-eat food products (Cagri, Ustunol, & Ryser, 2004). Over the last few years, consumer demand for food stuff of natural origin (termed as "Bio"), high quality, elevated safety, minimally processed, longer shelf-life, ease-to-eat with a fresh taste and appearance have turned out to be the cardinal importance (Sobrino-Lopez & Martin-Belloso, 2008). Currently, there is an escalating tendency to employ environmental friendly materials with the intention of substituting non-degradable materials, thus reducing the environmental pollution resulting from waste accumulation. To address the environmental issues, and concurrently extend the shelf-life and food quality, reducing packaging waste has catalysed the exploration of new bio-based packaging materials such as edible and biodegradable films (Burke, 2006; Tharanathan, 2003). One of the approaches is to use renewable biopolymers such as polysaccharides, proteins, gums, lipids and their complexes, derived from animal and plant origin (Ray & Bousmina, 2005). Such biodegradable/edible packaging not only ensures food safety but at the same instant are good source of nutrition (Reppas, Swidan, Tobey, Turowski, & Dressman, 2009).

Cellulose-based materials are being widely used as they offer the advantages like edibility, biocompatibility, barrier properties, aesthetic appearance, being non-toxic, non-polluting and having low cost (Vasconez, Flores, Campos, Alvarado, & Gerschenson, 2009). Hydroxypropyl methylcellulose edible films are attractive for food applications because it is a readily available non-ionic edible plant derivative shown to form transparent, odourless, tasteless, oil-resistant, water-soluble films with very efficient oxygen, carbon dioxide, aroma and lipid barriers, but with moderate resistance to water vapour transport (Villalobos, Chanona, Hernandez, Gutiaerrez, & Chiralt, 2005). HPMC is used in the food industry as an emulsifier, film former, protective colloid, stabilizer, suspending agent, or thickener. HPMC is approved for food uses by the FDA (21 CFR 172.874) and the EU (EC, 1995); its safety in food use has been affirmed by the JECFA (Burdock, 2007). The tensile strength of HPMC films is high and flexibility neither too high nor too fragile, which make them suitable for edible coating purposes (Brindle & Krochta, 2008).

Within the scope of natural food preservation, the application of antimicrobial peptides from lactic acid bacteria (LAB) in bioactive packaging films has received great attention (Cleveland,

* Corresponding author. Tel.: +33 3 83 59 58 80; fax: +33 3 83 59 57 72.
E-mail address: Stephane.Desobry@ensaia.inpl-nancy.fr (S. Desobry).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.02.021

Nancy-Université
INPL

AUTORISATION DE SOUTENANCE DE THESE
DU DOCTORAT DE L'INSTITUT NATIONAL
POLYTECHNIQUE DE LORRAINE

o0o

VU LES RAPPORTS ETABLIS PAR :
Madame Amparo CHIRALT BOIX, Professeur, Polytechnic University of Valencia

Monsieur Seamus FANNING, Professeur, Veterinary Science Centre, Belfield, Dublind

Le Président de l'Institut National Polytechnique de Lorraine, autorise :

Monsieur IMRAN Muhammad

à soutenir devant un jury de l'INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE, une thèse intitulée :

"Enrobages actifs contenants des peptides antimicrobiens nano-vectorisés. "

en vue de l'obtention du titre de :

DOCTEUR DE L'INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE

Spécialité : « **Procédés Biotechnologiques et Alimentaires** »

Fait à Vandoeuvre, le 13 avril 2011
Le Président de l'I.N.P.L.,
F. LAURENT

INSTITUT NATIONAL POLYTECHNIQUE - NANCY -

Institut National Polytechnique de Lorraine
inpl@inpl-nancy.fr - www.inpl-nancy.fr

2 av. de la Forêt de Haye - BP 3
54501 Vandœuvre-lès-Nancy Cedex
FRANCE
Tél. : +33(0)3 83 59 59 59
Fax : +33(0)3 83 59 59 55

Résumé

La nanotechnologie possède la potentialité d'améliorer la sécurité, les procédés, l'emballage alimentaire et le concept d'ingrédient fonctionnel. La nano-encapsulation d'agents actifs, est un concept innovant permettant de protéger les agents actifs d'une dégradation éventuelle pendant le procédé de fabrication de l'aliment et son stockage. Parmi les bactériocines, seule la nisine peut être utilisée en tant qu'additif alimentaire (E234). Le principal objectif de ce travail est de développer et d'optimiser une méthode de marquage fluorescent afin d'effectuer des études de transfert dans différents films de bio-polymères et dans l'aliment et de nano-encapsuler la nisine. Les obstacles scientifiques sont relatifs au greffage de fluorochrome de faible poids moléculaire sur la nisine et la réalisation de liposomes multi lamellaires afin d'obtenir un re-largage contrôlé de la nisine dans des matrices biologiques (aliments simulés et biomatériaux). Des films biodégradables à base de dérivés de cellulose (HPMC) contenant de la Nisaplin et 30% de glycérol ont des caractéristiques physico-chimiques compatibles avec celles demandés à un film d'emballage. La nano-encapsulation de la nisine dans différents nano-liposomes (Marin, végétaux ou pro-liposomes) par micro-fluidisation (CCDS) est une technique innovante pour la fabrication de nano-systèmes de re-largage. L'incorporation de nisine sous forme de nano-émulsion est une méthode efficace de contrôle des flores pathogènes sans altérer les caractéristiques des films d'HPMC. La nisine Z a été marquée par un composé fluorescent, et a une masse moléculaire de 3713,3. Des études en microscopie confocale ont permis de démontrer que l'interaction de la nisine avec les membranes bactériennes se situait au niveau de site de division de la cellule. La nisine marquée a permis l'étude des cinétiques de diffusion du composé actif à partir de différents biopolymères vers l'aliment. L'HPMC, le chitosane, le caséinate et l'acide poly-lactique agissent comme des réservoirs et libèrent progressivement la nisine afin d'obtenir un effet inhibiteur durable. Le choix du biopolymère affecte la biodispinibilité du composé à la surface et à l'intérieur de l'aliment. La prochaine révolution concernant la sécurité alimentaire par l'emballage mettra en avant le dernier concept technologique « 3-BIOs » qui se réfère aux notions Bioactif - Biodégradable - Bionanocomposite.
Mots Clés: Nano-biotechnologie, Bactériocines, Biopolymères, Re-largage contrôlé, Sécurité Alimentaire

Abstract

Food nanotechnology has the potential to improve food safety and bio-security, food processing, food packaging and functional ingredients. Nano-encapsulation of active agents is an innovative concept to protect them against possible denaturation during processing and storage. Bacteriocins can be interesting food bio-preservatives at industrial scale, but only nisin is allowed to be used as a food preservative (E234) by World Health Organization. The overall objective of the present work was to optimize and develop fluorescent labeling and encapsulation of nisin for molecular transfer study in different packaging based on biopolymers and in the food. The scientific obstacles were related to the grafting of low molecular weight fluorochrome on nisin, the structuring of multilamellar liposomes to obtain the controlled release kinetics of nisin in a biological matrix (simulated food and biomaterial). Biodegradable films based on cellulose derivative HPMC with Nisaplin® and 30% glycerol is a promising bioactive film due to its improved physico-chemical properties and good antimicrobial efficiency. Nanoencapsulation of nisin in different nanoliposomes (Marine-, Soy-, Pro-liposomes) by using continuous cell disruption system (CCDS) has provided an innovative method for nano-delivery systems fabrication. Incorporation of nisin in nano-emulsion form (encapsulated and free) can possibly be an effective approach to control pathogen without compromising the basic physico-chemical attributes of composite HPMC coatings. The fluorescently labeled nisin Z prepared had a molecular weight of 3717.3 Da. Confocal microscopic studies demonstrated the interaction of nisin with the bacterial membranes at the cell-division sites as possible mechanism of action against food borne pathogen. Fluorescently labeled nisin successfully characterize the controlled release rates of bioactive peptides. HPMC, CTS, SC and PLA packaging bio-membranes act as a reservoir and progressively release nisin to sustain a constant inhibitory effect. Choice of biopolymer is significant in providing requisite bioavailability of antimicrobial compounds at exterior surface and inside the food system. Based on the present study results, the emerging revolution concerning food safety through packaging possibly will rely on « 3-BIOs » blend with nanotechnology, which refers to Bioactive, Biodegradable and Bio-nanocomposite.
Key Words: Nano-biotechnology, Antimicrobials, Biopolymer packaging, Controlled release, Food Security