UNIVERSITE DE LILLE 1 - SCIENCES ET TECHNOLOGIES

Ecole Doctorale Science de la Matière, du Rayonnement et de l'Environnement

THESE DE DOCTORAT

Spécialité : Ingénierie des Fonctions Biologiques

Présentée par
Layal KARAM

Pour l'obtention du grade de
DOCTEUR DE L'UNIVERSITE DE LILLE 1

Etude des interactions aux interfaces entre peptides, matériaux et bactéries, pour la mise au point de surfaces antimicrobiennes et d'emballages alimentaires actifs

Study of surface interactions between peptides, materials, and bacteria for setting up antimicrobial surfaces and active food packaging

---

Préparée aux laboratoires :
ProBioGEM, UPRES EA 1026 - Polytech'Lille, France
UMET, UMR CNRS 8207 - Ecole Nationale Supérieure de Chimie de Lille, France

Soutenance le 8 juillet 2013 devant le jury composé de :

Directeurs de thèse

CHIHIB Nour-Eddine	**Maître de Conférences-HDR,** Université de Lille 1, France
DHULSTER Pascal	**Professeur,** Université de Lille 1, France
JAMA Charafeddine	**Professeur,** ENSC Lille, France

Rapporteurs

GALTAYRIES Anouk	**Maître de Conférences-HDR,** ENSC Paris, France
REVOL-JUNELLES Anne-Marie	**Professeur,** Université de Lorraine, France

Examinateurs

BERJEAUD Jean-Marc	**Professeur,** Université de Poitiers, France
DEGRAEVE Pascal	**Professeur,** Université Claude Bernard Lyon 1, France
HAMMOUTI Belkheir	**Professeur,** Université Mohammed 1erOujda, Maroc

© 2013 Tous droits réservés.

REMERCIEMENTS

Je tiens tout d'abord à remercier profondément mes directeurs de thèse, Prof. Pascal DHULSTER, Prof. Charafeddine JAMA et Dr. Nour-Eddine CHIHIB, pour m'avoir accueilli dans leurs équipes, d'avoir mis à ma disposition les moyens et l'encadrement nécessaires à l'avancement de mes idées, de m'avoir encouragé à établir des collaborations scientifiques très enrichissantes et de m'avoir accordé la liberté de gérer mon travail pour ainsi pouvoir développer mon autonomie et mon indépendance scientifique.

Mes sincères remerciements vont également au Prof. Anne-Marie REVOL-JUNELLES et au Dr. Anouk GALTAYRIES d'avoir accepté d'être rapporteurs de ce travail et aux Prof. Jean-Marc BERJEAUD, Prof. Pascal DEGRAEVE et Prof. Belkheir HAMMOUTI d'avoir accepté d'être membres du jury et examinateurs de ce mémoire.

Je remercie très chaleureusement Dr. Anne Sophie MAMEDE et Dr. Nicolas NUNS de l'UCCS pour m'avoir accompagné sur les analyses de mes nombreux échantillons, pour leur compétence, leur disponibilité et leur patience durant ces années de collaboration.

Je remercie vivement Prof. Guy LOUARN et Dr. Ahmad FAHS pour m'avoir accueilli au sein de l'IMN à Nantes et pour avoir mené une riche collaboration scientifique.

Je souhaite remercier également Dr. Guy DENIAU pour l'accueil chaleureux au sein de son équipe au CEA de Saclay, pour évaluer la possibilité d'une application de leurs procédés innovants de traitement de surface sur mes emballages.

Merci également à l'Ingénieur de Recherche Dominique DERESMES de l'IEMN à Lille pour son expertise et son suivi au cours du traitement de mes échantillons.

J'adresse mes chaleureux remerciements à Véronique LEVEL, responsable du service des relations internationales, pour m'avoir annoncé la bonne nouvelle de l'acceptation de mon dossier à l'Université de Lille 1, pour m'avoir ensuite accompagné

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

dans toutes les procédures administratives depuis mon départ du Liban jusqu'à mon arrivée à Lille et mon inscription à l'Université, pour sa gentillesse et sa disponibilité pour tout renseignement durant mon séjour. Je remercie particulièrement le programme Erasmus Mundus de l'Union Européenne pour avoir financé ma thèse.

Mes remerciements vont également aux entreprises DuPont Health and Nutrition en Angleterre et Polmeri Europa en France pour avoir offert, respectivement, la nisine pure et les emballages plastiques nécessaires à mes travaux de recherche.

Je remercie également tous les étudiants que j'ai encadré en stage et en particulier : Érica Christ PETTERLE, Samir BOUKLA, Marie CROMBET, Maud MILLET, Mélanie MORA et Illona DAGDALIANIDOU.

Je tiens à remercier tous les membres des laboratoires ProBioGEM et UMET. Je remercie particulièrement l'Ingénieur de Recherche Max BECHET et la secrétaire Cathy OUBLION pour leur aide pratique, leur énorme travail et leur gentillesse.

Un grand merci à tous les jeunes docteurs et doctorants de ProBioGEM avec qui j'ai partagé les bons et mauvais moments de ma thèse et qui ont réussi quelquefois à m'arracher de mon ordinateur pour aller prendre une petite pause-café avec eux.

J'exprime finalement mes plus chaleureux remerciements à ma famille, ma sœur et mes amis qui m'ont beaucoup manqué et soutenu durant ces longues années. A mon chéri qui m'a encouragé et soutenu mais surtout qui m'a supporté durant cette thèse qui prenait quasiment tout mon temps. Enfin un grand merci à mes amis à Lille qui ont enrichi et rendu ma nouvelle vie en France beaucoup plus agréable.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Liste des publications et communications scientifiques

I. Publications : Articles dans des journaux internationaux à comité de lecture

- L. Karam, C. Jama, N. Nuns, A.-S. Mamede, P. Dhulster, N.-E. Chihib, Nisin adsorption on hydrophilic and hydrophobic surfaces: evidence of its interactions and antibacterial activity, Journal of Peptide Science. 19 (2013) 377–385.

- L. Karam, C. Jama, P. Dhulster, N.-E. Chihib, Study of surface interactions between peptides, materials, and bacteria for setting up antimicrobial surfaces and active food packaging, Journal of Materials and Environmental Science. 4 (5) (2013) 798-821.

- L. Karam, C. Jama, N. Nuns, A.-S. Mamede, S. Boukla, P. Dhulster, N.-E. Chihib, Nisin-activated hydrophobic and hydrophilic surfaces: assessment of peptide adsorption and antibacterial activity against some food pathogens, Applied Microbiology and Biotechnology. ***(submitted)***

- L. Karam, C. Jama, A.-S. Mamede, A. Fahs, G. Louarn, P. Dhulster, N.-E. Chihib, Study of nisin adsorption on plasma-treated surfaces for setting up antimicrobial food packaging, Reactive and Functional Polymers. ***(submitted)***

II. Communications dans des conférences nationales et internationales

Communications orales

- Congrès « **Third international symposium on antimicrobial peptides (AMP 2012)**», organisé par « French Antimicrobial Peptides Society (FAPS) », Lille – France, 13 – 15 Juin 2012.
  L. Karam, C. Jama, A.-S. Mamède, N. Nuns, P. Dhulster, A. Fahs, G. Louarn, N.-E. Chihib, Study of nisin adsorption and interfacial interactions for setting up antimicrobial food packaging.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

- Congrès **« Journées Nord-Ouest Européennes des Jeunes Chercheurs (JNOEJC 2012) »**, organisé par la Société Chimique de France Nord-Pas de Calais-Picardie, la Société Chimique de France Normandie et le Groupe Français d'Études et d'Applications des Polymères du Nord-Pas de Calais, Villeneuve d'Ascq – France, 7 – 8 Juin 2012.
  L. Karam, C. Jama, A.-S. Mamède, N. Nuns, P. Dhulster, A. Fahs, G. Louarn, N.-E. Chihib, Emballages antimicrobiens: Innovation et sécurité pour nos produits.

- Congrès « **Journées Franco-Libanaises, Physique et Interfaces** », organisé par l'Université Lille 1 et l'Université Libanaise, Villeneuve d'Ascq – France, 18 – 21 Octobre 2011.
  L. Karam, C. Jama, A.-S. Mamède, N. Nuns, P. Dhulster, N.-E. Chihib, Caractérisation des interactions aux interfaces entre antibactériens, bactéries et matériaux pour la mise au point de surfaces et d'emballages antibactériens.

Communications par affiche

- Congrès « **SFGP 2011** », organisé par la Société Française de Génie des Procédés, Lille – France, 29 Novembre – 01 Décembre 2011.
  L. Karam, C. Jama, A.-S. Mamède, N. Nuns, P. Dhulster, N.-E. Chihib, Functionalization of low density polyethylene (LDPE) for the development of antimicrobial food packaging.

- Colloque « **Gestion des biofilms, enjeux industriels** », organisé par Adebiotech / Réseau National Biofilms, Paris – Romainville – France, 13 Octobre 2011.
  L. Karam, C. Jama, A.-S. Mamède, N. Nuns, P. Dhulster, N.-E. Chihib, Fonctionnalisation de polyéthylène basse densité pour la mise au point d'emballage alimentaire.

- Congrès **« Euromat 2011 »**, organisé par la Société Française de Métallurgie et de Matériaux et par Associazione Italiana di Metallurgia, Montpellier – France, 12 – 15 Septembre 2011.
  L. Karam, C. Jama, A.-S. Mamède, N. Nuns, P. Dhulster, N.-E. Chihib, Functionalization of low density polyethylene (LDPE) for the development of bioactive antilisterial food packaging.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

- Congrès **« The 18th International Colloquium on Plasma Processes (CIP 2011) »**, organisé par la Société Française du Vide, Nantes – France, 4 – 8 Juillet 2011.
  L. Karam, C. Jama, A.-S. Mamède, M. Casetta, P. Dhulster, N.-E. Chihib, Development of antilisterial active food packaging by nisin adsorption on low density polyethylene using plasma.

- Congrès **« Matériaux 2010 »**, organisé par la Fédération Française des Matériaux, Nantes – France, 18 – 22 Octobre 2010.
  L. Karam, C. Jama, M. Casetta, P. Dhulster, N.-E. Chihib, Fonctionnalisation de polyéthylène basse densité pour la mise au point d'emballage alimentaire bioactif antilisteria.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Etude des interactions aux interfaces entre peptides, matériaux et bactéries, pour la mise au point de surfaces antimicrobiennes et d'emballages alimentaires actifs

RESUME

La mondialisation du commerce alimentaire et les changements des modes de consommation présentent de nouveaux défis majeurs en sécurité alimentaire. La mise au point d'emballages alimentaires actifs, par adsorption de peptides antimicrobiens sur des matériaux, est une approche innovante et proactive pour améliorer la sécurité, la qualité et la durée de vie des produits emballés. L'adsorption de peptides en surface et l'activité antimicrobienne des supports fonctionnalisés dépendent principalement des propriétés de surface, des traitements de surface permettant de modifier ces propriétés et des interactions peptides-matériaux-bactéries. Dans cette thèse, le choix du peptide antimicrobien s'est porté sur la nisine, bactériocine à activité antilisteria, produite par des souches de *Lactococcus lactis* subsp. *lactis*. L'emballage choisi était le polyéthylène à basse densité, un support fréquemment utilisé dans le secteur agro-alimentaire. Plusieurs procédés de traitements plasma froid ont été mis au point pour développer des surfaces présentant des caractéristiques différentes et des fonctionnalités spécifiques nécessaires à l'étude des mécanismes d'adsorption. Des techniques physico-chimiques de caractérisation ont permis d'une part, de mettre en évidence la fonctionnalisation des supports par les traitements de surface et par la nisine et d'autre part, d'étudier les interactions aux interfaces. L'étude antimicrobienne a été menée pour comparer et confirmer l'activité antimicrobienne des différents emballages traités. Ces analyses ont également été effectuées contre des pathogènes alimentaires et à basse température pour évaluer une possible application industrielle de ces emballages.

Mots-clés : Emballage actif, emballage antimicrobien, nisine, traitement plasma, adsorption de peptides, interactions aux interfaces, techniques de caractérisation de surfaces, pathogènes alimentaires.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Study of surface interactions between peptides, materials, and bacteria for setting up antimicrobial surfaces and active food packaging

ABSTRACT

The globalization of food trade and changes in lifestyles present new major challenges for food safety. Setting up active food packaging, via antimicrobial peptide adsorption on materials, is an innovative and proactive approach to improve the safety, quality, and shelf-life of packaged foods. Peptide adsorption on surfaces and the antimicrobial activity of the functionalized materials depend mainly on surface properties, on surface treatments allowing the modification of such properties, and on peptides-materials-bacteria interactions. In this thesis, nisin, an antilisterial bacteriocin, produced by *Lactococcus lactis* subsp. *lactis,* was used as the antimicrobial peptide. The selected packaging was the low density polyethylene, a commonly used packaging in the food sector. Different cold plasma processes were optimized to develop surfaces with various characteristics and specific functionalities needed for the adsorption studies. Physico-chemical surface characterization techniques permitted from one side, to confirm the surface functionalization by surface treatments and by nisin and from another side, to study the surface interactions. The antimicrobial study was undertaken to compare and confirm the antimicrobial activity of the different treated packagings. This work was also carried out against some food pathogens and at refrigeration temperature in order to assess possible future food packaging applications.

Keywords: Active packaging, antimicrobial packaging, nisin, plasma treatment, peptide adsorption; surface interactions, surface characterization techniques, food pathogens.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Table of contents

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

ABBREVIATIONS

Å	Angströms
AA	Acrylic acid
AES	Auger electron spectroscopy
AFM	Atomic force microscopy
AITC	Allyl isothiocyanate
Ala	Alanine
AM	Antimicrobial agents
Ar	Argon
Asn	Asparagine
ATP	Adenosine 5'-triphosphate
B. cereus	*Bacillus cereus*
BCA	Bicinchoninic acid
BE	Binding energy
BHA	Butylatedhydroxyanisole
BHT	Butylatedhydroxytoluene
ca.	Around
CFU	Colony-forming units
Dha	Dehydroalanine
Dhb	Dehydrobutyrine
DP	Difference in population
EDTA	Ethylenediaminetetraacetate
e.g.	For example
ESCA	Electron spectroscopy for chemical analysis
EU	European Union
eV	Electron volt
FAO	Food and agriculture organization
FDA	Food and drug administration
FTIR	Fourier transform infrared
Gly	Glycine
GRAS	Generally recognized as safe
h	Hour

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

HCl	Hydrochloric acid
His	Histidine
HMT	Hexamethylenetetramine
HPMC	Hydroxyl-propyl-methyl-cellulose
Ile	Isoleucine
kDa	Kilodaltons
L. innocua	*Listeria innoucua*
L. monocytogenes	*Listeria monocytogenes*
LDPE	Low density polyethylene
Leu	Leucine
Lys	Lysine
MAP	Modified atmosphere packaging
Met	Methionine
MIC	Minimum inhibitory concentration
min	Minute
MO	Methyl orange
N_2	Nitrogen
NEXAFS	Near edge X-ray absorption fine structure
nm	Nanometer
ns	Nanosecond
O_2	Oxygen
OD	Optical density
PE-CVD	Plasma-enhanced chemical vapor deposition
PEO–PPO–PEO	Polyethylene oxide–polypropylene oxide–polyethylene oxide
PVC	Polyvinyl chloride
RF	Radio-frequency
RMS	Root-mean-squared
s	Second
S. aureus	*Staphylococcus aureus*
SEM	Scanning electron microscopy
Ser	Serine
sp.	Species
subsp.	Subspecies

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

TBHQ	Tertiary butylhydroquinone
TBO	Toluidine blue O
ToF-SIMS	Time-of-flight secondary ion mass spectrometry
US	United States
UV	Ultraviolet
Val	Valine
WHO	World health organization
XPS	X-ray photoelectron spectroscopy

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

INTRODUCTION

Food safety is recognized as a global public health priority and remains a current major challenge. Microbial food contamination causes many diseases, ranging from the most common diarrheal symptoms to severe illnesses and death. Unsafe foods not only significantly affect people's health and well-being, but they also have economic consequences for individuals, families, communities, businesses, and countries. As some foodborne diseases are controlled, others emerge as new threats. Globalization of the food trade has led to the rapid and widespread international distribution of foods, contributing thus to the risk of spread of pathogens and contaminants into new geographical areas. The distribution chains and the interval between processing and consumption of foods are longer. Travelers and immigrants may be exposed to unfamiliar foodborne hazards in new environments. Changes in microorganisms lead to the emergence of new pathogens, development of antibiotic resistance, and changes in virulence of known pathogens. Other challenges include changes in lifestyles such as eating meals prepared outside the home, requests for a wider variety of products, and demands for foods that are fresh, minimally processed, natural, and "preservative-free". The food production chain has become more complex, providing greater risks for contamination and growth of pathogens.

Traditional food preservation methods, as thermal processing, drying, freezing, irradiation and addition of chemical and food additives to products, present many disadvantages:

- High temperature treatments can compromise the nutritional, functional, and sensory characteristics of foods (Wan et al., 2009).
- Chemical preservatives are disapproved by consumers and present other health risks (Papagianni, 2003).
- The direct incorporation of food additives into product formulations may result in partial inactivation or dilution of the active substances by the food constituents. It is therefore expected to have only a limited effect on the microbial growth (Appendini and Hotchkiss, 2002; Coma, 2008).
- Some of these techniques cannot be applied to foods such as meats, fresh and ready-to-eat products (Coma, 2008; Kerry et al., 2006; Quintavalla and Vicini, 2002).

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

- These methods are not sufficient to meet new food safety challenges and to prevent still occurring foodborne outbreaks.

All these emerging challenges are the driving forces for the food industry to adapt to a changing environment, to innovate, and increasingly investigate new preservation methods that can be applied with respect to "hurdle technology" approach. Hurdle technology is a crucial concept based on the intelligent combinations of existing and novel preservation techniques ("hurdles") in order to achieve multi-target, mild but most effective preservation of foods (Leistner and Gorris, 1995). The different hurdles in a food (as low temperature, water activity, pH, packaging, bacteriocins) will not just have an additive effect on safety, but might act synergistically. In practical terms, this could mean that it is more effective to employ different preservative factors of small intensity than one preservative factor of larger intensity (Leistner, 2000). Thereby, the product safety is achieved without exposing it to extreme conditions, compromising its quality, as high temperatures or high preservative concentration. This approach is not only important for improving traditional preservation techniques but can also be more efficient when used for novel techniques. Active packaging technologies are considered as new "hurdles" that can play a major role in increasing the quality and safety of food products (Appendini and Hotchkiss, 2002; Leistner and Gorris, 1995). Such innovative packaging possesses attributes beyond basic barrier properties of traditional passive system. It is achieved by adding active ingredients in the packaging system and/or using functionally active polymers (Dutta et al., 2009). There are different types of active food packaging. The antimicrobial system is the version that acts directly to inhibit or retard the growth of microorganisms, decreasing thus the risk of foodborne illness and increasing the quality and shelf-life of foods (Suppakul et al., 2003). Antimicrobial peptide adsorption on surfaces presents an attractive method for setting up such system because it can use natural antimicrobials "peptides" meeting consumers' demands and it can prevent the adhesion of microorganisms not only on food packaging but also on other food contact surfaces.

For this purpose, the literature review will present briefly the different emerging technologies in active food packaging and the antimicrobials that can be used for setting up antimicrobial surfaces. The focus will be then on the study of peptide adsorption on surfaces. The research challenges, development steps, and surface treatment techniques that have potential and applications in this field are assessed.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

CHAPTER I:

LITERATURE REVIEW - ARTICLE I

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Study of surface interactions between peptides, materials, and bacteria for setting up antimicrobial surfaces and active food packaging

Layal Karam[1,2], Charafeddine Jama[2], Pascal Dhulster[1], Nour-Eddine Chihib[1,*]

[1]*Laboratoire ProBioGEM, Polytech'Lille, Université Lille1, Avenue Paul Langevin, 59655 Villeneuve d'Ascq, France*

[2]*Laboratoire UMET, UMR-CNRS 8207, Ecole Nationale Supérieure de Chimie de Lille, Université Lille 1, Avenue Dimitri Mendeleïev, 59655 Villeneuve d'Ascq, France*

* Corresponding author. E mail: Nour-Eddine.Chihib@univ-lille1.fr;
Tel: (+33) 03 59 63 21 75.

Abstract

Active food packaging and antimicrobial surfaces are innovative solutions for increasing food-borne diseases and changes in food habits. This review describes the different methods for setting up active packaging, with specific focus on antimicrobial surfaces developed by peptide adsorption. The key elements in the study of peptide adsorption on surfaces are the peptide nature, the surface properties and the interactions between them. Nisin, a well-known peptide, was reviewed and can be considered as a model for peptide studies. Plasma surface treatments were evaluated as potential versatile tools to provide specific functional groups and various surface characteristics needed to optimize and understand the adsorption behavior. The surface characterization methods were screened and discussed in terms of their relevance to investigate the interactions between peptides and surfaces, confirm each modification step and evaluate the antimicrobial activity of surfaces. For each factor, the advantages, drawbacks, applications, and further considerations were assessed in the fields of interaction studies and active food packaging technologies.

Keywords: Active food packaging; nisin; plasma treatment; peptide adsorption; surface interactions; surface characterization techniques.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

1. Introduction

Food-borne illnesses and microbial contamination are still issues of major worldwide concern despite the introduction of modern technologies and safety concepts in the food industry. Novel strategies for food preservation include setting up active packaging and antimicrobial surfaces. Active packaging can be defined as a mode of packaging in which the package, the product and the environment interact to prolong the shelf-life or enhance the safety or sensory properties of the product, while maintaining its quality [1]. Among the several types of available active packaging, the antimicrobial version is of great importance [2].

Various kinds of active substances can now be added to the packaging material to improve its functionality and give it new or additional function. Setting up antimicrobial surfaces by peptide adsorption presents many advantages. It permits to prevent surface contamination by killing the bacteria attempting to attach on surfaces. It is also of particular interest for basic studies of peptides-materials-bacteria interactions, which provide an essential basis for the development of other more sophisticated antimicrobial systems. Besides its potential for antimicrobial food packaging applications, it can be used to cover surfaces of food processing equipment so that they self-sanitize during use [3]. This method has in addition found applications in the biomedical sector to prevent microbial growth on medical and implanted devices [4-6].

The study of peptides adsorption on surfaces requires mainly the following: (1) choice of peptide and peptides characterization, (2) surface modification, and (3) surface characterization.

Of all the antimicrobial peptides known, nisin is presently the only one commercially available and approved by the FDA (Food and drug administration) and WHO (World health organization) [7]. Nisin is a bacteriocin, naturally produced by *Lactococcus lactis* subsp. *lactis.* This peptide is effective against a wide range of Gram-positive bacteria [8]. It has also shown the ability to retain its antibacterial activity in the adsorbed state [9]. Other bacteriocins have been isolated and can act as effectively as nisin with respect to particular foods/target bacteria. However, they have not been studied and exploited to the same extent as nisin. Therefore, nisin can serve as a model or case study encouraging the emergence of new bacteriocins and new potential bio-preservatives.

Peptides adsorption behavior is largely controlled by surface characteristics. This means that surface modification plays a vital role in the effectiveness of activated

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

materials. Surface modification can be achieved by chemical and physical methods. Wet chemical methods using strong acids and bases have been used industrially, but the disposal of hazardous waste leads to environmental and safety problems [7,10]. As a result, physical surface modification methods are preferred and include: flame, corona, irradiations, ultraviolet (UV), plasma, and laser treatments. Plasma treatment is probably the most versatile surface treatment technique. Different types of plasma can provide a wide range of surface modifications and can be used to create new specific surface functionalities, morphologies and chemistries that will result in different surface interactions [11,12]. This is highly valuable to study and enhance peptide adsorption on surfaces. They can in addition improve the wettability, sealability, adhesion, barrier, and many other characteristics of food packaging materials, while maintaining desirable bulk properties of the polymer [11].

After functionalization, characterization of the surface-engineered materials has profound scientific importance, leading to understanding the interactions taking place between peptides, materials, and bacteria. Examples of methods used for such purposes involve: X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (TOF-SIMS) to reveal the surface chemical structure of materials, atomic force microscopy (AFM) to investigate the topography of surfaces, colorimetric assays to quantify the amounts of surface functional groups and adsorbed peptides, as well as antimicrobial tests to assess the surfaces' antimicrobial activity.

In the first part of this paper, the various kinds of active packaging and the different classes of antimicrobials that can be used for antimicrobial packaging are briefly presented. The second part focuses on peptides adsorption on surfaces. Nisin's characteristics, mode of action, antibacterial activity, resistance, applications, as well as its specific adsorption on surfaces are discussed. Then, the general principles, advantages and disadvantages of different plasma processes that can find potential applications in food packaging and interactions studies are assessed. The following section describes the most widely used surface characterization methods and highlights their advantages, limitations and applications with respect to adsorption studies. Finally, general further considerations in this field are addressed.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

2. Different methods for setting up active food packaging

Active packaging concepts provide additional specific functions in food preservation as compared to traditional passive packaging limited to hold the food product and protect it from the external conditions [13]. A proposed classification of the different active packaging systems is shown in figure 1 and each type is briefly described below. Even though the active packaging systems are not all antimicrobials, they can contribute indirectly to food preservation and inhibition of bacterial growth.

2.1. Addition of sachets, pads or tablets containing the active substances into the packaging

The atmosphere within packaging can be changed by incorporating active substances into the package using a sachet, pad or tablet and allowing mechanisms like evaporation and absorption processes to inhibit the microbial growth. Such sachets are enclosed loose or attached to the interior of a package and their common disadvantages are the risks of sachet leakage and that the sachets can be accidentally ingested [7]. The most commercial applications include oxygen scavengers, carbon dioxide scavengers and generators, moisture absorbers, ethanol and chlorine dioxide generators.

2.1.1. Oxygen scavengers

Oxygen scavengers are primarily used to prevent oxidation, microbial growth, and spoilage reactions in foods [14]. Although they may not be intended to be antimicrobial, a reduction in oxygen inhibits the growth of aerobic bacteria and molds [3]. Oxygen scavenging technology may be used to remove residual O_2 after modified atmosphere packaging (MAP) or vacuum packaging and to absorb the oxygen that permeates through the packaging film [2]. However, under certain circumstances, their use can promote the growth of facultative or anaerobic microorganisms, which present another big threat to food safety [7]. Such types of packaging are relevant for meat, bakery, pasta, dairy, and produce industries [1,3,14].

2.1.2. Carbon dioxide scavengers and generators

Carbon dioxide generators are considered as antimicrobials too because of their inhibitory activity against a range of aerobic bacteria and fungi [2]. However, it has been reported

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

that a high concentration of carbon dioxide decreased the growth rate of *Clostridium botulinum* but greatly increased its production of toxin [15]. Thus, research into the safety risks associated with the use of carbon dioxide in packaging systems is necessary. Most applications for this packaging are for meat and poultry preservation [2].

Carbon dioxide absorbers may be used to remove carbon dioxide during storage in order to prevent bursting of the package. Possible applications include their use in packs of dehydrated poultry products and beef jerkey [14].

2.1.3. Moisture absorbers

Moisture absorbers are used to prevent water condensation and reduce the water activity of the product, thereby indirectly affecting microbial growth. They have been successfully used for moisture control in a wide range of foods, such as cheeses, meats, chips, nuts, popcorn, candies, gums, and spices [1,3].

2.1.4. Ethanol generators

Ethanol is used routinely in medical and pharmaceutical packaging applications, indicating its potential as an antimicrobial. Ethanol generators retard molds and prevent microbial spoilage of intermediate moisture foods as cheese, bakery, and dried fish products [1,3].

2.1.5. Chlorine dioxide generators

Chlorine dioxide has an antimicrobial activity against a broad spectrum of microorganisms including bacteria, spores, fungi, and viruses. Sustained and controlled release of chlorine dioxide is related to exposure to humidity greater than 80% and light. Applications for this technology are just beginning to unfold in the food industry for meat, poultry, fish, dairy, confectionery, and baked goods [2]. However it has an adverse effect on meat quality including color darkening [7].

2.2. Direct incorporation of the antimicrobial agents into the packaging material

Many antimicrobials can be directly incorporated into the packaging material, particularly films. Thermally stable antimicrobials as silver substituted zeolites and triclosan can be added in the melt for extrusion, co-extrusion or injection molding during the polymer/film

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

processing [16,17]. For heat-sensitive antimicrobials like enzymes, solvent compounding may be a more suitable method for their incorporation into polymers [3]. However, all those packaging materials must be in contact with the food and a migration of the antimicrobial agents to the surface is expected [2]. For volatile antimicrobials as sulfur dioxide and allyl isothiocyanate, precursor molecules are incorporated directly into the polymer or into carriers that may be extruded into packaging materials. The theoretical advantage of volatile compounds is that they can penetrate the bulk matrix of the food and that the contact between the food and the packaging is not necessary [3].

Antimicrobials can be incorporated into multilayers films (control layer/matrix layer/barrier layer) to achieve appropriate controlled release to the food surface. The inner layer controls the rate of diffusion of the active substance, while the matrix layer contains the active substance and the barrier layer prevents migration of the agent towards the outside of the package [3,18]. The main drawback of this method is that the embedded antimicrobials in the matrix layer will lack direct contact with the surrounding bulk, and will need to be efficiently released through diffusion to the interface. The diffusion process of the agents in the multilayer architecture is more complex than diffusion in solutions. Additional factors such as the tortuosity of the diffusion pathway, assembly thickness and peptide–polymer interactions can significantly impact the diffusion process. Furthermore, binding of bacteria on the top assembly layer may block the exit of bioactive molecules, which are still entrapped within the matrix [18].

Recent developments in nanotechnology allow also the incorporation of bioactive nano-compounds into the film for food packaging applications [7,19]. However the use of nano-composites has been a concern owing to the potential hazard of inhaled or ingested nano-materials and to the insufficient database and information on their toxicity [7].

2.3. Coating of the antimicrobial agents on the surface of the packaging material

An alternative to the incorporation of antimicrobial compounds during extrusion is to apply the antimicrobial additives as a coating. This has the advantage of placing the specific antimicrobial additive in a controlled manner without subjecting it to high temperature or shearing forces [2,3,14]. In addition, the coating can be applied at a later step, minimizing the exposure of the product to contamination. The coating can serve as a carrier for antimicrobial compounds in order to maintain high concentrations of preservatives on the surface of foods. Bioactive agents' activity may be based on migration or release by

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

evaporation in the headspace. Further research is required to establish the parameters for optimal antimicrobial efficiency, adhesion on packaging support, or the desorption procedure from the materials. Such factors as levels of antimicrobial agents, biocide purity, plastic formulation, and varying plastic composition will need to be evaluated [2].

2.4. Natural or modified antimicrobial polymers

Some polymers are inherently antimicrobials while others need to be modified to render them antimicrobials.

2.4.1. Natural antimicrobial polymers

Inherently antimicrobial polymers with film-forming properties provide bioactive films and can at the same time, be used as carriers of other antimicrobials. Cationic polymers such as chitosan and poly-L-lysine exhibit antibacterial activity since charged amines interact with negative charges on the cell membrane, causing leakage of intracellular constituents [2,3].

2.4.2. Modified antimicrobial polymers

In contrast to naturally antimicrobial polymers, some bioactive materials have been produced by modifying the surface composition of the polymer. A conversion of amide to amine groups of nylon by electron irradiation achieved an antimicrobial activity that inactivated target cells by contact [20]. Plasma treatments are under development as well. Ozdemir and co-workers [21] indicated that fluorine-based plasmas may be used to fluorinate the surface of polymers and form a packaging with "self-sterilization" or "self-pasteurization" capabilities. However, further research needs to be done to establish the effectiveness of such treatment.

2.5. Bioactive edible films and coatings

There is a growing interest in edible coatings due to factors such as environmental concerns, need for new storage techniques, and opportunities for creating new markets for under-utilized agricultural commodities with film-forming properties. Edible films and coatings prepared from polysaccharides, proteins, and lipids have a variety of advantages

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

such as biodegradability, edibility, biocompatibility, aesthetic appearance, and barrier properties against oxygen and physical stress [14,17]. For active packaging applications, the incorporated active agents are limited to edible compounds. The antimicrobials should be safe and approved as a food additive, because they have to be consumed with the coating layers and foods together. Cellulose derivatives such as hydroxyl-propyl-methyl-cellulose (HPMC) and alginate coatings are promising raw materials for edible coatings associated with antimicrobial entities [2,17]. The potential applications of edible films include preservation of fresh, frozen and processed meat and poultry products [2,7,17].

2.6. Attachment of the antimicrobials agents on the surface of the packaging material

Bioactive molecules can be attached onto polymers either chemically (via covalent immobilization) or physically (via adsorption) for setting up active antimicrobial surfaces. In the first category, the antimicrobial agent does not migrate to the surface of the food. In the second one, the antimicrobial effect is achieved with migration. Moreover such antimicrobial surfaces are intended not only for food applications but also for biomedical applications [22].

2.6.1. Antimicrobials covalent immobilization on surfaces

Chemical methods of immobilization involve the formation of at least one covalent bond between the bioactive molecules and the polymer matrix. This requires the presence of functional groups on both the antimicrobial and the polymer [3]. The antimicrobials with functional groups and with molecular structure large enough to retain activity in such applications are limited to enzymes or other antimicrobial proteins [17]. The most commercial polymers are inert with no reactive groups and they must thus undergo surface functionalization prior to attachment of a bioactive compound. Surface treatments using plasma techniques can be used for this purpose and will be discussed later. In addition, immobilization usually requires the use of cross-linkers or "spacer" molecules that link the functionalized polymer surface to the bioactive agent [3,22].

The advantages of this method are creating a stable bond between the compound and the functionalized polymer surface and providing activity during a sustained period of time. For active food packaging applications, it ensures that the bioactive compound will not migrate to the food and thus may offer the regulatory advantage of not requiring approval as a food additive but it still need to be accepted for direct contact with the food [2,22].

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

However, a consistent problem with the immobilized bioactive compounds is the decrease in their activity [18,22-24]. Covalent binding may alter the conformational structure and the active centers of the bioactive molecules as peptides and enzymes [24]. It may also restrict the mobility of peptides affecting thus their mode of action normally involving insertion and disruption of the cell membrane of the target micro-organism [25]. This reduced activity can significantly compromise the effectiveness of activated surfaces and shows the need for detailed fundamental studies to understand and optimize the parameters affecting the antimicrobial performance of immobilized bioactive agents. Such parameters include surface concentration of bound antimicrobials, spacer length, spacer flexibility, spacer cleavage due to polymer degradation reactions, and effect of peptide orientation at the interface on its biological activity [18].

2.6.2. Antimicrobials adsorption on surfaces

Physical methods of adsorption are mainly governed by hydrogen bonding, van der waals forces, electrostatic and hydrophobic interactions between the antimicrobials and the surfaces. Peptides are the most well-known antimicrobials with ability to adsorb on surfaces [25]. The factors affecting peptide adsorption on surfaces are numerous and entail: the peptide characteristics (type, charge, size, conformational stability…), the surface properties (nature, composition, charge, topography, roughness, hydrophobic/hydrophilic character…), the surrounding medium (pH, ionic strength), and the interactions between them. Moreover, peptide adsorption on surfaces can be optimized by the proper combination of treatment conditions such as contact time, peptide concentration, pH of the solution, and adsorption temperature [25-28]. However, such non-covalent methods are effective for short-term applications, because of the limited availability and relatively quick release of antimicrobials from the polymer. This method is the main focus of this review and will be further detailed for specific nisin (peptide) adsorption on surfaces.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

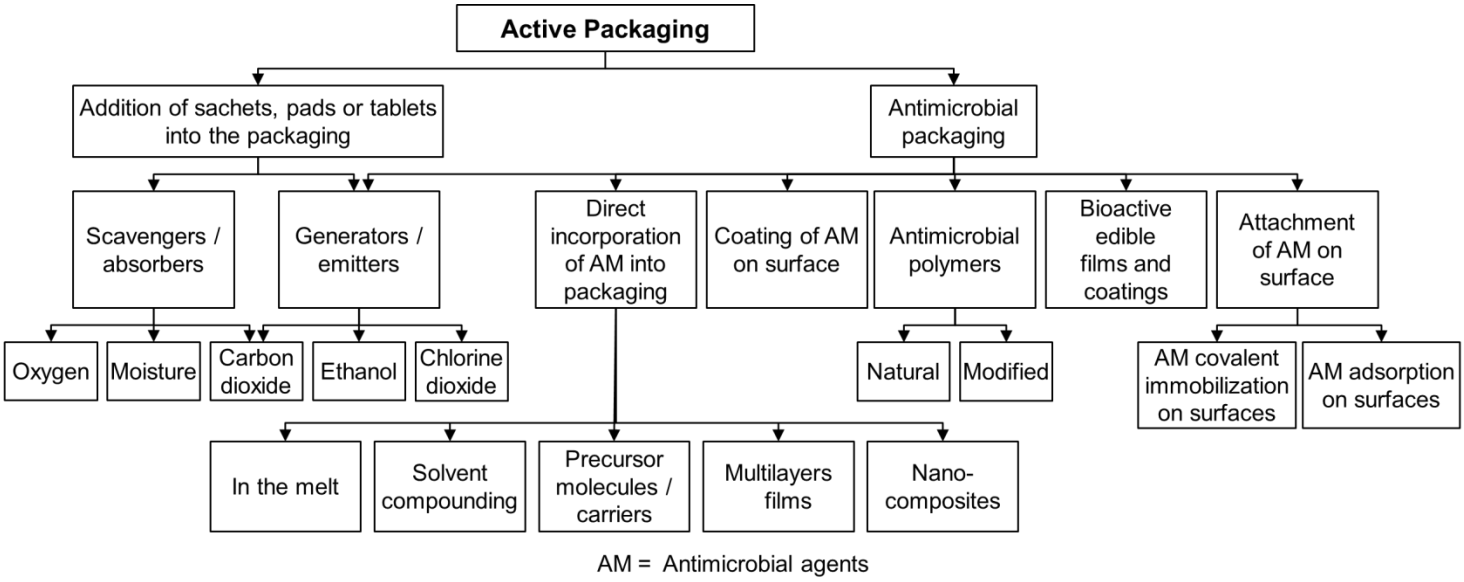

Figure 1: Classification of the different active packaging systems.

3. Classes of antimicrobials

Antimicrobial packaging is a form of active packaging that requires the presence of antimicrobials in order to be effective. The different antimicrobial agents that can be used in antimicrobial films, containers, and utensils are presented in previous reviews [1-3,7,17] and are listed in table 1.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Table 1: Examples of antimicrobial agents for potential use in food packaging materials

Antimicrobial agents classes	**Examples**
Organic acids	Acetic acid, benzoic acid, *p*-aminobenzoic acid, citric acid, lactic acid, malic acid, propionic acid, sorbic acid, succinic acid, tartaric acid
Organic acid salts	Potassium sorbate, sodium benzoate, potassium lactate
Organic acid anhydrides	Benzoic anhydride, sorbic anhydride
Inorganic acids	Phosphoric acid
Inorganic gases	Sulfur dioxide, chlorine dioxide
Alcohols	Ethanol
Amines	Hexamethylenetetramine (HMT)
Ammonium compounds	Silicon quaternary ammonium salt
Antibiotics	Natamycin
Antimicrobial peptides	Defensin, magainin, attacin, cecropin
Antioxidants	Butylatedhydroxyanisole (BHA)
Bacteriocins	Bavaricin, brevicin, carnocin, lacticin, mesenterocin, nisin, pediocin, sakacin, subtilin
Chelators	Citrate, conalbumin, ethylenediaminetetraacetate (EDTA), lactoferrin, polyphosphate
Enzymes	Chitinase, ethanol oxidase, β-glucanase, glucose oxidase, lysozyme, myeloperoxidase
Fatty acids	Lauric acid, palmitoleic acid, glycerol mono-laurate
Fatty acid ester	Monolaurin (lauricidin®)
Fungicides	Benomyl, imazalil

(continued on next page)

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Table 1: Examples of antimicrobial agents for potential use in food packaging materials (continued)

Antimicrobial agents classes	Examples
Metals	Copper, silver, zirconium, titanium oxide
Plant and spices extracts	Allyl isothiocyanate (AITC), grapefruit seed extract, bamboo powder, rheum palmatum, coptischinensis extracts, cinnamic acid, caffeic acid, *p*-coumaic acid
Essential oils and plant-volatile components	Carvacrol, cineole, cinnamaldehyde, citral, *p*-cymene, estragole (methyl chavicol), geraniol, Hinokitiol (β-thujaplicin), linalool, terpineol, thymol, oregano, lemongrass
Natural phenols	Catechin, *p*-cresol, hydroquinones
Phenolic compounds	Butylatedhydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ)
Parabens	Ethyl paraben, methyl paraben, propyl paraben
Polysaccharides	Chitosan, konjac glucomannan
Oligosaccharides	Chitooligosaccharide
Miscellaneous	Reuterin, triclosan, nitrites and sulphites, probiotics

4. Nisin

Among the different classes of antimicrobials, bacteriocins represent an attractive choice with regard to consumers demanding "natural and healthy products" and avoiding "artificial and chemical preservatives". Bacteriocins are antibacterial peptides produced by bacteria and can kill or inhibit the growth of other bacteria (usually closely related species) [29]. Many lactic acid bacteria produce a high diversity of different bacteriocins [30]. Although several bacteriocins have been characterized, nisin remains the most studied and the most commercially important bacteriocin because it is the only one approved for food applications and it has gained widespread application in the food industry. Nisin has been added to the GRAS (Generally recognized as safe) list by the United States FDA (Food

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

and drug administration) and to the positive list of food additives by the EU (European Union) where it was assigned the number E234. It has also been accepted by the FAO (Food and agriculture organization) and the WHO (World health organization). The peptide has been used as a food preservative in almost 50 countries for over 40 years because of its non-toxicity, high antibacterial activity, immediate digestibility in the intestine by enzyme α-chymotrypsin, heat stability at low pH, and absence of color and flavor [31,32].

4.1. Nisin characterization

Nisin is a ribosomally synthesized and post-translationally modified lantibiotic, produced by *Lactococcus lactis* subsp. *lactis* [33]. Lantibiotics are class I bacteriocins characterized by intramolecular rings formed by the unusual thioether amino acids lanthionine and 3-methyllanthionine, and also contain other rare dehydrated amino acids including dehydroalanine (Dha) and dehydrobutyrine (Dhb) [34,35]. Post-translational modification renders the lantibiotics biologically active [32]. In addition, nisin is a 3.5 kDa cationic amphiphilic peptide with a net positive charge. It contains 34 amino acids distributed in clusters of bulky hydrophobic residues at the N-terminus and hydrophilic residues at the C-terminus end [36] (Fig. 2). Nisin A is the originally isolated form of nisin and a further five natural variants have been described and differ by up to 10 amino acids (of 34 in total in nisin A). Nisin Z, F, and Q are like nisin A produced by *Lactococcus lactis*, while nisin U and U2 are produced by *Streptococcus* sp. [37].

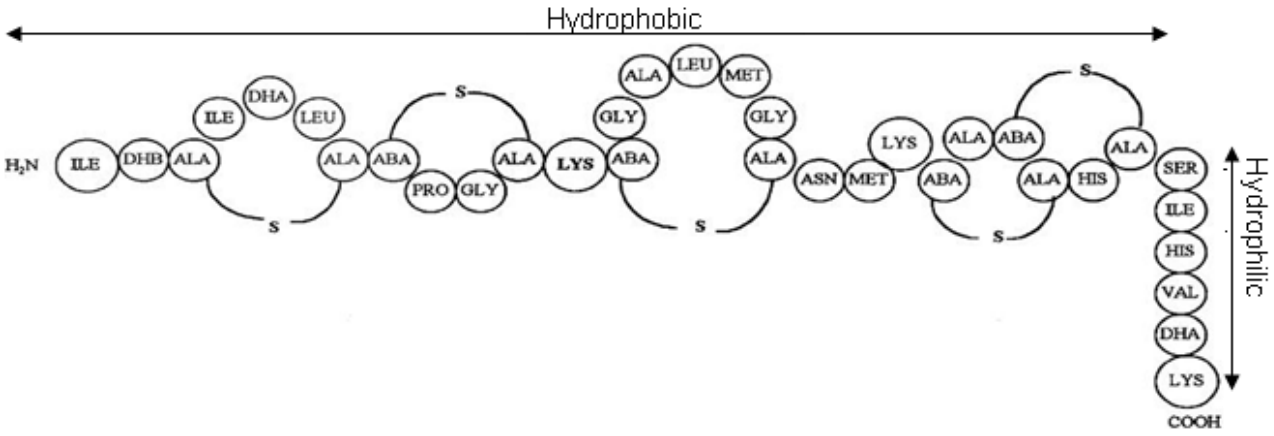

Figure 2: The primary structure of nisin A showing the distribution of amino acids in the hydrophilic and hydrophobic sides of the molecule (adapted from [38]).

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

4.2. Nisin mode of action

The antibacterial activity of bacteriocins is based on interaction with the targeted cell membrane of sensitive mainly Gram-positive bacteria. The prototype lantibiotic nisin is active at nanomolar concentrations through different killing mechanisms that are combined in one molecule. It inhibits cell wall biosynthesis and forms pores in the membrane through specific interactions with the cell wall precursor lipid II. The general steps involved in nisin activities include i) binding to the bacterial membrane, followed by ii) insertion into membrane, iii) pore formation, and iv) interactions with lipid II. We will describe briefly the steps responsible for nisin potential activity.

i) Nisin binding to the bacterial membrane

Binding with the target membrane is the first step in lantibiotic's mode of action. The positively charged C-terminus of nisin binds via electrostatic interactions with the anionic lipids of the bacterial cell membrane [29,32,39,40].

ii) Nisin insertion into membrane

After binding to the membrane, the hydrophobic interactions allow the amphiphilic peptide to insert its hydrophobic N-terminus into the lipid phase of the membrane, while the peptide adopts an overall orientation parallel to the membrane surface [29,39]. The most hydrophobic N-terminus of the peptide mainly affects nisin insertion and the whole peptide antimicrobial activity [40]. The presence and the increase of concentration of anionic lipids are essential for respectively efficient and deeper insertion of nisin in the lipid phase of the membrane [39,40].

iii) Pore formation by nisin

The inserted nisin subsequently obtains a trans-membrane orientation without losing contact with the membrane surface and thereby distorts the lipid bilayer to form a short-lived pore. The pore formation results in the rapid efflux of cellular materials (e.g. ions, amino acids, ATP), leading to the cell death [41]. The formed pores are of transient nature and the nisin-induced leakage is paralleled by translocation of the whole nisin molecule to the inside of the membrane [30,39]. It is also assumed that insertion is followed by aggregation and association of several molecules with the membrane to form a pore since lantibiotics are small peptides [40].

iv) Nisin interactions with lipid II

In addition to pore formation mechanism, a factor present in the target membrane dramatically increases the nisin activity and explains its effectiveness at nanomolar

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

concentrations. It was found that nisin uses lipid II, a peptidoglycan precursor, as a docking molecule for pore formation and binds with it as well for inhibiting cell wall biosynthesis [39,40]. Several experiments suggested that the N-terminus and the ring structures of nisin are important for its specific interaction with Lipid II [39].
Moreover, lipid II is the high affinity target for nisin. The dissimilar sensitivities of different indicator strains to nisin and the different minimum inhibitory concentration (MIC) values may be due to the presence of different lipid II contents among various microorganisms [29,32,39].

Lantibiotics are also effective against spores; using another distinct activity mechanism with a different structure-function relationship. The target of nisin for the inhibition of spore outgrowth is provided by reactive thiol groups on the spores [32]. Nisin activity against spores is attributed to the dehydroalanine residue in position 5 of the peptide and to binding with sulfhydryl groups on the exterior of the spores [31,32,40].
It is the combination of these properties that make nisin such a unique effective molecule.

4.3. Nisin antimicrobial activity

Nisin has shown to be effective against a wide range of Gram-positive bacteria, including many important foodborne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridium botulinum* [8,30,42]. In addition, it inhibits the outgrowth of spores from several *Bacillus* and *Clostridium* species [31]. However, it shows little or no activity against Gram-negative bacteria [43]. The described nisin mode of action showed that the cytoplasmic membrane is the target for nisin in vegetative cells and can thus explain such difference in activity. Unlike Gram-positive bacteria, Gram-negative species are covered and protected by the presence of an outer membrane carpeted by the lipopolysaccharide layer that acts as a barrier to the action of nisin on the cytoplasmatic wall [31]. Moreover, Gram-positive bacteria have relative higher concentrations of anionic lipid in their cytoplasmic membrane, facilitating nisin insertion, as compared to Gram-negative species [39].
Consequently, there is considerable interest in using nisin in combination with other antimicrobials and treatments to expand its spectrum of activity. For example, Gram-negative bacteria can be sensitized to nisin by exposure to chelating agents, sub-lethal heat, and to freezing [43]. When the outer membrane is impaired by agents such as the food-

© 2013 Tous droits réservés.
http://doc.univ-lille1.fr

grade chelator EDTA, it is disrupted rendering Gram-negatives sensitive to bacteriocins [30].

4.4. Resistance mechanisms

Today, bacteriocins are largely considered as a potential answer to the growing problem of resistance to conventional antibiotics [32,44]. However, when a new preservative is found to be safe and effective, it is critical to ensure the longevity of its use by preventing the proliferation of resistant cells. It is therefore important to understand the mechanism of resistance so that it can be avoided. Antibiotic resistance is usually associated with a genetic determinant, facilitating the transfer of resistance between cells, strains, and species. In contrast, bacteriocin resistance results mostly from a physiological adaptation and a change in the target cell membrane composition [45,46]. Moreover, several bacterial species can produce an enzyme, nisinase, which degrades nisin [47]. Consequently, the abuse of bacteriocins in food may induce cells adaptation and resistance and their usage should be optimized rather than maximized.

4.5. Nisin applications

4.5.1. Food applications

Nisin has found applications as a preservative and shelf-life extender in a broad range of food products which include processed and cottage cheese, milk products, dairy desserts, liquid egg, canned vegetables, salad dressings, high moisture hot baked flour products, fish and meat products, confectionary, beer and wine manufacture [41,43,44].

However various factors in food can affect or partially affect the action of nisin. For example nisin interactions with the food matrix and high fat content in foods can reduce its activity [2,43]. One of the advanced ways for using the bacteriocin is setting up nisin-activated antimicrobial packaging. Moreover, such systems can reduce the negative interactions or dilution of antimicrobial compounds induced by directly dispersing or mixing them with food. They will allow thus the peptide to be effective and maintained at higher concentrations on the food surface – where the microbial growth is mostly found – rather than lost in the food matrix [2,7].

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Few examples of such successful nisin applications were presented according to the method used for preparing the antimicrobial food packaging:

- Nisin adsorption on surface [26,27].
- Nisin-containing edible films [48,49].
- Direct nisin incorporation into plastic or multilayer films [50,51].
- Nisin covalent immobilization on surface [52].
- Nisin coating on the surface of the packaging materials [53-55].

Nisin was also used as part of a multi-preservation system known to food microbiologists as hurdle technology [56]. Other factors such as low temperature, pH, additives, and preservation techniques will be combined to ensure efficient destruction or inhibition of bacteria in foods. Antimicrobial packaging can be considered then as a "final-hurdle" in a food system where other hurdles already exist [30,57]. For example, nisin-activated films combined with modified atmosphere packaging and refrigeration temperatures permitted to reduce the population of lactic acid bacteria and to extend the shelf-life of sliced cheese and ham [58]. Siragusa and co-workers [50] showed that nisin impregnated packaging held at 4°C allowed to reduce the population of the psychrotrophic bacterium *Brochothrix thermosphacta* and to control the spoilage of beef carcass. The number of spoilage populations on beef cuts was also reduced by coupling storage at 1°C with antimicrobial packaging activated by nisin, HCl, and EDTA [59].

4.5.2 Other applications

Nisin has also found applications in agricultural, personal care products, clinical and veterinary therapies [31,32,60]. Some remarkable uses of nisin include: treatment of atopic dermatitis, oral decay, stomach ulcers, colon and enterococcal infections, control of respiratory tract infections caused by *Staphylococcus aureus*, inhibition of experimental vascular graft infection caused by methicillin-resistant *Staphylococcus epidermidis*, and more interestingly, nisin inhibits sperm motility, showing its potential as a contraceptive agent. It has also been used in health care products such as toothpaste and skin care products. In veterinary therapy, nisin is currently used as sanitizer against mastitis pathogens (*Staphylococcus* and *Streptococcus* species).

The success of adsorbed nisin on food contact surfaces prompted further studies to extend applications on medical devices [4]. Bower and co-workers [5] used nisin for setting up antimicrobial implantable medical devices. Nisin was adsorbed on polyvinyl chloride

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

(PVC) suction catheter tubing and was exposed to three species of Gram-positive bacteria. Nisin-treated PVC tubing demonstrated an ability to inhibit bacterial growth, while the untreated tubes allowed attachment and growth of the pathogens. They confirmed as well the ability of nisin to retain its activity in vivo and studied its activity when applied on implants placed in sheep and ponies. Nisin was also adsorbed on silica microspheres treated with trichlorovinylsilane to introduce hydrophobic vinyl groups, followed by self-assembly of the polyethylene oxide–polypropylene oxide–polyethylene oxide (PEO–PPO–PEO) triblock. The triblock-coated silica permitted the enhancement of nisin resistance to elution by fibrinogen blood proteins [6].

Although the main nisin applications are in food as natural agent preservative, research has verified its effectiveness for therapeutic purposes too. The peptide also showed its potential use for setting up antimicrobial surfaces for both the food and medical sectors.

4.6. Study of nisin adsorption on surfaces

Nisin has shown its ability to set up antimicrobial surfaces by adsorbing on surfaces and retaining its activity. However, its adsorption on hydrophilic and hydrophobic surfaces is still a matter of debate. Proteins and peptides adsorption on surfaces remains a complicated phenomenon governed by the interactions between the peptides and surfaces. Understanding the factors controlling such interactions is essential for setting up effective antimicrobial surfaces.

Hydrophilicity/hydrophobicity may be the initial parameter affecting peptides/protein adsorption. Previous studies showed that the highest activity was observed on the most hydrophobic nisin-activated films [26]. However, these results were in disagreement with reports showing that antimicrobial activity of adsorbed nisin depended upon surface hydrophobicity, with surfaces of low hydrophobicity retaining more nisin activity than the more hydrophobic surfaces [25,61-64]. Kim and co-workers [65] also found an increase in the antibacterial activity against *Listeria monocytogenes* when nisin was adsorbed onto a hydrophilic surface. Bower and co-workers [62] reported that the low-hydrophobicity surfaces generally displayed more nisin activity than higher-hydrophobicity surfaces, despite the finding that peptides adsorbed in greater amounts on the more hydrophobic surfaces. Similar studies on the adsorption of proteins on surfaces showed that the amount of adsorbed albumin and fibrinogen increases as the surface

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

becomes more hydrophobic, with the adsorbed protein undergoing greater conformational changes on hydrophobic as compared to hydrophilic surfaces [66,67]. This was inconsistent with other findings showing that the amount of adsorbed nisin was higher on the hydrophilic surface than on the hydrophobic one [9,61,68].

Other factors can also influence peptides/proteins adsorption on surfaces as the electrostatic attraction and repulsion between the charged surfaces and charged proteins. In the latter case, the pH of the surrounding medium can define the charge of the peptide (pH below or above the isoelectric point) and the charge of the surface (pH below or above the pKa of functional surfaces). In general, a negatively charged surface prevents adsorption of proteins with the same sign of charge, but accelerates adsorption of proteins with the opposite sign [25]. However, when the interaction forces were measured between an acrylic acid-grafted surface and probe tips with fixed albumin or lysozyme using an atomic force microscope, a significant adhesion force was observed not only with positively charged lysozyme, but also with negatively charged albumin at physiological pH [69].

The changes in surface topography and roughness could play a role too. The surface chemistry and topography can affect proteins adsorption on surfaces [70]. The change in surface roughness can enhance the anchoring effect and the adhesion properties of a surface [71].

Therefore studying these factors and modulating the material's surface properties such as chemical composition, hydrophilicity/hydrophobicity, surface charge and roughness, etc. are needed to preserve the antimicrobial activity of peptides and increase the activity of functionalized surfaces.

5. Surface modification by plasma treatments

Plasma treatments offer a wide range of processes that permit a tailor-made modification of surfaces for intended applications. Plasma is a partially or fully ionized gas with a net neutral charge and is often referred to as the fourth state of matter [72]. It can be divided into two main categories: Thermal plasmas (near-equilibrium plasmas) and cold plasmas (non-equilibrium plasmas). Thermal plasmas are composed of very high temperatures electrons and heavy particles, both charged and neutral, and they are close to maximal degrees of ionization (100%). Cold plasmas are characterized by low temperature heavy particles (atomic, molecular, ionic, neutral and radical species), relatively high temperature electrons, and they are associated with low degrees of ionization (10^{-4}–10%). The system

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

does not thus reach the equilibrium because of the difference in temperature between the particles [24]. Thermal plasmas will not be detailed in this review because they are not suitable for polymers' applications or for the processing and surface modification of organic materials. Cold plasmas are typically carried out at low pressures and they are generated and sustained by the transfer of energy to a gas environment. This energy can be in several forms including: thermal, electric or magnetic fields and direct current, radio or microwave frequencies [73]. Radio-frequency (RF) at 13.56 MHz is one of the most widely used sources [24]. The chemical reactions in cold plasma are initiated by the "hot" highly energetic electrons. The generated ionized and excited species induce plasma-surface reactions and change the surface properties of all polymer materials [12,74]. Concerning the plasma-surface interactions, further sub-categories exist and there are different views on how they can be classified. In this paper we will group them in the three most relevant processes for food packaging and surface analysis applications. They include: plasma functionalization of polymer surfaces, plasma-induced grafting, and plasma polymerization. For convenience, when "plasma" is used alone in this paper, it refers to "cold plasma", since thermal plasma is out of the scope of this work.

5.1. Plasma functionalization of polymer surfaces

When a polymeric material is exposed to plasma, many functional groups can be created on the surface. The active plasma species break the covalent bonds at the surface, leading to hydrogen abstraction and formation of surface radicals. The latter can then react with the gas-phase species to form different chemically active functional groups (mostly polar) at the surface [72] (Fig. 3). The type of functionalization imparted can be varied by selection of plasma gas and operating parameters.

Different reactive and inert gases are often used alone or in combination as air, argon, hydrogen, helium, oxygen, nitrogen, ammonia, carbon dioxide [21]. Oxygen and oxygen-containing plasmas are most commonly employed to produce a variety of oxygen functional groups, including C-O, C=O, O-C=O, C-O-O, and CO_3 at the polymer surfaces [10,71]. Oxygen and carbon dioxide plasmas can introduce carboxyl groups on surfaces [22]. Air and water plasmas oxidize surfaces, while water plasma can additionally incorporate hydroxyl functionality onto a material surface [10]. Ammonia and nitrogen plasmas are used to impart amine and nitrogen-containing functional groups to the surfaces [75,76]. Inert gases can be used to introduce radical sites on the polymer surface for

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

subsequent grafting or to initiate surface graft polymerization of vaporized monomers [22,77].

The surface modifications can also be controlled by plasma parameters such as the system design (reactor geometry, type of excitation, location of the substrates, etc.) and the selected experimental conditions (applied power, time, sample temperature, gas pressure and flow rate) [10,12].

The main advantages of such plasma surface treatments are [10,24,78]:

- Induced modification is confined to the surface layer without modifying the bulk properties of the polymer. Typically, the depth of modification is several hundred angstroms.
- Excited species in a gas plasma can modify the surfaces of all polymers, regardless of their structures and chemical reactivity.
- The plasma is a dry process and the problems encountered in wet chemical techniques, such as residual solvent on the surface and swelling of the substrate, are eliminated.
- Modification is uniform over the whole surface.
- Heat-sensitive polymeric materials can be successfully treated.
- Three-dimensional objects, such as food packages, can be treated without any difficulty.
- They are environmentally friendly.

However, one major drawback of plasma treatment is that the changes induced by the surface functionalization are time-dependent. This process called “aging” or “hydrophobic recovery” is attributed to migration of polar function groups into the polymer bulk and to structural rearrangement that buries chemical groups introduced at the surface [22,71,76]. Therefore, attention should be paid to carry out subsequent surface treatments and analysis directly after functionalization in order to reproduce accurate results. Moreover, aging of plasma-treated surfaces can be avoided or minimized by cross-linking the modified surface, by storing the activated surface at low temperature or in a polar solvent and by grafting or adsorbing other polymers and agents on the surfaces immediately after treatment [22,78].

Plasma functionalization can thus be used either directly for end-use applications or as a pretreatment for subsequent surface modification techniques and attachment of bioactive agents.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

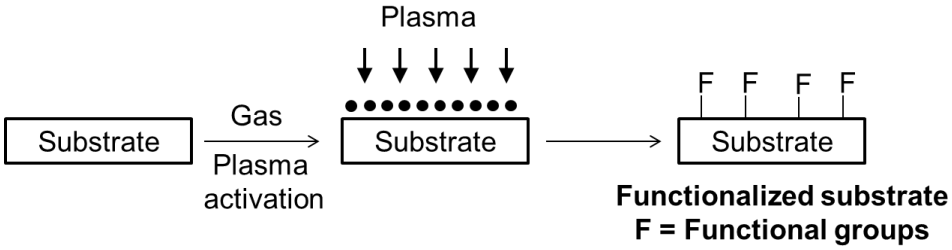

Figure 3: Schematic illustrating the concept of the plasma functionalization process.

5.2. Plasma-induced grafting

Polymer surfaces can also be modified by "plasma-induced grafting", which is a combination of plasma functionalization and conventional chemistry. It is based on grafting functional monomers (usually hydrophilic) onto surfaces and can be carried out in three main steps as described below and shown in figure 4:

i) Polymers are first exposed to the plasma (typically argon, oxygen or helium) to activate the surface and produce radicals [72].
ii) The substrate is then kept under air atmosphere. Most of the formed radicals are oxidized leading to oxygen and peroxide groups used to initiate the grafting of the monomer [79,80].
iii) Afterwards, the substrate is dipped in the monomer solution under inert atmosphere and the solution is heated [80]. Oxygen in the monomer solution should be avoided because it can inhibit the reactions and heating is needed to enhance peroxide decomposition rate [12,77].

In some cases grafting can also be done in vapor phase. When the plasma treatment on polymers is completed, the gas flow is cutoff and monomer vapor is introduced into the chamber [81].

Since the plasma only produces radicals close to the surface of the polymers, plasma-grafting is restricted to the upper surface layers [12].

Surface grafting provides versatile techniques for introducing functional groups such as amine, imine, hydroxyl, carboxylic acid, and epoxide onto a broad range of conventional polymeric substrates, most of which have a non-polar, less reactive surface. It is one method for decreasing the time-dependent effects of plasma treatment. The grafted monomers that are chemically bound to the surface are expected to prevent the

© 2013 Tous droits réservés.
http://doc.univ-lille1.fr

hydrophobic recovery, reduce surface rearrangement, and increase treatment stability [22,82]. Most importantly, the functional groups introduced can be used for further reactions with small or large molecules through covalent or non-covalent linkage. It is, therefore, of interest to understand how plasma and grafting operating parameters affect the type and quantity of the desired functional group [77,78]. Acrylic acid (AA) has been most widely used in the plasma-induced grafting method for introducing carboxylic acid functions [22,77,81]. AA has a strong affinity for proteins leading to the formation of an inter-polymer complex. The interaction is robust, because of multivalent hydrogen bonding of carboxylic acids with peptide bonds and other proton-accepting and -donating side groups possessed by amino acids [81].

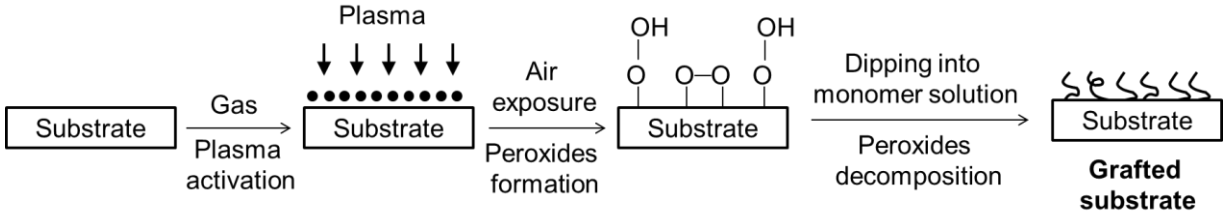

Figure 4: Schematic illustrating the concept of the plasma-induced grafting process.

5.3. Plasma polymerization/Plasma-enhanced chemical vapor deposition (PE-CVD)

Plasma polymerization is essentially a plasma-enhanced chemical vapor deposition process. It refers to the deposition of thin polymer films by vapor phase deposition through reactions of the plasma with an organic monomer gas (Fig. 5). The transformation of monomers into polymers encompasses plasma activation of monomers to radicals, recombination of the formed radicals and subsequent deposition and polymerization of the excited species on the surface of a substrate. Plasma polymers do not comprise repeating monomer units, but instead, complicated units containing cross-linked, fragmented, and rearranged units from the monomers. Consequently the materials formed by plasma polymerization possess unique chemical and physical properties different from that of conventional polymers [12]. The films can be formed on pretreated surfaces (plasma-activated) or on practically any substrate with good adhesion between the film and the

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

substrate. The process gas can be either a pure monomer gas or a small amount of monomer mixed with a carrier gas (e.g. argon). Low surface energy polymer films can be prepared from fluorine-containing monomers, such as C_2F_4 and C_3F_6 and from silicon-containing compounds, such as SiC_4, Si_2C_4O, and Si_2C_8O [10,12]. High surface energy polymer films can be formed from oxygen-containing monomers, such as acrylic acid, acetone, methanol, formic acid, and allyl alcohol [10].

The advantages of plasma polymers include:

- They are chemically inert.
- The films are pinhole-free.
- Films can be formed on a variety of substrates including polymers, metal, glass, and ceramics.
- Films with uniform thicknesses varying from microns down to nanometers and multilayer films can be formed.
- They have very good mechanical and barrier properties against various gases and water vapor.
- The variety of organic substances that can serve as monomers makes plasma polymerization an extremely versatile tool for the deposition of polymeric thin films.
- Aging is not a big problem in plasma polymerized films because of their highly cross-linked nature that decreases the mobility of polymer chains.

However, different process parameters are needed to adjust the deposition rate and the properties of the obtained thin film. Its structure is highly complex and depends on many factors, including reactor design, position of the substrate in the plasma, flux and energy of the ion bombardment, power level, substrate temperature, frequency, monomer structure, monomer pressure, and monomer flow rate [10-12].

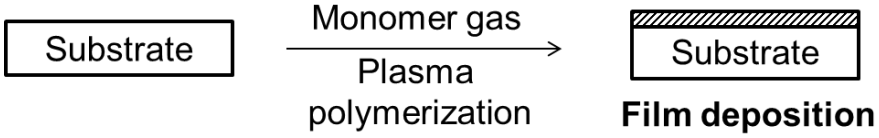

Figure 5: Schematic illustrating the concept of the plasma polymerization process.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

5.4. General plasma applications in food packaging

Polymer materials are inexpensive, easy to process, and exhibit excellent bulk and mechanical properties. However, their chemical inertness and their low surface energy represent generally a great barrier for their food packaging applications [21]. Plasma treatment has been used to expand the applications and transform these inexpensive materials into highly valuable finished products by:

- increasing polar groups, wettability and surface energy,
- improving dyeability, sealability, printability and adhesion to other polymers or metals,
- enhancing peel strength,
- improving gas and water barrier properties,
- reducing swelling tendency,
- increasing mechanical resistance and shear strength,
- reducing migration of plasticizers, and
- increasing stability [7,10,21,72,73].

These particular advantages and properties of plasma-treated films are highly desirable in food packaging applications to minimize leakage, reduce the risk of microbial contamination, prolong the shelf-life of foods, and improve package integrity.

Plasma treatments can also be used to clean and disinfect food contact surfaces. They can kill bacteria and viruses and are inexpensive, fast, and relatively safe [12]. Compared to conventional sterilizing methods using autoclave, chemical solutions, and gases (e.g. ethylene oxide, hydrogen peroxide), plasma treatments are effective near room temperature and in a shorter time (several minutes) without damaging or leaving toxic residues on the materials [72,83]. They are subsequently recognized as one of the most promising alternatives, particularly for heat sensitive materials which need to be kept sterile after processing [7]. However, the effectiveness of plasma to inactivate microorganisms on inert surfaces will depend greatly on the equipment design and operating conditions like gas type, flow rate, and pressure [73].

5.5. Plasma applications in protein adsorption studies and in setting up antimicrobial surfaces

Plasma has found applications in the study of protein adsorption on surfaces and in setting up antimicrobial surfaces. However, such applications are few in the food sector as

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

compared to the biomedical sectors. Therefore, examples will be presented in both sectors because of the similarities between the two disciplines. The plasma principle and process are the same for both applications and the interactions between the modified surfaces and the attached bioactive agents are also similar because the most used agents include proteins, peptides and enzymes. The only difference is the properties of bioactive compounds that change the targeted applications.

In adsorption studies, plasma treatments were used to evaluate the effect of surface characteristics on adsorption behavior. Sterrett and co-workers [84] studied the protein (albumin) adsorption on polyurethane elastomers modified by O_2, CH_4, CF_4, and C_2F_6 plasmas. Plasma treatments using CH_4 and/or C_xF_y increased the contact angle for both substrates while those with O_2 and O_2/CF_4 decreased the contact angle for the substrates considered. For substrates exhibiting smaller contact angles (hydrophilic surfaces), protein adsorption occurred to a greater extent. However, Kiaei and co-workers [85] showed that C_2F_4 plasma-treated surfaces with low energy surfaces retained a larger fraction of adsorbed albumin than the higher energy surfaces. The low energy plasma-treated surfaces should have high interfacial energies in water, with correspondingly high driving forces for protein adsorption through hydrophobic interactions [85].

For setting up antimicrobial surfaces, plasma treatments were used to functionalize polyester and polyethylene terephthalate woven fabrics prior to peptide-activation [28;86]. They were also used to generate the functional groups needed to covalently immobilize the bioactive agents on surfaces [87-89].

Such technologies present therefore a great potential for the attachment of antimicrobials or bioactive compounds on surfaces. If applied to food packaging and processing surfaces, they could allow for increased shelf-life and/or safety of food products. The purpose would then be to select and optimize the proper plasma process for the desired type of surface modification.

6. Surface characterization methods

The type of analytical tools used in characterizing surface modified polymers depends on the anticipated nature of the modification, the specificity required, and the resources available. The study of surface interactions between peptides, materials and bacteria for setting up antimicrobial surfaces requires using techniques capable of investigating the quantity of adsorbed peptides on the surfaces, the antimicrobial activity of the peptides

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

before and after the adsorption on surfaces, the surface modification at each step of the process, and most importantly, the interactions between the peptides and the materials. The most commonly used characterization methods for such studies are discussed briefly.

6.1. Peptide assays

Several methods exist for the determination of protein/peptides concentration in solution, including Biuret reaction, Lowry method, Bradford assay, and Bicinchoninic acid (BCA) assay. They are all based on the change of color upon complexing with protein but the BCA assay is more sensitive and applicable than either Biuret or Lowry procedures [90,91]. It also has less variability than the Bradford assay. The BCA assay has many advantages over other protein determination techniques because it is easy to use, the color complex is stable, there is less susceptibility to detergents and it is applicable over a broad range of protein concentrations. In this method, Cu^{2+} ions are reduced to Cu^{1+} ions by the peptide [90]. The amount of reduction is proportional to protein concentration. In alkaline environments, Cu^{1+} ion combines with BCA to form a purple-blue complex with strong absorbance at 562 nm. In addition to protein determination in solution, the BCA assay is suited for determining surface bound or adsorbed protein [22]. However, some interfering substances like buffer additives, chelating agents, and solvents can affect the accuracy of the method and they should be taken into consideration in the development of appropriate standard curves. The assay has been frequently used to determine the amount of nisin adsorbed or released from surfaces such as the food packaging poly(butylene adipate-co-terephthalate) film [92], bone cement [93], active polyethylene/polyamide/polyethylene film [94], agarose gel [95,96], and antimicrobial multilayer films (ethylcellulose/hydroxypropylmethylcellulose/ethylcellulose) [51].

6.2. Antimicrobial activity

In order to verify the efficacy of antimicrobial treated films, the antimicrobial activity should be assessed for the antimicrobial agent alone, for the films before treatment, after each surface treatment, and after the functionalization with the bioactive compounds. Such controls are needed to ensure that the peptides will preserve their activity after adsorption and that the activity of antimicrobial films is due to the presence of the bacteriocin and not to a change in the polymer surface chemistry during the modification process.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

The minimum inhibitory concentration (MIC) method is commonly used to check the activity of the antimicrobials alone such as peptides [97-99]. MIC can be determined by broth or agar dilution methods. The broth dilution method consists of seeding a microplate or a series of tubes containing growth medium with the target microorganism and with different concentrations of the antimicrobial. The microplates or tubes are incubated at a specific temperature and for a predetermined period of time. The optical density of the microplates is then measured with a microplate reader and the turbidity of the tubes is visually inspected for microbial growth. MIC is the lowest concentration of peptide resulting in the complete inhibition of growth of a test microorganism [98]. In the agar dilution method, the principle is the same but the tubes or microplates are replaced by agar plates and the MIC is determined as the lowest concentration of the agent resulting in a clear zone of inhibition [100].

To assess the antimicrobial activity of the films, agar diffusion assays and culturability loss tests have been the most widely used [3,23,58,63,101]. In the agar diffusion assay, the test film is placed on a solid agar medium inoculated with the test microorganism. The plate is then incubated and the inhibition of growth below or around the sample is used to determine the antimicrobial activity of the sample. The method is simple and effective but it gives only a qualitative assessment of the antimicrobial activity. Though, it can be considered quantitative if the diameter of the clear zones around the films is measured [3]. The size of the inhibition zone is proportional to the concentration of diffused agent from the film but it is affected by many factors, such as nisin-sensitive strains, amount of added agar and surfactant, growth rate of the indicator organism, diffusion of the antimicrobial agents, and pre-diffusion step [102]. The agar plate test method simulates the wrapping of foods and may suggest what can happen when films come into contact with contaminated surfaces and the antimicrobial agent migrates or diffuse from the film to the food. Therefore, this method is suitable for peptides capable of desorbing from surfaces but is not applicable for testing the activity of immobilized agents [23].

In the culturability loss tests, liquid media (buffer, growth media or foods) are inoculated with the target microorganisms and the antimicrobial film. The flasks are incubated with mild shaking and samples are taken over time and enumerated to measure the reduction of culturable cells [3,101]. However, the ratio of film surface area to volume (of product or media) must be considered in this test. From an antimicrobial standpoint, high

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

surface/volume ratios should increase the activity of antimicrobial films. But in real packaging applications, it may be impractical because the packaging surface area is predefined upon the products' size and volume requirements. Moreover, tests in buffer may be misleading since sensitive cells in nutrient-poor media may recover if nutrients are present as in growth media or foods.

Numerous studies concerning the antimicrobial activity of packaging materials have been reported and different methods of antimicrobial activity determination have been used. There is, however, no agreement upon standard methods to determine the effectiveness of antimicrobial food packaging. It is therefore difficult to compare the results of these studies because of substantial variations in the bioactive compounds, in the packaging matrix, in the test microorganisms, and in the test methods [3,23].

6.3. X-ray photoelectron spectroscopy (XPS)

XPS or electron spectroscopy for chemical analysis (ESCA) determines the atomic composition of the top few nanometers of the surface. It provides data in the *ca.* 1–10 nm surface layer [103]. The sample is bombarded by monochromatic X-ray photons and the binding energy of emitted photoelectrons is calculated. The resulting spectrum is a plot of intensity (arbitrary units) versus binding energy (eV). The binding energy can identify the element and its oxidation state. The intensity of the ejected photoelectrons relates directly to the material surface atomic distribution and can therefore be used to quantify the percent atomic concentration and the stoichiometric ratios [22,104]. In addition to determining and quantifying the surface atomic composition, this technique can be used to identify the presence of specific functional groups. The overall shape of XPS peak depends on the chemical environment of the element and is determined by the distribution and proportion of each existing functional group [104]. Those functional groups can also be used for the peak decomposition of mixed surfaces using curve-fitting models. For example, if a surface is composed of two polymers, such models allow the evaluation of the proportion of each polymer at the surface [105,106].

XPS has been extensively used to characterize and quantify the surface chemical composition of polymers and classical materials. Recent applications include biomaterials, biological, and bio-organic systems (peptides, proteins, microorganisms, polysaccharides, food) [61,52,107-110]. Therefore, XPS provides a promising tool in interactions studies for confirming and quantifying the surface chemical modification and the peptides adsorption.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

6.4. Time-of-flight secondary ion mass spectrometry (ToF-SIMS)

ToF-SIMS is a powerful technique for analyzing the chemical structure composition of the uppermost molecular or atomic layer of a solid surface. It analyzes the fragments emitted from the first 1–5 top monolayers [111]. It has unique features combining surface sensitivity, chemical specificity, and high spatial resolution [79,112,113].

In secondary ion mass spectrometry (SIMS), the sample surface is sputtered by an ion beam and the emitted secondary ions are analyzed by a mass spectrometer. Depending on the sputtering rate, we can differentiate between static and dynamic modes. In dynamic SIMS, a high-energy primary ion beam (> 10^{13} ions/cm^2) is applied for a relatively short time on the sample. This erodes away the material continuously from the surface to the bulk and allows a depth profiling of its chemical composition [79,104]. In static SIMS, the primary ion dose must not exceed 10^{13} ions/cm^2 to maintain sensitivity to the uppermost monolayers, to minimize sample damage and to promote the emission of large organic fragments [113]. ToF-SIMS is the main experimental variant of static SIMS that emerged as a technique of potential importance in surface science. For ToF-SIMS, a pulsed primary ion beam is used. The time of flight of the emitted secondary ions allows their separation in a mass spectrometer according to their mass to charge ratio. The resulting spectrum depicts signal intensity versus mass to charge ratio (m/z) and can be used to measure relative intensities of the chemical species.

ToF-SIMS can be used for surface chemical identification, imaging mode, and semi-quantitative analysis.

i) Surface chemical identification

The mass to charge ratio (m/z) of the species acquired in a ToF-SIMS experiment, yield positive and negative secondary ion mass spectra. By evaluating the masses of the signals, peaks can be often identified from the molecular ion of the analyte, fragments of the molecular ion, polymer repeat units, end groups and ions of any other components that may be in the samples [112,113]. Such characteristic peaks are used for the identification of the molecular structure and chemical composition of the surface.

ii) Imaging mode

Static ToF-SIMS can be done in imaging mode. The chemical composition of a sample can be mapped and a full mass spectrum is collected at each pixel in the image. After data acquisition, a specific secondary ion or a combination of ions can be selected and their

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

surface distribution mapped. In addition, a region of interest from the total ion image can be identified and the mass spectra from the pixels in that region can be summed, allowing spectral evaluation with restored sensitivity and dynamic range [113]. Such chemical maps can be also used for assessing surface homogeneity [22].

iii) Semi-quantitative analysis

ToF-SIMS is not a direct quantitative method due to the influence of the matrix effects on ion yields and the preferential ionization of one species over another. The intensity of a given fragment depends on its surrounding environment and is not necessarily directly proportional to its concentration on the surface. However, for most organic surfaces such as polymeric biomaterials and adsorbed protein films, these matrix effects are minimal [113]. Moreover, such analysis can be done by determining the relative amount of components at a surface of a sample and by rationing representative ions, elemental or molecular species [113,114].

However, ToF-SIMS data handling is complex and presents substantial challenges with interpretation. Imaging mode generates an enormous amount of data and a typical mass spectrum contains a huge number of peaks. Yet it is within this complexity that information about sample composition, molecular orientation, chemical bonding, and sample purity is contained. The challenge is how to extract this information from the spectra and images. In order to address and reduce such complexity, the multivariate analysis can be applied to process ToF-SIMS data. Examples of these methodologies include: principal component analysis, partial least squares, multivariate curve resolution, maximum auto-correlation factors, neural networks, latent profile analysis, mixture models, and discriminant analysis [113,115,116]. Another approach for ToF-SIMS data analysis is multi-method combination. Together static ToF-SIMS and XPS provide a powerful complementary approach to biomaterial surface analysis and represent the two most widely used surface analysis techniques [113]. ToF-SIMS can be used to complement XPS results by offering identification of chemical species and it may also be used to differentiate samples that have similar XPS spectra [22]. ToF-SIMS is more surface sensitive than XPS and characterizes the uppermost layers of the surface at lower sampling depth.

This technique has found many applications in the study of protein and peptide adsorption on surfaces [61,117-121].

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

6.5. Fourier-transform infrared (FTIR) spectroscopy

FTIR spectroscopy uses infrared radiation (IR) to determine the chemical functionalities present in a sample. When an infrared beam hits a sample, chemical bonds stretch, contract, and bend causing it to absorb IR radiation at specific wavenumber. The resulting plot is of absorbance (or transmittance) versus wavenumber. In this way, infrared spectra show absorption peaks that are characteristic of particular molecules and the way they are bonded to the surface. The advantages of this technique are that it does not require ultra-high vacuum conditions, as do XPS and ToF-SIMS, and an analysis can therefore be conducted in less than ten minutes. However, FTIR utility is limited by the micrometer range probed by the method, which is often too deep to detect modification or adsorbed agents at the uppermost layers of the surface. Modified surface layers with thickness from only several to tens of nanometers, cannot be observed by FTIR with sampling depth ranging from several hundred nanometers to more than 1 µm [79,112]. In such cases, other techniques like XPS and TOF-SIMS with much smaller sampling depths are needed. Consequently, this technique may not be relevant for the study of surface interactions and peptide adsorption but it can be used to monitor migration of functional groups to the polymer bulk, to determine the depth of surface modification, and to confirm functionalization of plasma-treated surfaces [10,122].

6.6. Dye assays

In a complementary approach to other techniques (FTIR, XPS, SIMS, etc.), dye assays or colorimetric methods are used to measure the amount of functional groups on a surface. Two frequently used methods for determining the surface density of carboxyl and amino groups on surfaces are respectively, toluidine blue O (TBO) and methyl orange (MO) dye tests. They are based on ion exchange mechanism and on electrostatic interaction between the functional group target and the dye. TBO is a positively charged molecule that can combine with a carboxyl group in alkaline solution to form a stable electrostatic complex. The complexed dye TBO molecules can be detached from the surface by dissolving in acetic acid or other organic solvents [77,79]. Similarly, MO is a negatively charged dye and can combine with positively charged amino groups on the material's surface under acidic conditions [79,123]. The combined MO molecules can be desorbed in potassium carbonate solution or other organic solvents. The amount of TBO and MO can be determined by measuring the optical density and using a standard calibration curve. The

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

amount of functional groups is calculated by assuming a combination ratio of 1:1 between TBO and –COOH and between MO and $–NH_2$. However, these methods remain less sensitive than XPS and SIMS and the pH control is critical to minimize error. TBO has been mostly used to determine the surface density of carboxylic acid functions on acrylic acid grafted and oxygen plasma-treated surfaces [77,78]. MO dye assay was used to measure the amino groups' density on ammonium plasma-treated surfaces [123].

6.7. Contact angle and surface energy measurements

Contact angle measurements are surface sensitive, providing information about the outermost few ångströms (≈ 5 Å) of the sample surface [78,81]. The contact angle measurement can be static or dynamic. The former is a measurement where the liquid is not in motion at the solid/liquid interface. The latter is a measurement where the liquid front is in motion with respect to the solid surface [81]. Most applications and especially in the scope of this review include the static measurement.

Water contact angle determines surface hydrophilicity/hydrophobicity by measuring how much a droplet of water spreads on a surface. The lower the contact angle, the more hydrophilic the surface is. When a surface has more polar groups introduced to it, hydrogen bonding with the water becomes easier and the droplet spreads along the hydrophilic surface, resulting in a lower contact angle. While contact angle is a simple and rapid method, it is limited by its inability to distinguish between different hydrophilic functional groups and by the measurement errors including difference in operator measurement, inconsistent water pH and hardness, and changes in environmental temperature and humidity.

When the contact angle is measured using two or three test liquids including polar (water) and non- polar (diiodomethane) liquids, the surface energy can be determined. The solid surface energy is the sum of polar and non-polar (dispersive) contributions. It permits to take into account the effect of these two contributions on the surface properties and interaction processes [71,124,125].

Both contact angles and surface energy measurements have been widely used to confirm the introduction of polar groups into surfaces, to determine the hydrophilic/hydrophobic character of plasma-treated surfaces and to show the effect of plasma treatment type and conditions on such character [11,71,78,124-127].

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

6.8. Zeta potential

Zeta potential is the most important technique used to acquire information on surface charge. When a charged solid surface is in contact with a liquid phase, an electrical potential develops at the interface. A double layer is established and divided into:

- the immobile (fixed) layer made of surface bound ionizable groups and tightly bound liquid phase ions of opposite charge, and
- the mobile (diffuse) layer made of loosely bound liquid phase ions of opposite charge.

A shear plane separates the layers from each other and the change in potential across this double layer is known as the electrokinetic or zeta potential [22,128] (Fig. 6). Commercial zeta potential or electrokinetic analyzers are available and bench top units can be set up as well.

The accumulation of surface charges (formation of the double layer) and the magnitude and sign of the zeta potential depend on:

- the type and the amount of dissociable functional groups on the solid surface,
- the pH of the electrolyte solution, and
- the interaction between the solid surface and the electrolyte solution.

Therefore, zeta potential measurements provide information not only about the surface charge but also about its chemical composition and acid-base character. However, they cannot be used to quantify the number or type of functional groups present and must be used in conjunction with other analytical tools to adequately describe changes in polymer surface chemistry. Zeta potential has found applications in confirming the introduction of cationic and anionic groups into plasma-treated or grafted surfaces [75,81,129] and in determining the charge and isoelectric points of surfaces [128,130]. Furthermore, it can be used to predict protein adsorption behaviors. The adsorbed mass can theoretically increase with increasing charge contrast between the surface and protein. Consequently, the surface charge would play an important role when electrostatic interactions are driving the adsorption phenomenon.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

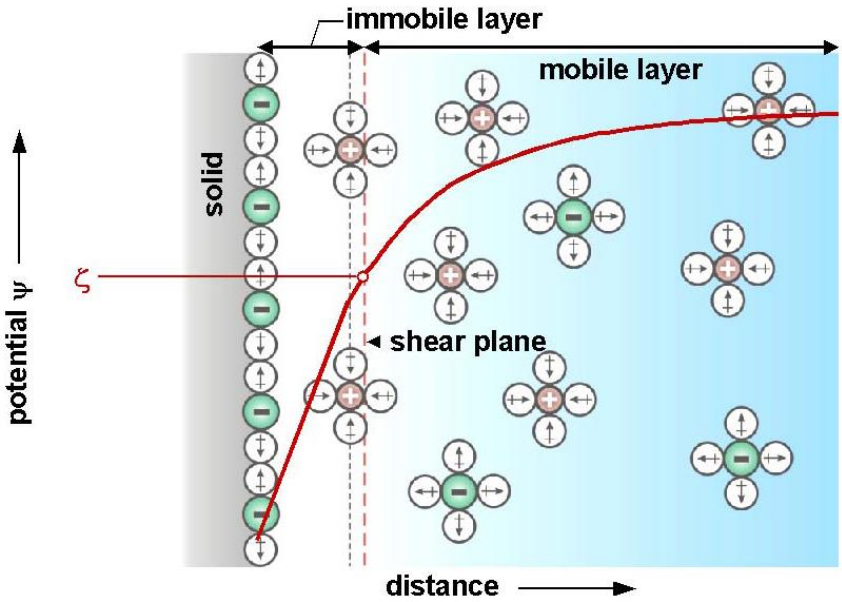

Figure 6: Schematic of zeta potential showing the double layer formation at the solid/liquid interface.

6.9. Atomic force microscopy (AFM)

AFM is used mainly for measuring surface topography and roughness. It is a very high resolution scanning probe method. An ultra-sharp AFM probe (tip) attached to a flexible cantilever, scans over the sample surface with sub-nanometer precision. Features on the sample surface, induce the cantilever deflection in the vertical and lateral directions as the sample moves under the tip. The AFM detects and records interactions between the tip and the surface. A surface topographical map is thereby generated and can be used for surface roughness calculation [22,131,132]. AFM can additionally provide high resolution three-dimensional images of solid surfaces and can work under different conditions such as ambient air, various gases, liquid, vacuum, low and high temperatures [81,112,132]. It has different modes including contact, tapping, and resonant or lateral modes, which can provide a deeper knowledge for different kinds of polymer surfaces [81,131]. The tapping mode (intermittent contact mode) is the most frequently used and is adapted for weakly adsorbed molecules and for soft surfaces because it minimizes the effects of friction and other lateral forces between the tip and the sample. In this mode, the cantilever/tip

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

assembly is sinusoidally vibrated by a piezo mounted above it, and the oscillating tip slightly taps the surface [131,132].

Disadvantages of AFM include limitations on the image maximum scanning area (around 150 x 150 µm). Another inconvenience is that at high resolution, the quality of the image is limited by the radius of curvature of the probe tip, and an incorrect choice of tip for the required resolution can lead to image artifacts [112]. Also, ultra-flat or rigid samples are desirable to obtain high atomic resolution [131].

AFM has been used to determine surface topography and roughness of plasma-treated surfaces [127,133]. It has also been used to study the surface topography of plastic films activated with bacteriocins [54]. In addition to evaluating surface topography, AFM can be used to investigate the interaction between the probe tip and the polymer-adsorbed or -grafted surface in an aqueous solution. Such applications permitted to measure the interaction forces between proteins and graft-polymerized surfaces [69].

In the scope of this review, AFM can be used to study the topography changes of surfaces after surface treatment and after peptide adsorption. Because of its high nanometer resolution in describing surface topography, AFM may help to explain the adsorption mechanisms of different antimicrobials on surfaces. It can also measure the force of interactions between the peptides and the surface.

6.10. Scanning electron microscopy (SEM)

SEM allows also the study of surface topography. When a sample is bombarded with electrons, it emits secondary electrons and X-rays. The intensity of the secondary electrons is detected to generate a high resolution three dimensional surface image. X-rays can be detected to conduct elemental analysis. SEM is not as surface sensitive as other techniques, and non-conducting polymers must be sputter-coated prior to analysis [22]. Nevertheless, it has often been used to measure surface topography of plasma-treated surfaces [71, 124,127,133,134].

6.11. Ellipsometry

Ellipsometry enables to study the kinetics of protein adsorption on surfaces and to characterize the thickness and structure of the adsorbed layer [79,112]. This is an optical technique that measures the changes in polarization state of a reflected light from its

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

incident light. When a monochromatic linear polarized light is reflected by a smooth surface, the polarization state will be changed, and will be further changed if a protein layer exists on the surface. The polarization state of the reflected light is related to many parameters including the protein layer thickness, which can be calculated through fitting the changes of polarization with a mathematical model. However, a great limitation of ellipsometry is that it cannot distinguish between polymers and proteins that have similar refractive indices. Only very smooth surfaces with strong reflection ability and different refractive index from that of proteins such as silicon or silicon dioxide can be used. Thus, the technique is not practical for the analysis of polymer surface materials [112,135] and has been used to study nisin adsorption on silicon surfaces [62,68].

7. Further considerations

Setting up active food packaging by adsorbing peptides on plasma-treated surfaces will need to take into consideration further challenges related to the peptides, plasma surface treatment, appropriate testing methods, applications in food, regulations, and cost.

7.1. Peptides

Peptides should be able to preserve their antimicrobial activity after adsorption on the surface and they should not change the film's performance or mechanical, barrier, and optical properties [3,17]. Their usage should be optimized or combined with other control factors to increase their effectiveness and to avoid bacteria cell resistance. The greatest restriction to the development and application of natural antimicrobial agents is cost.

7.2. Plasma surface treatment

The challenges encountered in plasma treatment can be summarized as follows:

- Most plasma treatments are performed under low-pressure and therefore require vacuum systems. This increases capital and operational costs of the plasma equipment and presents complications for continuous processes and for scaling up from laboratory size to a large scale industrial setting [22].
- The process parameters are highly system-dependent; the optimal parameters developed for one system usually cannot be adopted for another system.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Consequently, it is extremely difficult to repeat and compare results between laboratories and from a large variety of plasma systems [10,22].

- It is very difficult to control precisely the amount of a particular functional group formed on a surface [10]. Due to the multitude of elementary reactions occurring simultaneously in the plasma process, it is complicated to calculate in detail the plasma's physical and chemical behavior and especially its interactions with the surface.

Overcoming some drawbacks of plasma systems will increase their potential for surface treatment applications. In order to avoid vacuum conditions, the use of atmospheric pressure plasmas was considered as an alternative [24]. Atmospheric pressure plasmas can operate in a wide range of temperature and pressure. However, they can be controlled to operate like low-pressure cold plasmas without generating extensive heat in their surroundings [83]. As a result, they can also be suitable for the processing of organic compounds and for surface modification of polymer materials.

A solution for problems related to complex plasma processes requires a good knowledge of reactor hardware and design criteria [136], as well as future progress in simulation techniques and creation of sophisticated mathematical models able to predict real plasma processes.

7.3. Appropriate testing methods

There are many advanced techniques that have potential applications for surface analysis but there are still many unanswered questions in this field. The challenges rely on the choice of the adapted technique for the corresponding problematic, the proper combination of complementary techniques, as well as being able to process the complex data provided by those techniques. In addition, analyzing results from different combined techniques requires a thorough understanding of each method's principle, advantages, and limitations. For example FTIR, XPS, and ToF-SIMS have very different sampling depths and sensitivities that should be considered for interpreting the results correctly.

7.4. Applications in food

Testing the microbial growth in synthetic media should be followed by growth in the targeted foods. The food components can alter the antimicrobial activity of the bioactive

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

agents or inhibit their release from the packaging [2,3,17]. The storage of food under different temperatures and environmental conditions can also affect the activity of the antimicrobial films and proper simulation tests need to be carried out [1,17]. In addition, it is crucial to study the kinetics of release of the bioactive substances in order to understand or to modulate film activity and to investigate which type of food could be protected efficiently using these systems of active films.

7.5. Regulations

Regulations related to active packaging differ from country to country and are detailed in previous reports dedicated to this topic [137,138]. While active packaging is not subject to any special regulatory concern in the United States, the regulation of such packaging material in Europe is still evolving. In the US, antimicrobials in food packaging that may migrate to food are considered food additives and must meet the food additive standards [3,7]. Unlike the US, EU countries regulate substances added to or used in packaging separately from food additives [7]. To be on the European market, such systems should comply with specific regulations concerning the active packaging [2,137,138]. Consequently the regulatory issues should be addressed too because they present complications limiting the commercial availability of antimicrobial packaging.

7.6. Cost

Even though there is no published data on the cost of antimicrobial films, they can be expected to be expensive due to the cost of plasma technological investment and the usage of natural antimicrobial peptides. This may restrict options for commercialization, especially for small and medium sized businesses. However, such costs are negligible compared to the economic loss associated with foodborne illness outbreaks. Moreover, these cost increases are counterbalanced by benefits such as:

- Reductions in waste due to the improved quality and shelf-life of products.
- Lower waste disposal expenses of chemicals generated by wet chemistry processes.
- Environmental considerations associated with such environmentally benign processes.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

- Replacement or simplification of aseptic packaging process and chemical sterilization of the packages. Packaging materials will have self-sterilizing abilities due to their own antimicrobial effectiveness.
- Improved additional functional properties of plasma-treated food packaging films (See section“5.4.”).

Therefore, a comprehensive assessment of specific costs and benefits is an essential next step in establishing the commercial application of innovative packaging systems. Recognition of the benefits of active packaging technologies by the food industry and increased consumer satisfaction will open new frontiers for active packaging technology. As interest in the field grows; so will availability and cost effectiveness of antimicrobial packaging.

8. Conclusions

Antimicrobial surfaces and packaging are gaining interest from researchers and industry due to their potential to provide quality and safety benefits. In this review, we summarized the methodology, advantages, disadvantages, testing methods, applications, and considerations related to antimicrobial food packaging systems in general, and specifically to peptide adsorption on plasma-treated surfaces. Obviously, there is much work to be done. The methods for setting up antimicrobial packaging are still in the developmental stages and the peptide/protein adsorption on surfaces remain a complicated unclear phenomenon that need to be studied further. In order to advance in this field, investigating the complex interactions at the interfaces is a prerequisite for later successful packaging applications. This implies the need for continued research in setting up new functionalized surfaces, better modulating surface treatment processes, and developing surface characterization methods. More importantly, the effective participation and collaboration of research institutions (materials scientists, plasma physicists, chemists, biologists, bioengineers…), industry, and government regulatory agencies are imperative.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

References

1. Suppakul, P., Miltz, J., Sonneveld, K., Bigger, S. w., *J. Food Sci.* 68 (2003) 408.
2. Coma, V., *Meat Sci.* 78 (2008) 90.
3. Appendini, P., Hotchkiss, J.H., *Innovative Food Sci. Emerging Technol.* 3 (2002) 113.
4. Bower, C.K., Bothwell, M.K., McGuire, J., *Colloids Surf., B* 22 (2001) 259.
5. Bower, C.K., Parker, J.E., Higgins, A.Z., Oest, M.E., Wilson, J.T., Valentine, B.A., Bothwell, M.K., McGuire, J., *Colloids Surf., B* 25 (2002) 81.
6. Ryder, M.P., Schilke, K.F., Auxier, J.A., McGuire, J., Neff, J.A., *J. Colloid Interface Sci.* 350 (2010) 194.
7. Lee, K.T., *Meat Sci.* 86 (2010) 138.
8. Jack, R.W., Tagg, J.R., Ray, B., *Microbiol. Rev.* 59 (1995) 171.
9. Daeschel, M.A., McGuire, J., Al-Makhlafi, H., *J. Food Prot.* 55(9) (1992) 731.
10. Chan, C.-M., Ko, T.-M., Hiraoka, H., *Surf. Sci. Rep.* 24 (1996) 1.
11. Ozdemir, M., Yurteri, C.U., Sadikoglu, H., *Food Technol.* 53(4) (1999) 54.
12. Chu, P.K., Chen, J.Y., Wang, L.P., Huang, N., *Mater. Sci. Eng., R* 36 (2002) 143.
13. Devlieghere, F., Vermeiren, L., Debevere, J., *Int. Dairy J.* 14 (2004) 273.
14. Kerry, J.P., O'Grady, M.N., Hogan, S.A., *Meat Sci.* 74 (2006) 113.
15. Lövenklev, M., Artin, I., Hagberg, O., Borch, E., Holst, E., Rådström, P., *Appl. Environ. Microbiol.* 70 (2004) 2928.
16. Cooksey, K., *Addit. Polym.* 2001 (2001) 6.
17. Quintavalla, S., Vicini, L., *Meat Sci.* 62 (2002) 373.
18. Onaizi, S.A., Leong, S.S.J., *Biotechnol. Adv.* 29 (2011) 67.
19. Azeredo, H.M.C. de, *Food Res. Int.* 42 (2009) 1240.
20. Cohen, J.D., Erkenbrecher, C.W., Haynie, S.L., Kelley, M.J., Kobsa, H., Roe, A.N., Scholla, M.H., United States patent US 5,428,078 (1995).
21. Ozdemir, M., Yurteri, C.U., Sadikoglu, H., *Crit. Rev. Food Sci. Nutr.* 39 (1999) 457.
22. Goddard, J.M., Hotchkiss, J.H., *Prog. Polym. Sci.* 32 (2007) 698.
23. Kugel, A., Stafslien, S., Chisholm, B.J., *Prog. Org. Coat.* 72 (2011) 222.
24. Denes, F.S., Manolache, S., *Prog. Polym. Sci.* 29 (2004) 815.
25. Daeschel, M.A., Mcguire, J., *Biotechnol. Genet. Eng. Rev.* 15 (1998) 413.
26. Leung, P.P., Yousef, A.E., Shellhammer, T.H., *J. Food Saf.* 23 (2003) 1.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

27. Guerra, N.P., Macías, C.L., Agrasar, A.T., Castro, L.P., *Lett. Appl. Microbiol.* 40 (2005) 106.
28. Kerkeni, A., Behary, N., Dhulster, P., Chihib, N.-E., Perwuelz, A., *J. Appl. Polym. Sci.* 129 (2013) 866.
29. Cleveland, J., Montville, T.J., Nes, I.F., Chikindas, M.L., *Int. J. Food Microbiol.* 71 (2001) 1.
30. Deegan, L.H., Cotter, P.D., Hill, C., Ross, P., *Int. Dairy J.* 16 (2006) 1058.
31. De Arauz, L.J., Jozala, A.F., Mazzola, P.G., Vessoni Penna, T.C., *Trends Food Sci. Technol.* 20 (2009) 146.
32. Asaduzzaman, S.M., Sonomoto, K., *J. Biosci. Bioeng.* 107 (2009) 475.
33. Tremp, A., *Nat. Rev. Microbiol.* 4 (2006) 322.
34. Kuwano, K., Tanaka, N., Shimizu, T., Nagatoshi, K., Nou, S., Sonomoto, K., *Int. J. Antimicrob. Agents* 26 (2005) 396.
35. Suda, S., Hill, C., Cotter, P.D., Ross, R.P., *Bioeng. Bugs* 1 (2010) 345.
36. Van De Ven, F.J.M., Van Den Hooven, H.W., Konings, R.N.H., Hilbers, C.W., *Eur. J. Biochem.* 202 (1991) 1181.
37. Piper, C., Hill, C., Cotter, P.D., Ross, R.P., *Microb. Biotechnol.* 4 (2011) 375.
38. Gross, E., Morell, J.L., *J. Am. Chem. Soc.* 93 (1971) 4634.
39. Breukink, E., de Kruijff, B., *Biochim. Biophys. Acta, Biomembr.* 1462 (1999) 223.
40. Bauer, R., Dicks, L.M.T., *Int. J. Food Microbiol.* 101 (2005) 201.
41. Chihib, N.-E., Monnerat, L., Membré, J.M., Tholozan, J.-L., *J. Appl. Microbiol.* 87 (1999) 438.
42. Pol, I.E., Smid, E.J., *Lett. Appl. Microbiol.* 29 (1999) 166.
43. Delves-Broughton, J., Blackburn, P., Evans, R.J., Hugenholtz, J., *Antonie van Leeuwenhoek* 69 (1996) 193.
44. Papagianni, M., *Biotechnol. Adv.* 21 (2003) 465.
45. Crandall, A.D., Montville, T.J., *Appl. Environ. Microbiol.* 64 (1998) 231.
46. Chihib, N.-E., Crepin, T., Delattre, G., Tholozan, J.-L., *FEMS Microbiol. Lett.* 177 (1999) 167.
47. Jarvis, B., *J. Gen. Microbiol.* 47 (1967) 33.
48. Nguyen, V.T., Gidley, M.J., Dykes, G.A., *Food Microbiol.* 25 (2008) 471.
49. Sanjurjo, K., Flores, S., Gerschenson, L., Jagus, R., *Food Res. Int.* 39 (2006) 749.
50. Siragusa, G.R., Cutter, C.N., Willett, J.L., *Food Microbiol.* 16 (1999) 229.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

51. Guiga, W., Swesi, Y., Galland, S., Peyrol, E., Degraeve, P., Sebti, I., *Innovative Food Sci. Emerging Technol.* 11 (2010) 352.
52. Héquet, A., Humblot, V., Berjeaud, J.-M., Pradier, C.-M., *Colloids Surf., B* 84 (2011) 301.
53. Mauriello, G., De Luca, E., La Storia, A., Villani, F., Ercolini, D., *Lett. Appl. Microbiol.* 41 (2005) 464.
54. Storia, A.L., Ercolini, D., Marinello, F., Mauriello, G., *J. Food Sci.* 73 (2008) T48.
55. Ho Lee, C., Soon An, D., Cheol Lee, S., Jin Park, H., Sun Lee, D., *J. Food Eng.* 62 (2004) 323.
56. Leistner, L., *Int. J. Food Microbiol.* 55 (2000) 181.
57. Leistner, L., Gorris, L.G.M., *Trends Food Sci. Technol.* 6 (1995) 41.
58. Scannell, A.G.M., Hill, C., Ross, R.P., Marx, S., Hartmeier, W., Arendt, E.K., *Int. J. Food Microbiol.* 60 (2000) 241.
59. Ercolini, D., Ferrocino, I., La Storia, A., Mauriello, G., Gigli, S., Masi, P., Villani, F., *Food Microbiol.* 27 (2010) 137.
60. Reddy, K.V.R., Yedery, R.D., Aranha, C., *Int. J. Antimicrob. Agents* 24 (2004) 536.
61. Karam, L., Jama, C., Nuns, N., Mamede, A.-S., Dhulster, P., Chihib, N.-E., *J. Pept. Sci.* 19 (2013) 377.
62. Bower, C.K., McGuire, J., Daeschel, M.A., *Appl. Environ. Microbiol.* 61 (1995) 992.
63. Bower, C.K., McGuire, J., Daeschel, M.A., *J. Ind. Microbiol.* 15 (1995) 227.
64. Bower, C.K., Daeschel, M.A., McGuire, J., *J. Dairy Sci.* 81 (1998) 2771.
65. Kim, Y.-M., An, D.-S., Park, H.-J., Park, J.-M., Lee, D.S., *Packag. Technol. Sci.* 15 (2002) 247.
66. Elwing, H., Welin, S., Askendahl, A., Lundström, I., *J. Colloid Interface Sci.* 123 (1988) 306.
67. Lu, D.R., Park, K., *J. Colloid Interface Sci.* 144 (1991) 271.
68. Lakamraju, M., Mcguire, J., Daeschel, M., *J. Colloid Interface Sci.* 178 (1996) 495.
69. Kidoaki, S., Nakayama, Y., Matsuda, T., *Langmuir* 17 (2001) 1080.
70. Raffaini, G., Ganazzoli, F., *Langmuir* 29 (2013) 4883.
71. Sanchis, M.R., Blanes, V., Blanes, M., Garcia, D., Balart, R., *Eur. Polym. J.* 42 (2006) 1558.
72. Bogaerts, A., Neyts, E., Gijbels, R., van der Mullen, J., *Spectrochim. Acta, Part B* 57 (2002) 609.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

73. Wan, J., Coventry, J., Swiergon, P., Sanguansri, P., Versteeg, C., *Trends Food Sci. Technol.* 20 (2009) 414.
74. D' Agostino, R., Favia, P., Oehr, C., Wertheimer, M.R., *Plasma Processes Polym.* 2 (2005) 7.
75. Tušek, L., Nitschke, M., Werner, C., Stana-Kleinschek, K., Ribitsch, V., *Colloids Surf., A* 195 (2001) 81.
76. Kull, K.R., Steen, M.L., Fisher, E.R., *J. Membr. Sci.* 246 (2005) 203.
77. Gupta, B., Hilborn, J.G., Bisson, I., Frey, P., *J. Appl. Polym. Sci.* 81 (2001) 2993.
78. Lee, K.T., Goddard, J.M., Hotchkiss, J.H., *Packag. Technol. Sci.* 22 (2009) 139.
79. Ma, Z., Mao, Z., Gao, C., *Colloids Surf., B* 60 (2007) 137.
80. Legeay, G., Poncin-Epaillard, F., Arciola, C.R., Int. J. Artif. Organs 29 (2006) 453.
81. Kato, K., Uchida, E., Kang, E.-T., Uyama, Y., Ikada, Y., *Prog. Polym. Sci.* 28 (2003) 209.
82. Kang, E.T., Tan, K.L., Kato, K., Uyama, Y., Ikada, Y., *Macromol.* 29 (1996) 6872.
83. Muranyi, P., Wunderlich, J., Heise, M., *J. Appl. Microbiol.* 103 (2007) 1535.
84. Sterrett, T.L., Sachdeva, R., Jerabek, P., *J. Mater. Sci.: Mater. Med.* 3 (1992) 402.
85. Kiaei, D., Hoffman, A.S., Horbett, T.A., *J. Biomater. Sci., Polym. Ed.* 4 (1993) 35.
86. Behary, N., Kerkeni, A., Perwuelz, A., Chihib, N.-E., Dhulster, P., *Text. Res. J.* (2013).
87. Vreuls, C., Zocchi, G., Thierry, B., Garitte, G., Griesser, S.S., Archambeau, C., Weerdt, C.V. de, Martial, J., Griesser, H., *J. Mater. Chem.* 20 (2010) 8092.
88. Conte, A., Buonocore, G.G., Sinigaglia, M., Lopez, L.C., Favia, P., D'Agostino, R., Del Nobile, M.A., *J. Food Prot.* 71(1) (2008) 119.
89. Vartiainen, J., Rättö, M., Paulussen, S., *Packag. Technol. Sci.* 18 (2005) 243.
90. Wiechelman, K.J., Braun, R.D., Fitzpatrick, J.D., *Anal. Biochem.* 175 (1988) 231.
91. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., *Anal. Biochem.* 150 (1985) 76.
92. Bastarrachea, L., Dhawan, S., Sablani, S.S., Powers, J., *J. Food Eng.* 100 (2010) 93.
93. Van Staden, A.D., Brand, A.M., Dicks, L.M.T., *J. Appl. Microbiol.* 112 (2012) 831.
94. Guiga, W., Galland, S., Peyrol, E., Degraeve, P., Carnet-Pantiez, A., Sebti, I., *Innovative Food Sci. Emerging Technol.* 10 (2009) 203.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

95. Sebti, I., Blanc, D., Carnet-Ripoche, A., Saurel, R., Coma, V., *J. Food Eng.* 63 (2004) 185.
96. Carnet Ripoche, A., Chollet, E., Peyrol, E., Sebti, I., *Innovative Food Sci. Emerging Technol.* 7 (2006) 107.
97. Neetoo, H., Ye, M., Chen, H., *Int. J. Food Microbiol.* 123 (2008) 220.
98. Turgis, M., Vu, K.D., Dupont, C., Lacroix, M., *Food Res. Int.* 48 (2012) 696.
99. Mota-Meira, M., Lapointe, G., Lacroix, C., Lavoie, M.C., *Antimicrob. Agents Chemother.* 44 (2000) 24.
100. Kaur, G., Singh, T.P., Malik, R.K., Bhardwaj, A., *Probiotics Antimicrob. Proteins* 4 (2011) 11.
101. Song, H.-J., Richard, J., *Int. J. Food Microbiol.* 36 (1997) 155.
102. Pongtharangkul, T., Demirci, A., *Appl. Microbiol. Biotechnol.* 65 (2004) 268.
103. Song, J., Gunst, U., Arlinghaus, H.F., Vancso, G.J., *Appl. Surf. Sci.* 253 (2007) 9489.
104. Butt, H.-J., Physics and chemistry of interfaces, Wiley-VCH, (2003).
105. Gurdak, E., Rouxhet, P.G., Dupont-Gillain, C.C., *Colloids Surf., B* 52 (2006) 76.
106. Zuyderhoff, E.M., Dekeyser, C.M., Rouxhet, P.G., Dupont-Gillain, C.C., *J. Colloid Interface Sci.* 319 (2008) 63.
107. Rouxhet, P.G., Genet, M.J., *Surf. Interface Anal.* 43 (2011) 1453.
108. Rouxhet, P.G., Misselyn-Bauduin, A.M., Ahimou, F., Genet, M.J., Adriaensen, Y., Desille, T., Bodson, P., Deroanne, C., *Surf. Interface Anal.* 40 (2008) 718.
109. Ahimou, F., Boonaert, C.J.P., Adriaensen, Y., Jacques, P., Thonart, P., Paquot, M., Rouxhet, P.G., *J. Colloid Interface Sci.* 309 (2007) 49.
110. Rouxhet, P.G., Mozes, N., Dengis, P.B., Dufrêne, Y.F., Gerin, P.A., Genet, M.J., *Colloids Surf., B* 2 (1994) 347.
111. Muramoto, S., Brison, J., Castner, D.G., *Anal. Chem.* 84 (2012) 365.
112. Kim, M.S., Khang, G., Lee, H.B., *Prog. Polym. Sci.* 33 (2008) 138.
113. Belu, A.M., Graham, D.J., Castner, D.G., *Biomaterials* 24 (2003) 3635.
114. Fletcher, J.S., Henderson, A., Biddulph, G.X., Vaidyanathan, S., Lockyer, N.P., Vickerman, J.C., *Appl. Surf. Sci.* 255 (2008) 1264.
115. Graham, D.J., Wagner, M.S., Castner, D.G., *Appl. Surf. Sci.* 252 (2006) 6860.
116. Tyler, B., Rayal, G., Castner, D., *Biomaterials* 28 (2007) 2412.
117. Schilke, K.F., McGuire, J., *J. Colloid Interface Sci.* 358 (2011) 14.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

118. Sanni, O.D., Wagner, M.S., Briggs, D., Castner, D.G., Vickerman, J.C., *Surf. Interface Anal.* 33 (2002) 715.
119. Poleunis, C., Rubio, C., Compère, C., Bertrand, P., *Appl. Surf. Sci.* 203–204 (2003) 693.
120. Henry, M., Dupont-Gillain, C., Bertrand, P., *Langmuir* 19 (2003) 6271.
121. Aoyagi, S., Hayama, M., Hasegawa, U., Sakai, K., Tozu, M., Hoshi, T., Kudo, M., *J. Membr. Sci.* 236 (2004) 91.
122. Nitschke, M., Meichsner, J., *J. Appl. Polym. Sci.* 65 (1997) 381.
123. Hartwig, A., Mulder, M., Smolders, C.A., *Adv. Colloid Interface Sci.* 52 (1994) 65.
124. Shin, G.H., Lee, Y.H., Lee, J.S., Kim, Y.S., Choi, W.S., Park, H.J., *J. Agric. Food Chem.* 50 (2002) 4608.
125. Abbas, A., Vivien, C., Bocquet, B., Guillochon, D., Supiot, P., *Plasma Processes Polym.* 6 (2009) 593.
126. Grover, N., Singh, H., Gupta, B., *J. Appl. Polym. Sci.* (2010) 3498.
127. Gröning, P., Collaud Coen, M., Schlapbach, L., *Chimia* 55 (2001) 171.
128. Lin, W.-C., Lee, S.-H., Karakachian, M., Yu, B.-Y., Chen, Y.-Y., Lin, Y.-C., Kuo, C.-H., Shyue, J.-J., *Phys. Chem. Chem. Phys.* 11 (2009) 6199.
129. Mochizuki, A., Ogawa, T., Okamoto, K., Nakatani, T., Nitta, Y., *Mater. Sci. Eng., C* 31 (2011) 567.
130. Schweiss, R., Welzel, P.B., Werner, C., Knoll, W., *Colloids Surf., A* 195 (2001) 97.
131. Jandt, K.D., *Surf. Sci.* 491 (2001) 303.
132. Bhushan, B., Nanotribology and nanomechanics: an introduction, Springer, (2005).
133. Ataeefard, M., Moradian, S., Mirabedini, M., Ebrahimi, M., Asiaban, S., *Plasma Chem. Plasma Process.* 28 (2008) 377.
134. Kim, K.S., Ryu, C.M., Park, C.S., Sur, G.S., Park, C.E., *Polym.* 44 (2003) 6287.
135. Malin, M., Vedam, K., *Surf. Sci.* 56 (1976) 49.
136. Favia, P., d' Agostino, R., *Surf. Coat. Technol.* 98 (1998) 1102.
137. Dainelli, D., Gontard, N., Spyropoulos, D., Zondervan-van den Beuken, E., Tobback, P., *Trends Food Sci. Technol.* 19 (2008) S103.
138. Restuccia, D., Spizzirri, U.G., Parisi, O.I., Cirillo, G., Curcio, M., Iemma, F., Puoci, F., Vinci, G., Picci, N., *Food Control* 21 (2010) 1425.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

OBJECTIVES

Research and development in the field of antimicrobial surfaces and interaction studies is interdisciplinary in nature, associating mainly microbiology, materials sciences, chemistry, and physics.

The literature review revealed the high relevance of antimicrobial surfaces but presented also many research gaps that need to be fulfilled for successful development of this sector. It permitted to highlight the challenges related to peptide adsorption and interactions on surfaces. This mechanism is not clearly understood and nisin adsorption and antibacterial activity on hydrophilic and hydrophobic surfaces are still a matter of debate. Peptide adsorption on surfaces are highly governed by surface properties and plasma surface treatments can be promising for modifying those properties needed to understand and to improve this complex phenomenon. Moreover, surface characterization techniques have limited capabilities so far in investigating such surface interactions.

This work concept involved therefore nisin adsorption on hydrophilic and hydrophobic surfaces, using plasma for modifying surface properties, and combining different surface analysis techniques for surface characterization.
Preliminary studies were carried out first to set up and optimize nisin adsorption protocol, as well as different types of plasma treatments processes.

The first part will focus on the characterization of hydrophilic and hydrophobic interactions on nisin-activated surfaces for further correlation with antibacterial activity. This entails determining the composition, molecular structure, conformation, orientation, and spatial distribution of all chemical species and biomolecules present on the surface. The combination of sophisticated analysis techniques will be tailored for such purposes.

The second part will address the debate and study in details the antibacterial activity and the amount of nisin adsorbed on hydrophilic and hydrophobic surfaces. Challenge tests against some food pathogens and simulation tests at refrigeration temperature will be also evaluated.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

The third part will use plasma surface modification to study the various factors affecting nisin adsorption and its antibacterial activity on surfaces. It will assess as well the potential of applying plasma processes for setting up antimicrobial food packaging systems.

The thesis is presented in the form of four articles including a review and three published or submitted research papers. It was the result of fruitful collaboration between five laboratories: ProBioGEM[1] for nisin-surface functionalization and antimicrobial study, UMET[2] for plasma surface treatment and some analysis techniques, UCCS[3] for XPS and ToF-SIMS analysis, IMN[4] and IEMN[5] for AFM analysis.

[1] Procédés Biologique et Génie Enzymatique et Microbien, Université de Lille1
[2] Unité de Matériaux et Transformations, Université de Lille1
[3] Unité de Catalyse et de Chimie du Solide, Université de Lille1
[4] Institut des Matériaux Jean Rouxel, Université de Nantes
[5] Institut d'électronique de microélectronique et de nanotechnologie, Université de Lille1

© 2013 Tous droits réservés.
http://doc.univ-lille1.fr

CHAPTER II:

NISIN ADSORPTION ON HYDROPHILIC AND HYDROPHOBIC SURFACES: EVIDENCE OF ITS INTERACTIONS AND ANTIBACTERIAL ACTIVITY

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

1. Introduction

The antimicrobial performance of surface-adsorbed peptides is a key factor that will determine the success of their applications. This performance is dependent on the peptide interaction with the surface materials and particularly on hydrophilic and hydrophobic interactions. However, such interpretations are generally based on predictions and theoretical models in literature (Lakamraju et al., 1996). Showing the evidence of these interactions, require methods capable of probing the orientation and conformation of peptides on surfaces, which is far from being a direct simple process.

In this paper, we attempted to combine ToF-SIMS and XPS techniques to address these challenges. XPS is less sensitive than ToF-SIMS but both methods can be used in a complementary approach for chemical identification and quantification. XPS has been used to determine and quantify the atomic composition of the surfaces (Kim et al., 2008). ToF-SIMS has been mostly used to study the surface composition, molecular structure, and for chemical mapping (Goddard and Hotchkiss, 2007). For peptides-adsorbed surface, the ion fragments produced by the bombardment of the surface are characteristic of different specific amino acids (Sanni et al., 2002). Therefore, the intensity ratio of different amino acid mass fragments provides useful information for the identification of the adsorbed peptides. Moreover, nisin adsorbs in a multilayer system at high nisin concentrations (Lakamraju et al., 1996). Due to its low sampling depth, ToF-SIMS can characterize the outer 1–5 nm of the peptide layers (Muramoto et al., 2012). As the type of the surface or nisin concentration change, the peptide adjusts to the surface and changes its conformation or orientation too. New regions of the nisin layers with different amino acid compositions will be thus exposed to the static SIMS sampling depth and be detected. Consequently, it would be possible that the relative intensities of the detected amino acid fragments are sensitive to the orientation of nisin on the surface and to its degree of conformational alteration.

Based on those assumptions, the following work aims to answer the following two main questions: (1) Are ToF-SIMS and XPS capable of showing nisin orientation and interactions on hydrophobic and hydrophilic surfaces? (2) Are those interactions correlated with the antibacterial activity?

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

2. Article II

Nisin adsorption on hydrophilic and hydrophobic surfaces: evidence of its interactions and antibacterial activity

Layal Karam[a,b], Charafeddine Jama[b], Nicolas Nuns[c], Anne-Sophie Mamede[c], Pascal Dhulster[a] and Nour-Eddine Chihib[a,1]

[a] Laboratoire ProBioGEM, Polytech'Lille, Université Lille1, Avenue Paul Langevin, 59655 Villeneuve d'Ascq, France

[b] Laboratoire UMET, UMR-CNRS 8207, Ecole Nationale Supérieure de Chimie de Lille, Université Lille 1, Avenue Dimitri Mendeleïev, 59655 Villeneuve d'Ascq, France

[c] Unité de Catalyse et de Chimie du Solide, UMR-CNRS 8181, Ecole Nationale Supérieure de Chimie de Lille, Université Lille 1, Avenue Dimitri Mendeleïev, 59655 Villeneuve d'Ascq, France

Abstract

Study of peptides adsorption on surfaces remains a current challenge in literature. A complementary approach, combining X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (ToF-SIMS), was used to investigate the antimicrobial peptide nisin adsorption on hydrophilic and hydrophobic surfaces. The native low density polyethylene (LDPE) was used as hydrophobic support and it was grafted with acrylic acid (AA) to render it hydrophilic. XPS permitted to confirm nisin adsorption and to determine its amount on the surfaces. ToF-SIMS permitted to identify the adsorbed bacteriocin type and to observe its distribution and orientation behavior on both types of surfaces. Nisin was more oriented by its hydrophobic side to the hydrophobic substrate and by its hydrophilic side to the outer layers of the adsorbed peptide, in contrast to what was observed on the hydrophilic substrate. A correlation was found between XPS and ToF-SIMS results, the types of interactions on both surfaces and the observed antibacterial

[1] Corresponding author. Fax: +33 3 28 76 73 81 (N.E. Chihib).
Email address: Nour-Eddine.Chihib@univ-lille1.fr (N.E. Chihib).

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

activity. Such interfacial studies are crucial for better understanding the peptides interactions and adsorption on surfaces and must be considered when setting-up antimicrobial surfaces.

Keywords: Peptides adsorption; peptides interactions; antimicrobial surfaces; nisin; peptides characterization; hydrophobic surfaces; hydrophilic surfaces; materials' interfaces; X-ray photoelectron spectroscopy (XPS); time-of-flight secondary ion mass spectrometry (ToF-SIMS)

Abbreviations: XPS, X-ray photoelectron spectroscopy; ToF-SIMS, time-of-flight secondary ion mass spectrometry; LDPE, low density polyethylene; AA, acrylic acid; HCl, hydrochloric acid; BE, binding energy; ns, nanosecond; Gly, Glycine; Ala, Alanine; Ser, Serine; Met, Methionine; Val, Valine; Lys, Lysine; Leu, Leucine; Ile, Isoleucine; Asn, Asparagine; His, Histidine; GRAS, Generally Recognized As Safe; FDA, Food and Drug Administration; AFM, atomic force microscopy; SEM, scanning electron microscopy; AES, auger electron spectroscopy; FTIR, fourier transform infrared; NEXAFS, near edge X-ray absorption fine structure.

Introduction

The use of antimicrobial peptides adsorbed onto surfaces is one of the possible innovative and proactive approaches to prevent contaminations and infections. This is highly relevant for setting up antimicrobial food packaging that acts to reduce, inhibit or retard the growth of microorganisms that may be present in the packed food or packaging material itself [1]. Such improved antimicrobial strategies might also benefit the biomedical industry where the bacterial infection and implants can cause life-threatening illnesses. In the pharmaceutical and cosmetic fields, the sterilization of high value containers is often not practical, and the need for peptide shield that possesses antimicrobial properties is crucial too. Low density polyethylene (LDPE) is a well-known polymer due to its applications in many fields, for example in the agro-food sector as packaging, trays, plastic bags, utensils, housings, in the medical field as covers, various containers for surgical tools…

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Adsorption of peptides, such as nisin, on a surface and their interactions, remain a complicated phenomenon that depends on many factors as the nature of the surface. When adsorbed on hydrophilic or hydrophobic surfaces, proteins and peptides adopt different orientation, interaction and structural rearrangement [2]. Such conformational changes can significantly impact the antimicrobial activity and the performance of biomaterials [3]. For this reason, to advance in any of these areas, it will be essential to gain a more thorough understanding of the relationship between surface properties and how antimicrobial peptides behave and interact with materials. This entails characterizing the biomaterial surface in detail by determining the composition, molecular structure, interactions, conformation, orientation, and spatial distribution of all chemical species and biomolecules present on the surface. Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, is a 3.5 kDa antibacterial peptide approved as Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA) [4] and is effective against pathogens like *Listeria monocytogenes*, *Staphyloccoccus aureus* and *Clostridium perfringens* [5,6]. Nisin has been widely used in the food industry as a safe and natural preservative [7] and has found some applications in the medical sector too [8,9]. In addition, previous studies showed its ability to retain stable antimicrobial activity in the adsorbed state [10].

A wide range of surface analysis techniques has been recently used to characterize the surface properties of biomaterials [11-16]. These include X-ray photoelectron spectroscopy (XPS), time-of-flight secondary ion mass spectrometry (ToF-SIMS), atomic force microscopy (AFM), scanning electron microscopy (SEM), auger electron spectroscopy (AES), contact angle methods, reflection fourier transform infrared spectroscopy (FTIR), and near edge X-ray absorption fine structure (NEXAFS). Among them, ToF-SIMS and XPS are valuable tools because they are capable of probing the surfaces at different sensitivities and sampling depths, which is very informative for interfacial studies.

The current challenge is to relate the extensive information provided by those techniques to surface molecular structure, to the peptide conformation and orientation on surfaces. Each technique has its own advantages and limitations; therefore the purpose of this study is to develop a complementary approach combining ToF-SIMS and XPS analytical methods, to understand nisin adsorption and antibacterial activity on hydrophilic and hydrophobic LDPE surfaces. This film, hydrophobic in nature, was grafted with

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

acrylic acid (AA) to generate hydrophilic surfaces and nisin was adsorbed on both surfaces.

Materials and methods

Film preparation

Low density polyethylene (LDPE) of 70 μm thickness (Polimeri Europa, France SAS) was cut into 2 x 2 cm^2 and washed with ethanol in an ultrasonic bath to remove possible dusts, oily compounds or any chemicals and wetting agents adsorbed on the film surface, then it was dried in an oven at 55°C for 3 h. Those films were either used directly or treated for nisin adsorption.

Plasma and acrylic acid (AA) treatment

In order to elaborate hydrophilic surfaces, grafting of acrylic acid (AA) monomers (99.5%, Acros Organics, Belgium) was done using the optimized grafting in solution method of Gupta and co-workers [17]. Before grafting, LDPE films were pre-treated by Ar/O_2 (95/5%) plasma. Plasma treatments were performed in a radio-frequency cold plasma reactor of 350 liters capacity (Europlasma CD1200, Belgium) at an excitation frequency of 13.56 MHz. The preselected vacuum working pressure was 30 mTorr. An experimental design was set to optimize Ar/O_2 (95/5%) plasma treatment parameters and the following optimal conditions were retained: gas flow rate of 1000 sccm (standard cubic centimeter per minute), generator power of 420 Watts and an exposure time of 245 s. The films were then exposed to laboratory atmosphere for approximately 30 min in order to generate oxygen and peroxide groups on the surface before immersion in 25 ml of AA solution. The grafting experiments were carried out at 50°C, in water jacketed glass reactors, under nitrogen atmosphere and slight shaking. After 6 h, the films were removed from the solution and ultrasonically washed in ethanol to remove ungrafted AA. They were then dried at room temperature and kept in Petri dishes for further treatment or analysis. The amount of grafting on the AA treated film was determined using Toluidine Blue O method [17]. The surface concentration of AA was calculated according to a mole-to-mole complex between the dye and carboxylic acid groups and was recorded at 6.5 ± 0.6

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

nmol/cm². The water contact angle decreased from 101.8 ± 1.4° to 44.2 ± 2.1° after surface treatment. Therefore, in the present study, the native LDPE was used as hydrophobic surface and the AA treated film as hydrophilic one.

Nisin preparation

A pure grade of nisin A was donated by Danisco (Beaminster Dorset, United Kingdom). Activity was indicated as 5.2 x 10^7 IU/g. Nisin solutions were prepared by dissolving 1.0 mg/ml of nisin in HCl (0.01 M). Solutions were freshly prepared and filtered (0.22 μm) before each experiment.

Nisin adsorption on films

Nisin adsorption was carried out on native and AA treated films. Each film was immersed in 20 ml of nisin solution (1.0 mg/ml) and it was agitated at 8°C for 16 h. After that the samples were removed from solution and briefly rinsed by immersion for 10 seconds in sterile distilled water to remove non adsorbed nisin. Those high nisin concentration films were the representative surfaces used for all the tests to compare both types of surfaces. For interfacial studies, the latter treated films were rinsed for 6 h by immersion in sterile HCl (0.01 M) under slight shaking, to generate films with lower nisin concentrations. Preliminary experiments permitted to choose the optimized rinsing conditions needed for analyzing two concentrations high and lower on both types of surfaces. Those surfaces, needed only for interactions studies, were characterized by XPS and semi-quantitative ToF-SIMS. All the tests were done after drying the films in sterile Petri dishes at 25°C for 24 h.

Surface characterization

X-ray photoelectron spectroscopy (XPS)

XPS experiments were carried out using a Kratos Analytical AXIS Ultra^{DLD} spectrometer (United Kingdom). A monochromatized aluminium source (Al Kα = 1486.6 eV) was used for excitation. The analyzer was operated in constant pass energy of 40 eV using an

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

analysis area of approximately 700 µm x 300 µm. Charge compensation was applied to compensate for the charging effect occurring during the analysis. The C 1s hydrocarbon (285.0 eV) binding energy (BE) was used as internal reference. The spectrometer BE scale was initially calibrated against the Ag $3d_{5/2}$ (368.2 eV) level. Pressure was in the 10^{-10} Torr range during the experiments. The homogeneity of a sample was checked by analyzing three different surface areas. Quantification and simulation of the experimental photopeaks were carried out using CasaXPS software. Quantification took into account a non-linear Shirley [18] background subtraction.

Time – of – flight secondary ion mass spectrometry (ToF-SIMS)

ToF-SIMS spectra measurements were carried out using a ToF-SIMS V instrument (ION-TOF GmbH, Germany). This instrument is equipped with a Bi liquid metal ion gun (LMIG). Pulsed Bi^{3+} primary ions have been used for analysis (25 keV, 0.4 pA). Mass spectra and images were taken for each sample, from an area of 500 µm x 500 µm (30 scans) using 256 x 256 pixel random rasters. These experimental conditions allowed staying within the static conditions since primary ion dose did not exceed 10^{12} ions/cm^2. Pulsed low energy flood gun (20 eV) were used for charge neutralization. The hydrogen pulse width varied from 0.8 to 1 ns depending on the samples studied. Average mass resolution was about 4000 at *m/z* = 86.16 ($C_5H_{12}N^+$) which allowed the separation of organics ion fragments under investigation.

Bacterial culture and growth conditions

The antibacterial tests were carried out against *Listeria innocua* LMG 11387 (BCCM, Belgium). Pre-cultures were performed by inoculating a single colony in 10 ml of Brain Heart broth (Biokar Diagnostics, Beauvais, France). The cultures were made by inoculating 10 ml of Brain Heart broth with 100 µl of the pre-culture. Pre-cultures and cultures were incubated at 37°C for 24 h.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Assessment of the antibacterial activity of nisin-functionalized films

Qualitative antibacterial tests of nisin-functionalized films were done using a modified agar diffusion assay [19]. Mueller Hinton agar medium (Biokar Diagnostics, Beauvais, France) was seeded with the indicator micro-organism, *Listeria innocua.* The face up of the film to be tested was placed on the agar surface. Bioassay plates containing experimental samples were kept at 4°C for 4 h to initiate nisin diffusion and were then incubated at 37°C for 24 h. Nisin activity was assessed as an inhibition of the indicator bacterium growth under and around the film.

Results and discussion

Previous studies on nisin adsorption [20] reported that the bacteriocin adopts a multilayer coverage on hydrophilic and hydrophobic surfaces when working with high nisin concentration (1.0 mg/ml). In addition, nisin held in the outer layers is loosely bound and removable upon rinsing. Therefore, in order to understand adsorption behavior and peptide conformation in a multilayer system, studying the outer layers (the 2^{nd} or the 3^{rd} layer) is not sufficient and cannot be easily predictable. Then it is necessary also to work at lower peptide concentration, at the inner layers (peptide – substrate interface) or closer to the first layer of contact between the peptide and the film as suggested by Henry and co-workers [2]. For all those purposes, films with high and lower peptide concentrations were prepared (see section "Nisin Adsorption on Films") and studied by XPS and ToF-SIMS. XPS was used to determine the elemental composition of the films, the functional groups and then the amount of nisin adsorbed on the surfaces. ToF-SIMS was used to identify nisin and to study its adsorption behavior and distribution on surfaces.

XPS evidence of nisin adsorption on hydrophilic and hydrophobic surfaces

Quantification of nisin adsorption

The elemental composition was expressed as atomic percentage ratio of oxygen, nitrogen and sulfur with respect to carbon (Table 1). Before nisin adsorption, traces of oxygen impurities were detected on the surface of the native film but O/C ratio increased from

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

0.006 to 0.170 due to the formation of oxygenated functional groups after AA treatment. Those ratios increased also on both types of surfaces after nisin adsorption due to the peptide additional contribution (Table 1). In contrast to both bare substrates, nisin contains nitrogen and sulfur with atomic S/N ratio of 0.117 consistent with S/N ratio of 0.166, calculated from the known composition of nisin. Therefore its adsorption on the surface and its amount were highlighted by the detection of these two elements. Hydrophilic AA treated films exhibited higher ratios of N/C and S/C and therefore higher quantity of nisin than the hydrophobic native films (Table 1). In addition, higher quantities of nisin were detected on the briefly rinsed films than on the 6 h rinsed ones for both types of surfaces. On hydrophilic film, nisin was partially removed from the surface with a decrease of N/C and S/C from 0.163 and 0.013 to 0.057 and 0.003 respectively. On hydrophobic film, N/C ratio decreased from 0.047 to 0.005 and S/C ratio from 0.005 to below the detection limit (Table 1). This confirmed the adsorption of nisin on each surface at high and lower concentration levels.

Correlation between nisin adsorbed amount and surface layer analyzed

The distribution and proportion of each existing functional group, determine the overall shape of C 1s peaks of the different samples. The C 1s decomposition by curve-fitting was used to estimate the proportions of both nisin and substrates in mixed surfaces (Figure 1). By fitting the experimental XPS spectra, obtained after nisin adsorption, with reference spectra of pure nisin and bare substrates, we found out that the amount of nisin decreased from 78 to 20% on the high and lower concentration AA treated films (Figure 1a and b), respectively and from 23 to 5% for the same concentrations on the native films (Figure 1c and d). Subsequently, the substrate proportion was as following: the AA treated film proportion increased from 22 to 80% for the high and lower concentration films respectively (Figure 1a and b) and the native film proportion increased from 77 to 95% for the same films (Figure 1c and d). XPS analysis provides data in the *ca.* 1–10 nm surface layer [21] and an increase in the proportion of the substrates was observed with the decrease of nisin quantity on the surfaces. Taking into account that all the samples were investigated at the same depth, that XPS analysis of different areas of the surfaces showed the same composition in all the sites and that nisin was totally covering both surfaces as observed by ToF-SIMS (see section “Nisin distribution on surfaces by ToF-SIMS imaging

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

mode"); a higher amount of nisin can induce a higher thickness of nisin layer and vice versa. This is consistent with a multilayer adsorption, as reported by Lakamraju and co-workers [20]. They showed also that the mass of nisin adsorbed on hydrophilic and hydrophobic surfaces decreased after rinsing in buffer for 1 h and that the first nisin layer is tightly bound to the surface, with much of the loosely bound outer layer removable upon rinsing. In addition, similar work [22] reported that adsorption of β-lactoglobulin and ovalbumin to chromium oxide surfaces results in a protein bilayer in which the first layer is tightly attached to the surface, whereas the second layer desorbs upon rinsing. The observed decrease in adsorbed nisin amount upon rinsing is in good agreement with such a structure. We can then postulate that analyzing the interfaces of the high concentration films provides information about the outer layers of nisin adsorbed on the surface, while the lower concentration films, rinsed for 6 h, give information about the inner or bottom layers where nisin is adsorbed in a thinner layer or smaller amount and is consequently present closer to the substrate interface. This is relevant for ToF-SIMS that is more surface sensitive than XPS and characterizes the uppermost layers of the surface at lower sampling depths; it analyzes the fragments emitted from the first 1–5 top monolayers [23].

Table 1. XPS surface chemical composition of: pure nisin, native and AA treated films, before and after nisin adsorption at high and lower concentrations.

LDPE Samples	Atomic percentage (%)				Atomic ratio		
	C	N	S	O	N/C	S/C	O/C
Native film	99.4	−	−	0.6	−	−	0.006
AA treated film	85.5	−	−	14.5	−	−	0.170
Pure Nisin	69.8	14.5	1.7	14.0	0.208	0.025	0.200
Native film + high nisin concentration	90.6	4.3	0.4	4.7	0.047	0.005	0.052
Native film + lower nisin concentration	98.6	0.5	−	0.9	0.005	−	0.009
AA treated film + high nisin concentration	68.7	11.2	0.9	19.2	0.163	0.013	0.279
AA treated film + lower nisin concentration	81.0	4.6	0.3	14.1	0.057	0.003	0.174

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

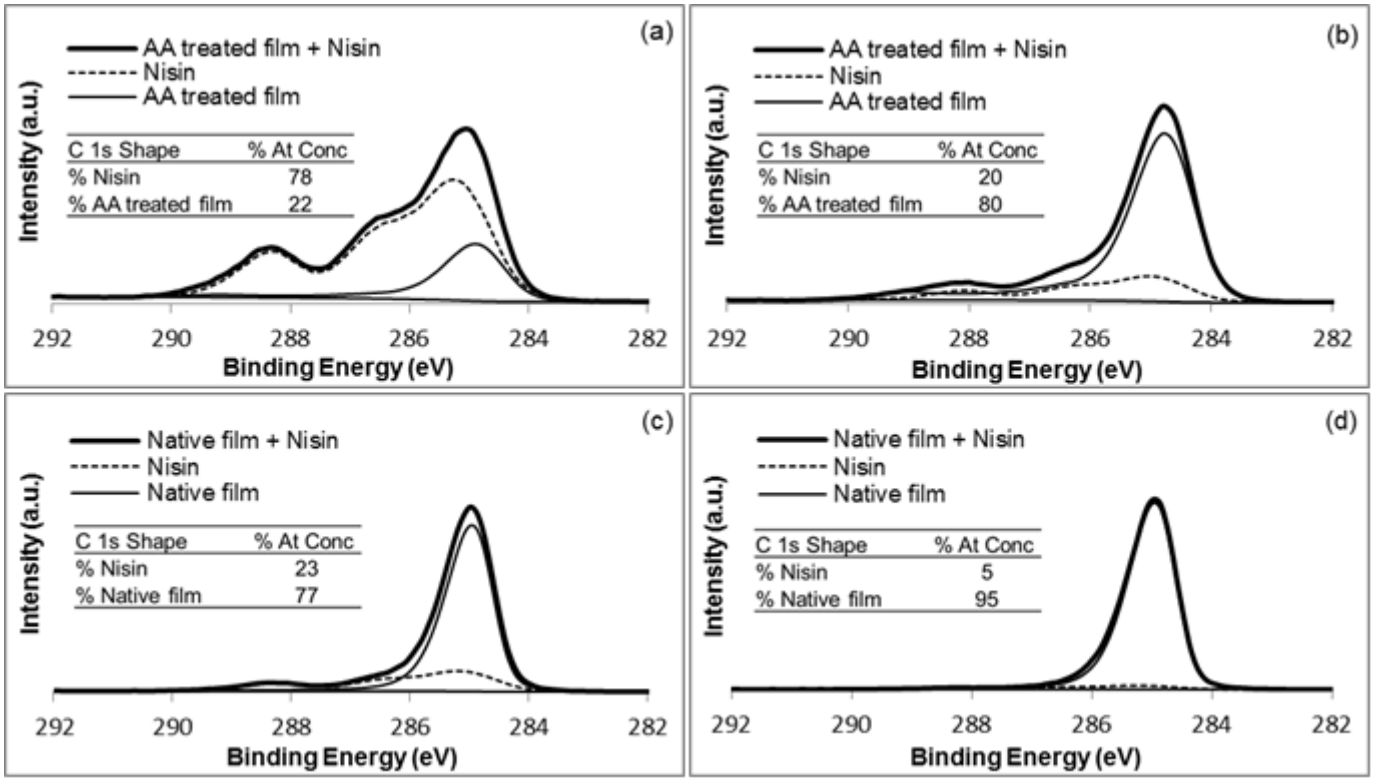

Figure 1. C 1s peaks curve fitting using models defined from XPS data of pure nisin and bare films: C 1s peak of AA treated film with high nisin concentration (a) and lower nisin concentration (b), C 1s peak of native film with high nisin concentration (c) and lower nisin concentration (d).

ToF-SIMS evidence of nisin adsorption on hydrophilic and hydrophobic surfaces

Identification of nisin characteristic ions

ToF-SIMS generates a huge amount of information about the chemical composition and the structure of surfaces. In the current study, we attempted to analyze nisin adsorption on native LDPE and AA treated surfaces. The positive nisin peaks were used because negative ion spectra provide little peak information for most amino acids [12] and there are few nisin characteristic peaks in negative mode. The positive spectra obtained in this experiment (Figure 2) and previous work done on nisin and proteins [2,24,25] allowed us to find out the characteristic fragment peaks of nisin with their associated amino acids (Table 2 and Figure 3).

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Representative spectra from 0 to 120 *m/z* and the peak assignments are presented for each of the different surface treatments. Native samples exhibited a large number of (CxHy) peaks characteristic of LDPE films (Figure 2a). After AA grafting, oxygenated species induced by this treatment, appeared on the surface as CH_3O and C_2H_5O (Figure 2b). Moreover, two peaks characteristic of AA were detected on the surface at *m/z* = 73 in the positive ion mode and *m/z* = 71 in the negative ion mode (spectra not shown). Acrylic acid has the molecular structure $C_3H_4O_2$. The presence of the molecular parent ion fragments $C_3H_5O_2^+$ (*m/z* = 73) and $C_3H_3O_2^-$ (*m/z* = 71) can be associated to the AA grafting [27]. After nisin adsorption several nitrogen and sulfur containing species (CxHyN and CxHyS), characteristic of nisin, appeared on both native (Figure 2c) and AA treated films mass spectrums (Figure 2d). In addition, high intensity peaks, related to repeated amino acids in nisin molecule, were observed such as CH_4N^+ (*m/z* 30.03), $C_2H_6N^+$ (*m/z* 44.04) and $C_5H_{12}N^+$ (*m/z* 86.16), associated to Gly, Ala and Leu, Ile respectively.

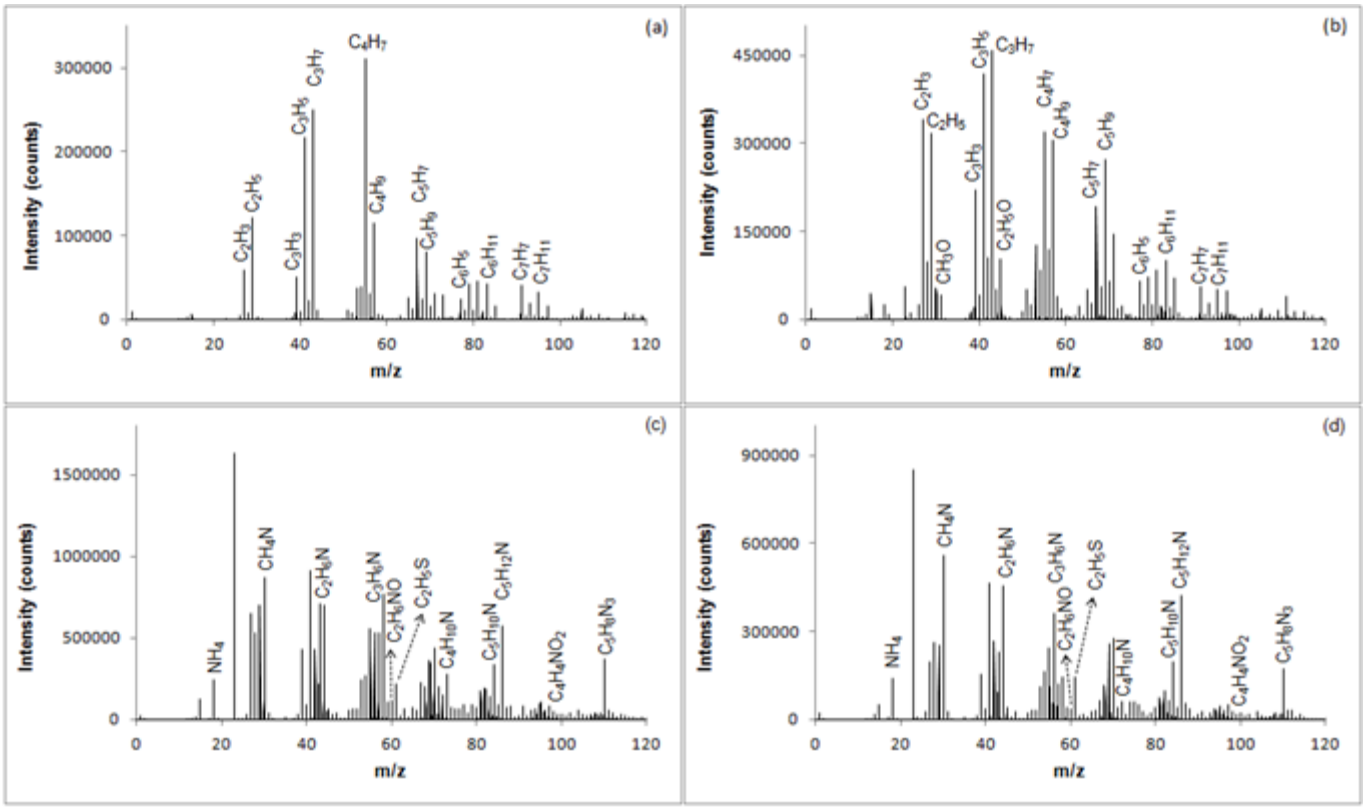

Figure 2. Positive ion ToF-SIMS spectra of the native films before (a) and after nisin adsorption (c) and of the AA treated films before (b) and after (d) nisin adsorption.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Table 2. List of characteristic positive ions, detected on the films after nisin adsorption, with their associated amino acids.

m/z	Characteristic secondary ion	Associated amino acid	Number of amino acids in nisin molecule	References
18.03	NH_4^+	All		[2,24,25]
30.03	CH_4N^+	Glycine (Gly) and other amino acids		[2,24,25]
44.04	$C_2H_6N^+$	Alanine (Ala)	8	[2,24,25]
56.09	$C_3H_6N^+$	Lysine (Lys)	3	[2]
60.04	$C_2H_6NO^+$	Serine (Ser)	1	[2,24,25]
61.01	$C_2H_5S^+$	Methionine (Met)	2	[2,24,25]
72.08	$C_4H_{10}N^+$	Valine (Val)	1	[2,24,25]
84.14	$C_5H_{10}N^+$	Lysine (Lys)	3	[2]
86.16	$C_5H_{12}N^+$	Leucine (Leu),	2	[2]
		Isoleucine (Ile)	3	
98.02	$C_4H_4NO_2^+$	Asparagine (Asn)	1	[2,24,25]
110.07	$C_5H_8N_3^+$	Histidine (His)	2	[2,24,25]

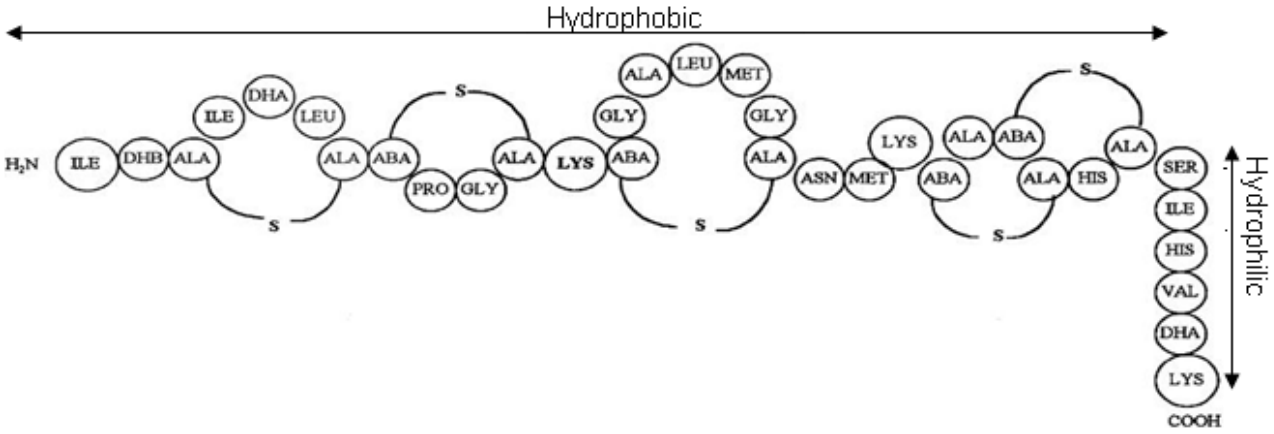

Figure 3. The primary structure of nisin A showing the distribution of amino acids in the hydrophilic and hydrophobic sides of the molecule (adapted from [26]).

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Nisin adsorption behavior: orientation and interactions

In our experiments, ToF-SIMS analysis of the uppermost surfaces of nisin adsorbed films, allowed to detect all the listed amino acids (Table 2) on both types of surfaces and for both levels of concentration. Those findings confirmed the presence of nisin on the surfaces but were not sufficient to explain the bacteriocin conformation.

Nisin structure and amino acids distribution in the molecule

Nisin has an amphiphilic character, with a cluster of bulky hydrophobic residues at the N-terminus and hydrophilic residues at the C-terminal end [28] (Figure 3). In the list of characterized nisin fragments (Table 2), Ser and Val are the only amino acids available in the hydrophilic domain but not in the hydrophobic one. His, Lys and Ile are available in the hydrophilic side too but another His is also present at the end of the hydrophilic domain and the beginning of the hydrophobic domain while two other Lys and Ile are present in the main part of the latter domain. Moreover Ile cannot be separated from Leu in ToF-SIMS spectra and since nisin contains five amino acids of Leu, Ile of which four are available in the hydrophobic side, they can be more associated to the latter side of the peptide. The remaining amino acids can either be associated to all amino acids in the molecule as NH_4^+ and CH_4N^+ or are only available in the hydrophobic domain as Ala, Met, Leu and Asn.

Nisin adsorption behavior by semi-quantitative ToF-SIMS

Direct quantitative ToF-SIMS analysis is difficult because the secondary ion yields depend on several factors and are not directly proportional to their concentration in the sample. This is due to the influence of the matrix effects on ion yields and the preferential ionization of one species over another. In other words, comparing the concentration of one species to another in the same spectrum is not possible. However for similar materials, we can compare spectrum to spectrum species after intensity normalization. This can be achieved by characterizing the relative amount of components at a surface of a sample and rationing representative ions, elemental or molecular species [29]. For this purpose, we normalized the peak intensities to the total intensity (counts) of the spectrum, in order to

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

eliminate differences in total secondary ion yield from spectrum to spectrum and we compared the relative intensity of each nisin characteristic fragment for the different samples and treatments. To understand adsorption behavior, the study of peptide conformation was based on the type of the surface, the surface layer analyzed and on the distribution of nisin amino acids according to their presence in the hydrophilic or hydrophobic side of the molecule (Figure 3). We determined for this purpose, for each type of surface, the contribution of each amino acid to the outer nisin layers (high nisin concentration films) and to the inner or bottom nisin layers (lower nisin concentration films). This was done by calculating the following intensity ratios for the native and AA treated films*:*

$$\frac{(Ix)\,outer\ layers}{(Ix)\ outer\ layers + (Ix)\ inner\ layers} \quad (1)$$

and

$$\frac{(Ix)\ inner\ layers}{(Ix)\ outer\ layers + (Ix)\ inner\ layers} \quad (2)$$

where I represents, the normalized intensity for each nisin characteristic amino acid (x) listed in Table 2. Figure 4 showed in general, that the distribution of amino acids, presented for both types of surfaces, similar trends in the inner layers of one surface and the outer layers of the second one (Figure 4a and d; Figure 4b and c). However, opposite trends were observed for specific amino acids between the inner and outer layers of the same surface (Figure 4a and b; Figure 4c and d) as well between the inner layers (Figure 4a and c) or the outer layers (Figure 4b and d) of the two different surfaces. When we compared the outer and inner layers of the hydrophobic native films, we observed that, on the inner layers as opposed to the outer ones, the lowest contributions of amino acids were for Ser, Val, His (hydrophilic side of nisin) and the highest ones for Ala, Met, Asn (hydrophobic side of nisin) (Figure 4c and d). This suggests that the molecule is oriented by its hydrophobic side to the hydrophobic substrate and by its hydrophilic side to the outer layers. On the hydrophilic AA treated films, the amino acids characteristics of the hydrophilic side of the molecule (Ser, Val) had the highest intensity ratios in the inner layers. Also the amino acids associated to the hydrophobic side of nisin (Met, Asn, Leu)

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

had the lowest intensity ratios in the same layers (Figure 4a). The inverse was exactly observed on the outer layers of the same surface (Figure 4b). This can be explained by an orientation of the molecule by its hydrophilic side to the hydrophilic substrate and by its hydrophobic side toward the outer layers. The amino acids present in both sides of the molecule and mostly in the larger hydrophobic one are not of much relevance for the orientation study. They presented in general parallel behavior and were more contributing to the hydrophobic side of the molecule.

Those observations can confirm the theoretical model predicted by Lakamraju and co-workers [20]. They suggested that on a hydrophobic support and due to hydrophobic interactions, hydrophobic domain of the peptide may be oriented toward the hydrophobic support and the hydrophilic domain having less contact. The inverse would be observed on hydrophilic surfaces, due to hydrogen bonding or electrostatic interactions [20].

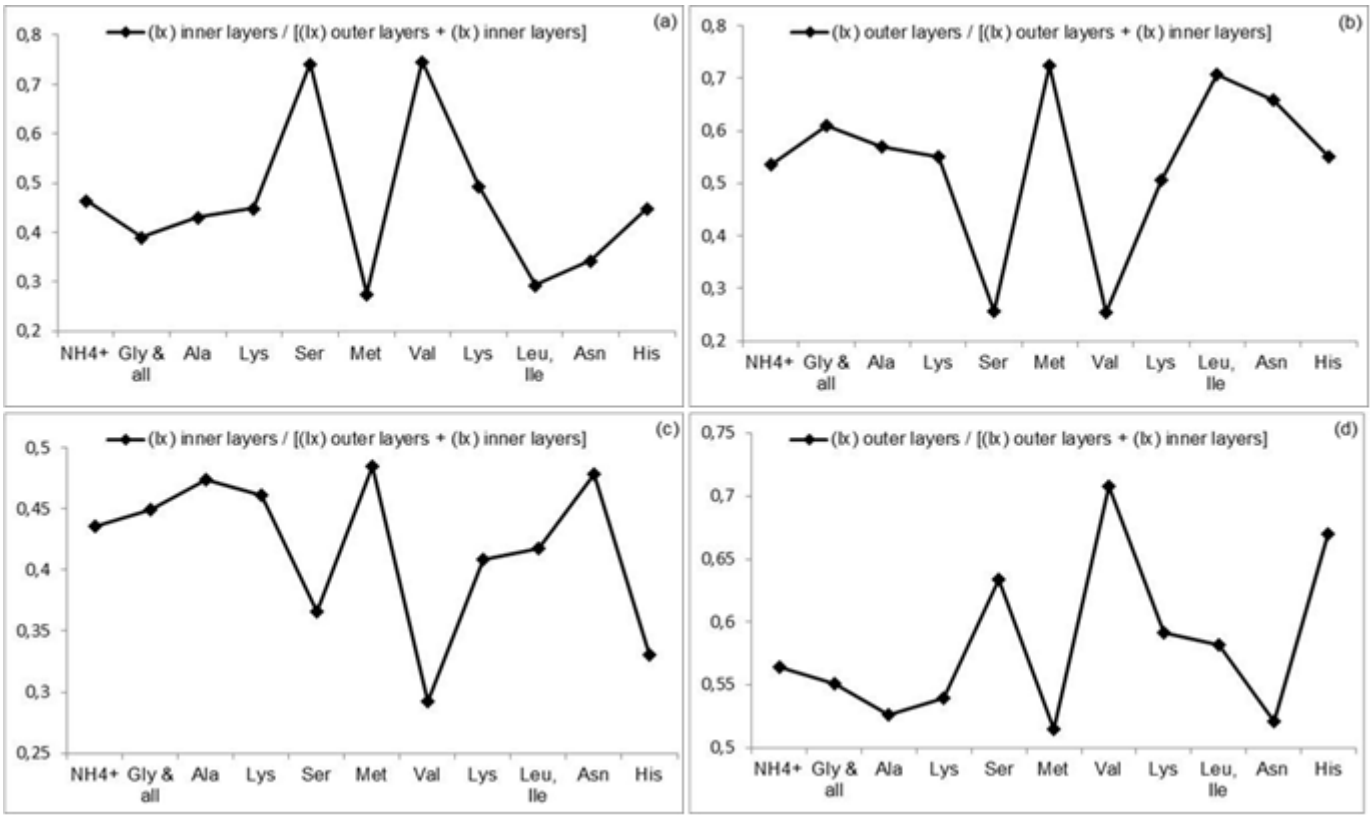

Figure 4. Normalized peak intensities (I) ratios, characterizing the contribution of each amino acid (x) to the inner and outer layers of nisin adsorbed on both surfaces: nisin inner (a) and outer layers (b) on the AA treated film; nisin inner (c) and outer layers (d) on the native film.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Nisin distribution on surfaces by ToF-SIMS imaging mode

In order to generate a chemical map of the surface, the imaging mode was used and the surface distribution of nisin characteristic ions, was mapped (Figure 5).

SIMS images showed differences in nisin distribution on the native (hydrophobic) and AA treated (hydrophilic) films. The hydrophobic surface (Figure 5a) presented ‘‘dark spots” suggesting a non-uniformity of nisin distribution on the film while the hydrophilic one (Figure 5b) showed an even peptide distribution on the total surface. For more confirmation, we analyzed separately for the hydrophobic film, the composition of observed dark spots area and the one for the equivalent light area (without dark spots). This was done by reconstruction of ToF-SIMS spectra from the above mentioned regions of interest (Figure 6a and b). All amino acids were detected in both areas with slightly higher normalized intensity peaks in the light area than in the dark area (Figure 6c); indicating that nisin was covering the entire surface of this film but not uniformly in all the sites.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

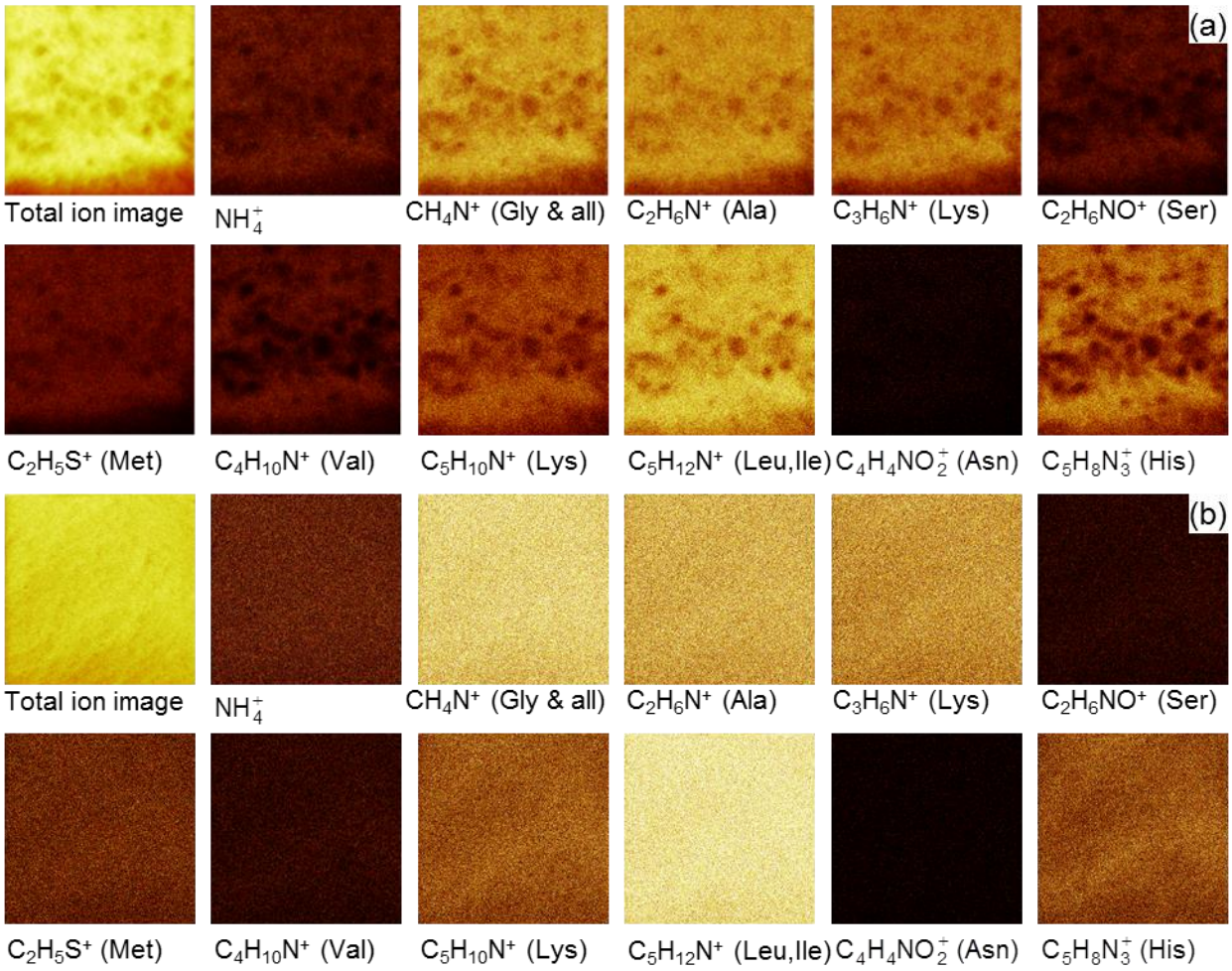

Figure 5. ToF-SIMS images (500 x 500 µm, positive ion mode) of total ion and of nisin characteristic secondary ions, showing non-uniform nisin distribution on the native films (a) versus uniform one on the AA treated films (b).

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

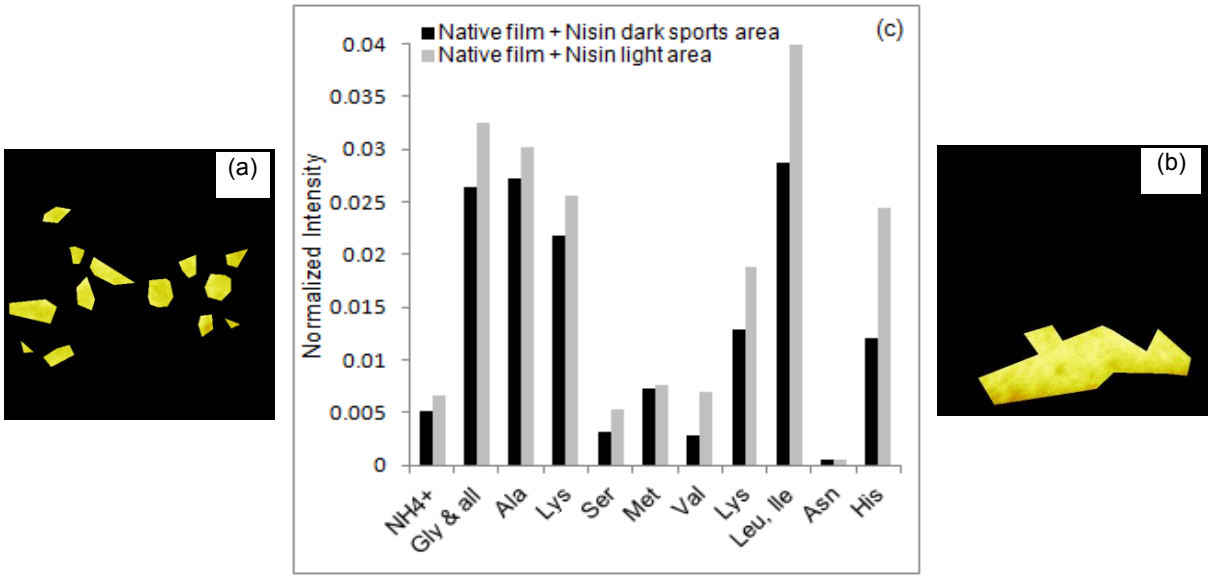

Figure 6. Investigation of the two areas in ToF-SIMS non-uniform image (Figure 5a): nisin dark spots area (a) and equivalent nisin light (without dark spots) area (b) of the native film.

Normalized peak intensities of characteristic amino acids, showing nisin presence in dark spots and light areas of the native films (c).

Assessment of the antibacterial activity of nisin-functionalized films

The antibacterial activity of nisin-functionalized films, against *Listeria innocua*, was shown in Figure 7. The control films had no antibacterial activity since no inhibition was observed for native (Figure 7a) and AA treated films (Figure 7b). However, after nisin adsorption, native LDPE films (hydrophobic) showed irregular localized antibacterial activity on different regions under the film (Figure 7c). By contrast, the AA treated films (hydrophilic) showed a uniform activity with clear inhibition zone under and around the film (Figure 7d). Those results can be correlated to the lower amount of nisin detected on hydrophobic surfaces versus the hydrophilic ones, to the nisin distribution on both surfaces as shown by ToF-SIMS (Figure 5) as well to the types of interactions on those surfaces. Despite its total presence on the hydrophobic surface, the peptide was not totally "available" for the antibacterial activity and its irregular activity can be related to its non-uniform distribution. The opposite was observed on the hydrophilic surface and nisin

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

seemed to be more “available” or freely desorbed from this film than from the hydrophobic one. Those findings are in agreement with previously reported work [2,3] and can be explained by the force of interactions on both surfaces and by the higher peptide conformational change on hydrophobic surfaces relative to hydrophilic ones [3]. Such changes in nisin conformation can affect its functionality [2].

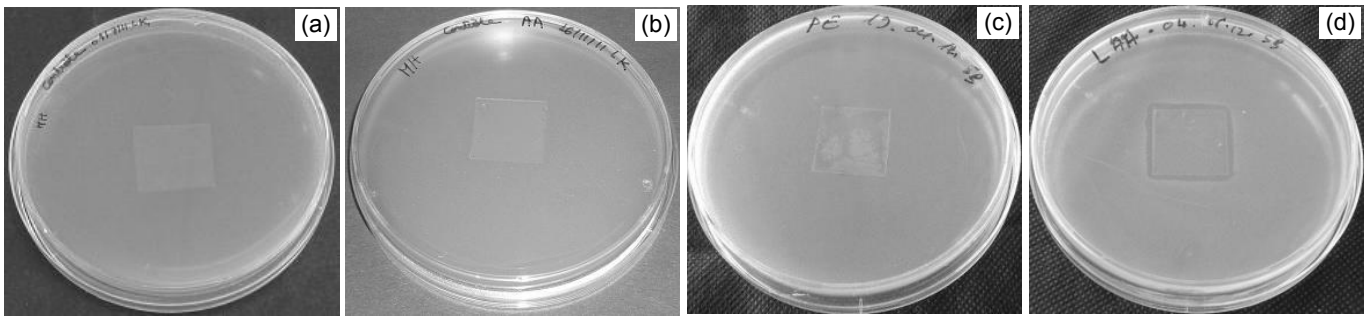

Figure 7. Antibacterial activity assay of nisin-functionalized films against *Listeria innocua:* control samples: native (a) and AA treated films (b); nisin-functionalized samples: native film + nisin (c) and AA treated film + nisin (d).

Conclusions

Advances using interfacial techniques permitted so far to study the chemical composition, molecular structure, surface coverage and topography of materials without being able to clearly correlate this information with the peptides orientation and conformation on surfaces. This work allowed us by combining ToF-SIMS and XPS techniques to understand nisin adsorption behavior on a hydrophobic surface, the native LDPE and on a hydrophilic surface, after AA treatment. Those observations were clearly associated with the detected antibacterial activity.

From our results, we can conclude the following:

i. On the hydrophilic surface, nisin was detected at higher amount, it was more oriented by its hydrophilic side to the hydrophilic substrate and by its hydrophobic side to the outer layers of the peptide, it had a uniform distribution on the surface, it was able to

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

preserve its biologically active structure after adsorption and therefore its antibacterial activity.

ii. On the hydrophobic surface, nisin was detected at lower amount, it was more oriented by its hydrophobic side to the hydrophobic substrate and by its hydrophilic side to the outer layers of the peptide, it had a non-uniform distribution on the surface, it had a higher conformational change and therefore less functionality or antibacterial activity.

Nisin adsorption on AA treated LDPE films, would be very promising for setting up antibacterial surfaces, unlike its adsorption on native LDPE. A surface presenting localized antibacterial activity in some areas of the surface but not on all the surface, is not interesting nor effective for food packaging or for biomedical applications. Future advances in materials sciences, life sciences, biology, biomedical and food sectors rely on understanding the adsorption behavior of peptides and proteins as well as bacteria adhesion on surfaces and can benefit enormously from these developments. Further work can focus on combining similar interfacial techniques and using different types of peptides, proteins, surfaces and applications, as well as working on adsorption at controlled low concentatrions or self-assembled monolayers to provide an in-depth analysis of the phenomena at the interfaces.

Acknowledgements

This work was supported financially by the “Action de Recherche Concertée d’Initiative Régionale (ARCIR) de la région Nord Pas de Calais”(France) and by a PhD fellowship within the Erasmus Mundus Program. The Fonds Européen de Développement Régional (FEDER), CNRS, Région Nord Pas-de-Calais and Ministère de l’Education Nationale de l’Enseignement Supérieur et de la Recherche were also acknowledged for funding XPS/LEIS/ToF-SIMS spectrometers within the Pôle Régional d’Analyses de Surface.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

References

1 Appendini P, Hotchkiss JH. Review of antimicrobial food packaging. *Innovative Food Science & Emerging Technologies* 2002; **3**: 113–126.

2 Henry M, Dupont-Gillain C, Bertrand P. Conformation Change of Albumin Adsorbed on Polycarbonate Membranes as Revealed by ToF-SIMS. *Langmuir* 2003; **19**: 6271–6276.

3 Daeschel MA, Mcguire J. Interrelationships between protein surface adsorption and bacterial adhesion. *Biotechnol. Genet. Eng. Rev.* 1998; **15**: 413–438.

4 Anon. Nisin preparation: affirmation of GRAS status as a direct human food ingredient. *Fed. Regist.* 1988; **54**: 11247.

5 Benkerroum N, Sandine WE. Inhibitory Action of Nisin Against Listeria monocytogenes. *J. Dairy Sci.* 1988; **71**: 3237–3245.

6 Hurst A, in *Advances in Applied Microbiology* (Eds: Perlman D, Laskin AI) Academic Press, **1981**, pp. 85–123.

7 Delves-Broughton J, Blackburn P, Evans RJ, Hugenholtz J. Applications of the bacteriocin, nisin. *Antonie van Leeuwenhoek* 1996; **69**: 193–202.

8 Bower CK, Parker JE, Higgins AZ, Oest ME, Wilson JT, Valentine BA, Bothwell MK, McGuire J. Protein antimicrobial barriers to bacterial adhesion: in vitro and in vivo evaluation of nisin-treated implantable materials. *Colloids Surf. B Biointerfaces* 2002; **25**: 81–90.

9 Ryder MP, Schilke KF, Auxier JA, McGuire J, Neff JA. Nisin adsorption to polyethylene oxide layers and its resistance to elution in the presence of fibrinogen. *J. Colloid Interface Sci.* 2010; **350**: 194–199.

10 Bower CK, Daeschel MA, McGuire J. Protein Antimicrobial Barriers to Bacterial Adhesion. *J. Dairy Sci.* 1998; **81**: 2771–2778.

11 Somorjai GA. From surface materials to surface technologies. *MRS Bull.* 1998; **23**: 11–29.

12 Vickerman JC, Swift AJ, in *Surface Analysis – The Principal Techniques* (Ed: Vickerman JC) John Wiley & Sons Ltd., Chichester, **1997**, pp. 135–214.

13 Rohrer H. Scanning tunneling microscopy: a surface science tool and beyond. *Surf. Sci.* 1994; **299–300**: 956–964.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

14 Zhuang H, Gardella Jr. JA. Spectroscopic characterization of polymer surfaces. *MRS Bull.* 1996; **21**: 43–48.

15 Ratner BD. Advances in the analysis of surfaces of biomedical interest. *Surf. Interface Anal.* 1995; **23**: 521–528.

16 Ratner BD, Chilkoti A, Castner DG. Contemporary methods for characterizing complex biomaterial surfaces. *Clin. Mater.* 1992; **11**: 25–36.

17 Gupta B, Hilborn JG, Bisson I, Frey P. Plasma-induced graft polymerization of acrylic acid onto poly(ethylene terephthalate) films. *J. Appl. Polym. Sci.* 2001; **81**: 2993–3001.

18 Shirley DA. High-Resolution X-Ray Photoemission Spectrum of the Valence Bands of Gold. *Phys. Rev. B.* 1972; **5**: 4709–4714.

19 Scannell AGM, Hill C, Ross RP, Marx S, Hartmeier W, Arendt EK. Development of bioactive food packaging materials using immobilised bacteriocins Lacticin 3147 and Nisaplin®. *Int. J. Food Microbiol.* 2000; **60**: 241–249.

20 Lakamraju M, Mcguire J, Daeschel M. Nisin adsorption and exchange with selected milk proteins at silanized silica surfaces. *J. Colloid Interface Sci.* 1996; **178**: 495–504.

21 Song J, Gunst U, Arlinghaus HF, Vancso GJ. Flame treatment of low-density polyethylene: Surface chemistry across the length scales. *Appl. Surf. Sci.* 2007; **253**: 9489–9499.

22 Arnebrant T, Ivarsson B, Larsson K, Lundström I, Nylander T. Bilayer formation at adsorption of proteins from aqueous solutions on metal surfaces. *Prog. Colloid Polym. Sci.* 1985; **70**: 62–66.

23 Muramoto S, Brison J, Castner DG. Exploring the Surface Sensitivity of TOF-Secondary Ion Mass Spectrometry by Measuring the Implantation and Sampling Depths of Bi_n and C_{60} Ions in Organic Films. *Anal. Chem.* 2012; **84**: 365–372.

24 Sanni OD, Wagner MS, Briggs D, Castner DG, Vickerman JC. Classification of adsorbed protein static ToF-SIMS spectra by principal component analysis and neural networks. *Surf. Interface Anal.* 2002; **33**: 715–728.

25 Schilke KF, McGuire J. Detection of nisin and fibrinogen adsorption on poly(ethylene oxide) coated polyurethane surfaces by time-of-flight secondary ion mass spectrometry (TOF-SIMS). *J. Colloid Interface Sci.* 2011; **358**: 14–24.

26 Gross E, Morell JL. Structure of nisin. *J. Am. Chem. Soc.* 1971; **93**: 4634–4635.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

27 Ji R, Liew T, Chong TC. Effect of acrylic acid vapour on lubricated recording hard-disk media surfaces. *Appl. Surf. Sci.* 2006; **252**: 6683–6685.

28 Van De Ven FJM, Van Den Hooven HW, Konings RNH, Hilbers CW. NMR studies of lantibiotics. *Eur. J. Biochem.* 1991; **202**: 1181–1188.

29 Fletcher JS, Henderson A, Biddulph GX, Vaidyanathan S, Lockyer NP, Vickerman JC. Uncovering new challenges in bio-analysis with ToF-SIMS. *Appl. Surf. Sci.* 2008; **255**: 1264–1270.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

3. Conclusions

Our findings allowed to confirm the theoretical model predicted in literature and to show nisin orientation on hydrophilic and hydrophobic surfaces. The observed antibacterial activity confirmed the importance effects of such interactions on the performance of bioactive materials. New applications and perspectives were also found for ToF-SIMS technique increasing its potential for interfacial studies. Advances in this research field are highly dependent on the development of surface characterization methods too.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

CHAPTER III:

NISIN-ACTIVATED HYDROPHOBIC AND HYDROPHILIC SURFACES: ASSESSMENT OF PEPTIDE ADSORPTION AND ANTIBACTERIAL ACTIVITY AGAINST SOME FOOD PATHOGENS

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

1. Introduction

After showing the evidence of hydrophilic and hydrophobic interactions on surfaces, other debates remain in the literature on the antibacterial activity and the amount of nisin adsorbed on hydrophilic and hydrophobic surfaces.

The first part of this paper will address this debate in relation to our research findings.

The second part will assess the nisin-functionalized films for potential future food applications. The effectiveness of both packaging materials will be evaluated against some food pathogens and at refrigeration storage temperature.

The food pathogens used for this study will be briefly presented below because they are not detailed in the article:

Listeria monocytogenes is a Gram-positive, facultative anaerobic rod. It has become a major concern to the food industry because of its implication in several outbreaks of foodborne disease. Listeriosis, with a mortality rate of about 24%, can cause abortion and neonatal death in pregnant women and their fetuses and may lead to septicemia (blood poisoning) and meningitis in infants, elderly, and immuno-compromised persons (Farber and Peterkin, 1991). *L. monocytogenes* is ubiquitous in the environment and has been isolated from soils, plants, sewage, and water (Abee et al., 1994). It is extremely resistant, surviving refrigeration temperatures, low pH, and high salt concentrations (Boziaris and Nychas, 2006; Farber and Peterkin, 1991; Chihib et al., 2003; Neunlist et al., 2005). *L. monocytogenes* can be found in a wide variety of raw and processed foods. Various meat products, seafood, milk, soft cheeses, and other dairy products have been associated with *Listeria* contamination (Bower et al., 1995; Kaur et al., 2011).

Bacillus cereus is a Gram-positive, facultative anaerobic, spore-forming rod. There are two types of *B. cereus* food poisoning. The first type is caused by an emetic toxin produced by growing cells in the food and results in vomiting. The second type is caused by enterotoxins produced during vegetative growth of *B. cereus* in the small intestine and results in diarrhea. In a small number of cases both types of symptoms are recorded, probably due to the production of both types of toxins (Granum and Lund, 1997). Psychrotrophic strains have been implicated in outbreaks of food-borne illness and are

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

capable of producing enterotoxins (Beuchat et al., 1997). *B. cereus* is found in soil and groundwater and frequently on plants and animals at the point of harvest or slaughter. Several researchers have documented its presence in raw and processed meat, vegetables, rice, dairy products, dry dessert mixes, infant foods, spices and seasonings, and a wide range of ready-to-serve foods (Beuchat et al., 1997; Periago and Moezelaar, 2001).

Staphylococcus aureus is a Gram-positive, facultative anaerobic coccus that is often involved in food poisoning, due to staphylococcal enterotoxins being produced in foodstuffs (Hennekinne et al., 2012; Solano et al., 2013). The ingested toxins cause vomiting, diarrhea, nausea, abdominal cramps, and pain (Solano et al., 2013). *S. aureus* can be found in the air, dust, sewage, water, environmental surfaces, humans, and animals. Its growth and its enterotoxin production are inhibited at low temperatures (Hennekinne et al., 2012). Nevertheless, it remains a major cause of food poisoning because it can contaminate food products during preparation and processing and was associated with dairy, meat, and fish products (Solano et al., 2013).

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

2. Article III

Nisin-activated hydrophobic and hydrophilic surfaces: assessment of peptide adsorption and antibacterial activity against some food pathogens

Layal Karam[a,b], Charafeddine Jama[b], Anne-Sophie Mamede[c], Samir Boukla[a], Pascal Dhulster[a] and Nour-Eddine Chihib[a,1]

[a] Laboratoire ProBioGEM, Polytech'Lille, Université Lille1, Avenue Paul Langevin, 59655 Villeneuve d'Ascq, France

[b] Laboratoire UMET, UMR-CNRS 8207, Ecole Nationale Supérieure de Chimie de Lille, Université Lille 1, Avenue Dimitri Mendeleïev, 59655 Villeneuve d'Ascq, France

[c] Unité de Catalyse et de Chimie du Solide, UMR-CNRS 8181, Ecole Nationale Supérieure de Chimie de Lille, Université Lille 1, Avenue Dimitri Mendeleïev, 59655 Villeneuve d'Ascq, France

Abstract

An effective antimicrobial packaging or food contact surface should be able to kill or inhibit micro-organisms that cause food-borne illnesses. Setting-up such systems, by nisin adsorption on hydrophilic and hydrophobic surfaces, is still a matter of debate. For this purpose, nisin was adsorbed on two types of low density polyethylene (LDPE): the hydrophobic native film and the hydrophilic acrylic acid (AA) treated surface. The antibacterial activity was compared for those two films and it was highly dependent on the nature of the surface and the nisin adsorbed amount. The hydrophilic surfaces presented higher antibacterial activity and higher amount of nisin than the hydrophobic surfaces. The effectiveness of the activated surfaces was assessed against *Listeria innocua* and the food pathogens *Listeria monocytogenes, Bacillus cereus* and *Staphylococcus aureus*. *Staphylococcus aureus* was more sensitive than the three other test bacteria toward both

[1] Corresponding author. Fax: +33 3 28 76 73 81 (N.E. Chihib).
Email address: Nour-Eddine.Chihib@univ-lille1.fr (N.E. Chihib).

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

nisin-functionalized films. Simulation tests to mimic refrigerated temperature showed that the films were effective at 20 and 4°C with no significant difference between the two temperatures after 30 min of exposure to culture media.

Key-words: Antibacterial activity, nisin adsorption, *Listeria*, *Bacillus*, *Staphylococcus*, hydrophilic surface.

Introduction

Food-borne diseases have been and remain a worldwide major concern. Active antimicrobial packaging is one of the innovative food packaging concepts that have been introduced as a response to the continuous changes in current consumer demands and market trends (Quintavalla and Vicini 2002). It acts to reduce inhibit or retard the growth of micro-organisms that may be present in the packed food or packaging material itself (Appendini and Hotchkiss 2002).

As a part of hurdle technology (Leistner 2000), active food packaging in combination with other hurdles such as low temperature, present a proactive approach to increase the shelf-life of foods and reduce the risk of food pathogens contamination related to minimally processed, easily prepared and ready-to-eat 'fresh' products. One way of setting-up such types of packaging is to adsorb antimicrobial peptides on surfaces.

Nisin, a bacteriocin produced by strains of *Lactococcus lactis* has found practical applications in the food industry (Delves-Broughton et al. 1996) because of its bactericidal effect against a broad range of Gram-positive bacteria, including many species of *Listeria*, *Staphylococcus* and spore-forming bacteria like *Bacillus* and *Clostridium* (Jack et al. 1995; Pol and Smid 1999).

Despite the fact that nisin has been used for decades as a food preservative and studied thoroughly in literature, its adsorption on hydrophobic and hydrophilic surfaces is still a matter of debate. Due to its amphiphilic nature, nisin is able to adsorb on both types of surfaces and retain stable activity in the adsorbed state (Daeschel et al. 1992). Disagreements were reported on the nisin' antibacterial activity when adsorbed on hydrophilic or hydrophobic films (Leung et al. 2003; Daeschel and Mcguire 1998; Bower et al. 1998; Bower et al. 1995a and b). Some reports agreed on the antibacterial activity of the two kinds of films but disagreed on the amount of peptides adsorbed on each surface (Bower et al. 1995a; Lakamraju et al. 1996; Daeschel et al. 1992). Thus, two questions arise and remain not clearly answered. Does nisin have a better antibacterial activity on

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

hydrophilic or hydrophobic surfaces? And does nisin adsorb in a higher amount on hydrophilic or hydrophobic surfaces?
Moreover, it has been reported that nisin's effectiveness depends on temperature (Abee et al. 1994; Thomas and Wimpenny 1996; Beuchat et al. 1997; Pol and Smid 1999; Periago and Moezelaar 2001). The third question is: are the nisin-functionalized films effective at refrigeration temperature?

The major objective of this study has been to assess the antibacterial activity of two nisin-activated hydrophilic and hydrophobic films and to study how this activity was correlated to the nature of the surface and to the amount of nisin adsorbed on surfaces. The effectiveness of both packaging materials was also evaluated against different food pathogens and at refrigeration temperature.

Materials and methods

Film preparation

Low density polyethylene (LDPE) (Polimeri Europa, France SAS), commonly used in the food packaging sector, was cut into 2 x 2 cm^2 and washed with ethanol in an ultrasonic bath to remove possible dusts, oily compounds or any chemicals and wetting agents absorbed on the film surface, then it was dried in an oven at 55°C for 3 h. Those films were either used directly or treated for nisin adsorption.

Acrylic acid (AA) treatment

In order to elaborate hydrophilic surfaces, the native LDPE, hydrophobic in nature, was pre-treated by Ar/O_2 (95/5%) plasma and subsequently grafted with acrylic acid (AA) monomers (99.5%, Acros Organics, Belgium) as described in our previous work (Karam et al. 2013). The amount of grafting on the AA treated film was determined using Toluidine Blue O method (Gupta et al. 2001). The surface concentration of AA was calculated according to a mole-to-mole complex between the dye and carboxylic acid groups and was recorded at 6.5 ± 0.6 nmol/cm². The water contact angle decreased from 101.8 ± 1.4° to 44.2 ± 2.1° after surface treatment. Therefore, in the present study, the native LDPE was used as hydrophobic surface and the AA treated film as hydrophilic one.

© 2013 Tous droits réservés.
http://doc.univ-lille1.fr

Nisin preparation

A pure grade of nisin A was donated by Danisco (Beaminster Dorset, United Kingdom). Activity was indicated as 5.2 x 10^7 IU/g. Nisin solutions were prepared by dissolving 1.0 mg/ml of nisin in HCl (0.01 M). Solutions were freshly prepared and filtered (0.22 μm) before each experiment.

Nisin adsorption on films

Nisin adsorption was carried out on native and AA treated films. Each film was immersed in 20 ml of nisin solution (1.0 mg/ml) and it was agitated at 8°C for 16 h. After that, the samples were removed from solution and briefly rinsed with sterile distilled water to remove non adsorbed nisin. All the tests were done after drying the films in sterile Petri dishes at 25°C for 24 h.

X-ray photoelectron spectroscopy (XPS)

XPS experiments were carried out using a Kratos Analytical AXIS Ultra^{DLD} spectrometer (United Kingdom). A monochromatized aluminium source (Al Kα = 1486.6 eV) was used for excitation. The analyzer was operated in constant pass energy of 40 eV using an analysis area of approximately 700 μm x 300 μm. Charge compensation was applied to compensate for the charging effect occurring during the analysis. The C 1s hydrocarbon (285.0 eV) binding energy (BE) was used as internal reference. The spectrometer BE scale was initially calibrated against the Ag $3d_{5/2}$ (368.2 eV) level. Pressure was in the 10^{-10} Torr range during the experiments. Quantification and simulation of the experimental photopeaks were carried out using CasaXPS software. Quantification took into account a non-linear Shirley background subtraction (Shirley 1972).

Nisin quantification by bicinchoninic acid (BCA) assay

The amount of nisin adsorbed on the films was determined by release test. Each film was placed in sterile HCl (0.01 M) under slight shaking for 72 h. Preliminary experiments were done to verify that no additional amount of nisin was released after 72 h and the dried films

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

showed no remaining antibacterial activity after the desorption assay (data not shown). The concentration of nisin released in solution was quantified using a colorimetric method BCA (QBCA, Sigma, France) (Wiechelman et al. 1988; Smith et al. 1985). 500 µL of sample and 500 µL of QBCA reagent were placed in micro-centrifuge tubes, mixed and incubated for 1 h at 60°C. The absorbance was measured at 562 nm within 10 min using a UV-VIS spectrophotometer (UVmini-1240, Shimadzu, Japan). Triplicate tests were performed for each sample. The calibration curve used for the determination of nisin concentration had the following equation:

$\text{Absorbance}_{(562\ nm)} = 0.0326\ [\text{nisin}_{\mu g/ml}]$

Bacterial cultures and growth conditions

Listeria innocua LMG 11387 (BCCM, Belgium), *Listeria monocytogenes* ATCC 35152 (LM/NCTC, United Kingdom), *Bacillus cereus* ATCC 14579 (LGC Standards, France) and *Staphylococcus aureus* CIP 4.83 (CRBIP, France) were used in this study. Pre-cultures were performed by inoculating a single colony in 10 ml of Brain Heart broth (Biokar Diagnostics, France). The cultures were made by inoculating 10 ml of Brain Heart broth with 100 µl of the pre-culture. Pre-cultures and cultures were incubated at 37°C for 24 h.

Minimum inhibitory concentrations (MICs) assay

The MICs were determined using a microplate assay to test *L. innocua*, *L. monocytogenes*, *B. cereus* and *S. aureus* susceptibilities to nisin. Each well of a sterile 96 microtiter plate (Corning Costar 96-well Cell Culture Plates 3799; Corning Incorporated, USA) was filled with 100 µl of sterile Mueller Hinton broth (Biokar Diagnostics, France). The first column of wells received 100 µl of nisin solution. After mixing, 100 µl was transferred to the next column of wells in a process of two-fold serial dilutions to cover the nisin concentration range from 250 to 0.244 µg/ml. Each well was then inoculated with 100 µl of the bacterial culture diluted to approximately 10^6 CFU/ml in sterile Mueller Hinton broth. The microtiter plates were incubated at 37°C for 24 h. After the incubation period, the optical density (OD) was measured at 630 nm with a microplate reader (model MRX II; Thermo Labsystems, USA). A sterile Mueller Hinton medium incubated with the target bacterium (no antimicrobial agent) was used as a positive control of growth. A sterile Mueller Hinton medium incubated under the same condition (no inoculum, no antimicrobial agent) was

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

used as a blank. The MICs were calculated from the highest dilution showing complete growth inhibition of the tested micro-organisms (OD equals OD of the blank) (Neetoo et al. 2008; Turgis et al. 2012). Experiments were repeated four times.

Assessment of the antibacterial activity of nisin-functionalized films

Agar diffusion assay

The antibacterial tests of nisin-functionalized films were done using a modified agar diffusion assay (Scannell et al. 2000). Mueller Hinton agar medium (Biokar Diagnostics, France) was seeded with the indicator micro-organisms: *L. innocua, L. monocytogenes, B. cereus* and *S. aureus.* The face up of the film to be tested was placed on the agar surface. Bioassay plates containing experimental samples were kept at 4°C for 4 h to initiate nisin diffusion and were then incubated at 37°C for 24 h. Nisin activity was assessed as an inhibition of the indicator bacterium growth under and around the film.

Culturability loss test at 20 and 4°C

The culturability loss tests were carried out for both types of nisin-functionalized surfaces by putting a film in contact with 5 ml of cell suspension diluted to *ca.*10^6 CFU/ml. After 30 min of contact time at 20 and 4°C, samples were enumerated by making serial dilutions in tryptone-physiological salt solutions, followed by plating on Luria Bertani agar (Biokar Diagnostics, France) and incubation for 24 h at 37°C. In each experiment, a control of bacterial culture, without the test film, was realized under the same conditions. The results were expressed in terms of logarithm of the difference in population (DP) according to the equation (Song and Richard 1997): $\log DP = \log (N_0/N) = (\log N_0) - (\log N)$; where N_0 and N are, respectively, the bacterial population (CFU/ml) before and after exposure of bacteria culture to nisin-functionalized films for 30 min. Those experiments were made in triplicates and all the values were presented as a mean value +/- the standard deviation.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Results

Assessment of nisin adsorbed amount on surfaces

XPS analysis was performed to determine the chemical changes and the amount of nisin adsorbed on the surfaces. The chemical composition of the films, expressed as atomic concentrations was presented in figure 1.

The results showed that the native film contained 99.4% of C and traces of O impurities. The AA treated films showed an increase in oxygen atomic concentration from 0.6 to 14.5% due to the formation of oxygenated functional groups after the grafting process. After nisin adsorption on both surfaces, XPS analysis revealed two new contributions of N and S (Fig. 1). Unlike the bare substrates, nisin contains nitrogen and sulfur which were used to identify and quantify the presence of the peptide on the surface. Nitrogen is related to the peptide's amino acids and sulfur is the signature of methionine lateral chain and lanthionine sulphur bridges, constituting nisin molecule. Hydrophilic AA treated films exhibited higher concentrations of N and S and therefore higher amount of nisin than the hydrophobic native films (Fig. 1).

The decomposition of the carbon C 1s peak brings another proof of surface treatment and nisin adsorption by providing details on the chemical bonds environment and the functional groups available on the surface. This peak can be decomposed, according to the surface composition, into the following four components: i) the first at 285.0 eV assigned to carbon only bound to carbon and/or hydrogen, the second at 286.2 eV assigned to carbon making a single bond with oxygen or nitrogen, the third at 288.1 eV assigned to carbon making two single bonds or one double bond with oxygen or nitrogen and the fourth at 289.3 eV associated to carbon making one double bond and one single bond with oxygen and attributed to carboxyl functions (Rouxhet et al. 1994). The native film contained only carbon bound to carbon and/or hydrogen (C–C, C-H) consistent with the chemical structure of LDPE films (Fig. 2b). After AA treatment, oxygenated functional groups were obtained as C-O, C=O, O=C-O and were associated to the effect of the plasma treatment and acrylic acid grafting (Fig. 2d). After nisin adsorption, all nisin characteristic functions were observed on the films. Those functions are mainly C-C, C-H, C-N, C-O and the amide peptide bond O=C-N (Fig. 2a). Only an additional O=C-O function was detected on the AA nisin-treated films and can be due to the AA substrate contribution to the final signal (Fig. 2e). The component at 288.1 eV characteristic of carbon in peptide bonds was also at higher percentage on the hydrophilic films as compared to hydrophobic ones (Fig.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

2c,e). We can conclude that XPS permitted to confirm nisin adsorption and to determine its amount on each type of surface.

The amount of nisin adsorbed on surfaces was also indirectly calculated by BCA method by releasing the peptide adsorbed on the surfaces in HCl at pH 2. Nisin was recorded at 38.2 ± 2.6 µg on the hydrophilic film versus 14.7 ± 0.7 µg on the hydrophobic one and those release test values were in accordance with the recorded amounts by XPS.

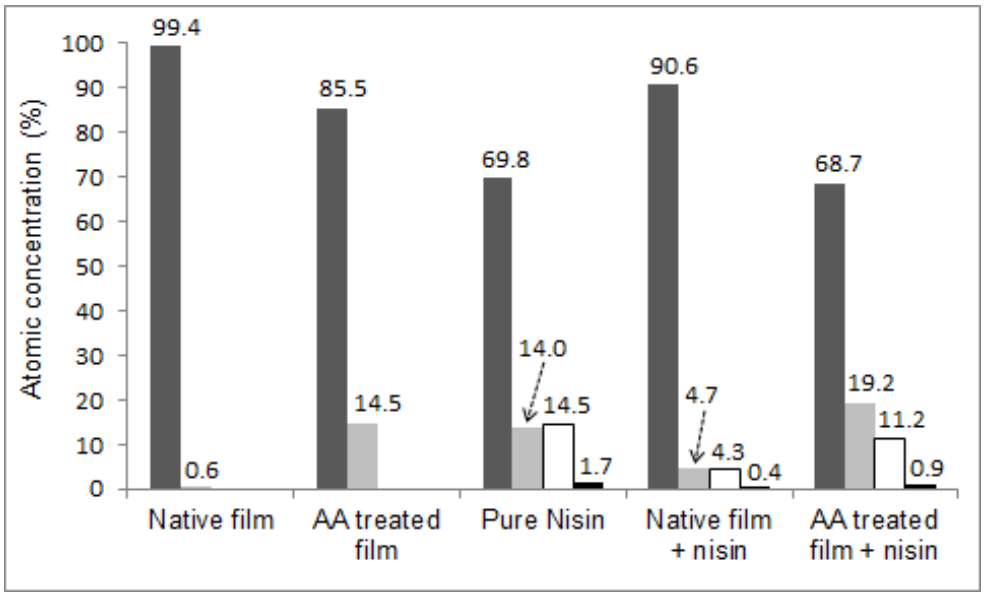

Fig. 1 Surface elemental composition from XPS analysis of pure nisin, native and AA treated films, before and after nisin adsorption: C (■), O (■), N (□), and S (■)

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

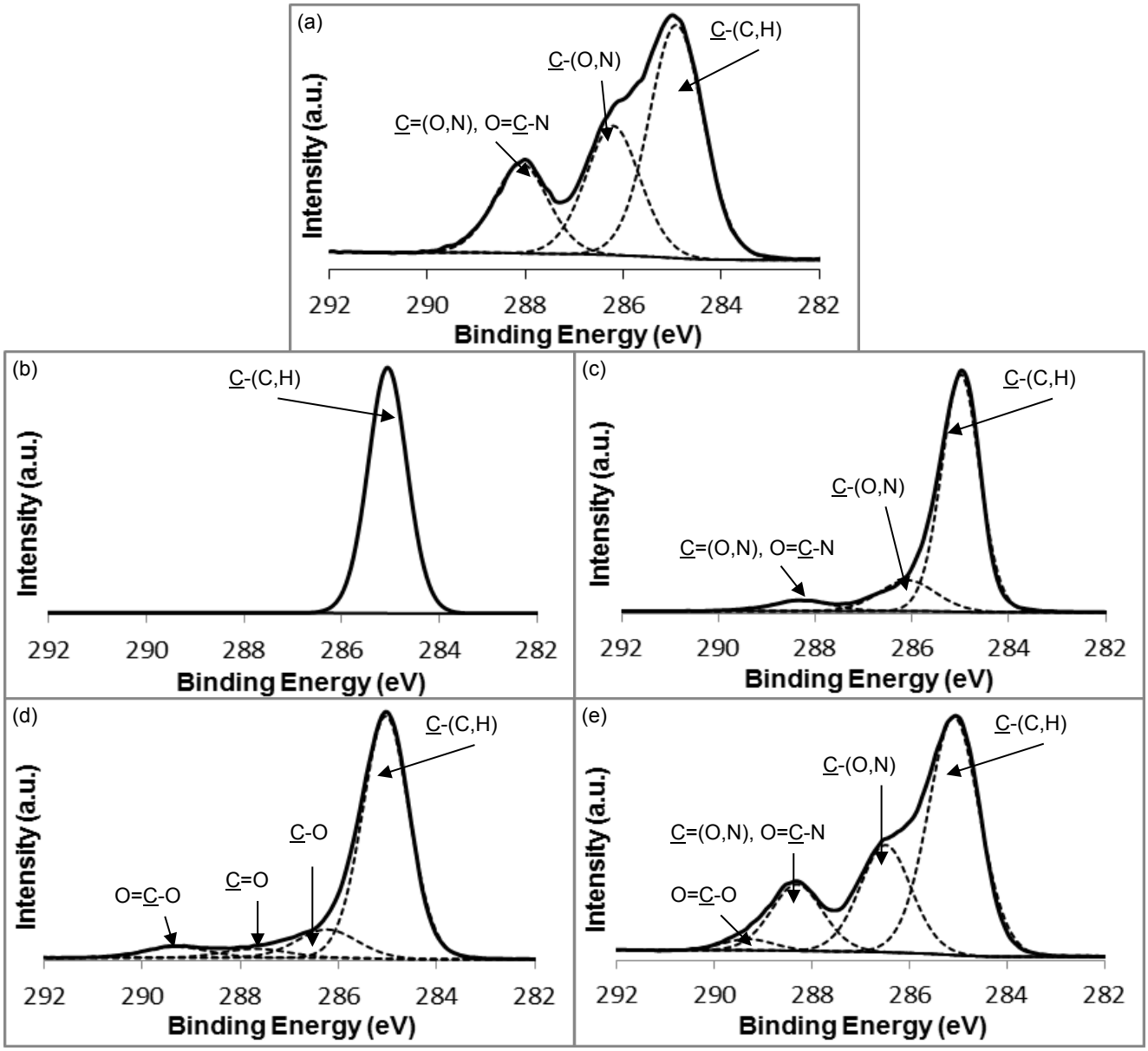

Fig. 2 C 1s XPS spectra of pure nisin (a), native film (b), native film + nisin (c), AA treated film (d), AA treated film + nisin (e). The different chemical functions contributing to the signal are indicated by arrows. The spectra were normalized to obtain the maximum of the main peak at the same height

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Assessment of the antibacterial activity of nisin-functionalized films

The sensitivity of *L. innocua*, *L. monocytogenes*, *B. cereus* and *S. aureus* toward nisin was first evaluated by determining the MIC values (Table 1). Then, the antibacterial activity of nisin-functionalized films was assessed by the agar diffusion assay against the listed micro-organisms (Table 2 and Fig. 3). It was affected by the types of both the surface and the test bacteria. The control films had no antibacterial activity since no inhibition was observed for native and AA treated films (data not shown). However, after nisin adsorption, native LDPE films (hydrophobic) showed irregular localized antibacterial activity on different regions under the film against the four tested bacteria (Fig. 3a, b, c, d). By contrast, the AA treated films (hydrophilic) showed in the four cases, higher and uniform activity with clear inhibition under and around the films (Fig. 3e, f, g, h). Moreover both films showed differences in the antibacterial activity against the tested bacteria and food pathogens. The sensitivity of tested bacteria was highlighted by an increase of the localized irregular inhibition area for the hydrophobic surfaces (Fig. 3a, b, c, d) and by an increase of zones of inhibition around the films for the hydrophilic surfaces (Table 2). *S. aureus* showed the highest sensitivity toward nisin-treated films as compared to the three other test micro-organisms. Although no significant difference was observed amongst *L. innocua, L. monocytogenes and B. cereus*, the general observed bacterial sensitivity to nisin-functionalized films can be ranked from the highest to the lowest sensitivity as following: *S. aureus* > *B. cereus* > *L. monocytogenes* > *L. innocua.*

Table 1 Minimum inhibitory concentrations (MICs) of nisin against *L. innocua, L. monocytogenes, B. cereus* and *S. aureus*

Test bacteria	**MIC (µg/ml)**
L. innocua	7.8
L. monocytogenes	7.8
B. cereus	7.8
S. aureus	3.9

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Table 2 Antibacterial activity against various types of bacterial populations, after nisin adsorption on native and AA treated films[a]

Test bacteria	Native film + nisin	AA treated film + nisin
L. innocua	0	1.9 ± 0.2
L. monocytogenes	0	2.0 ± 0. 1
B. cereus	0.4 ± 0.7	2.3 ± 0.2
S. aureus	0.9 ± 0.4	4.5 ± 0.4

[a] Values are expressed as zones of inhibition (mm) around the films, followed by their standard deviation

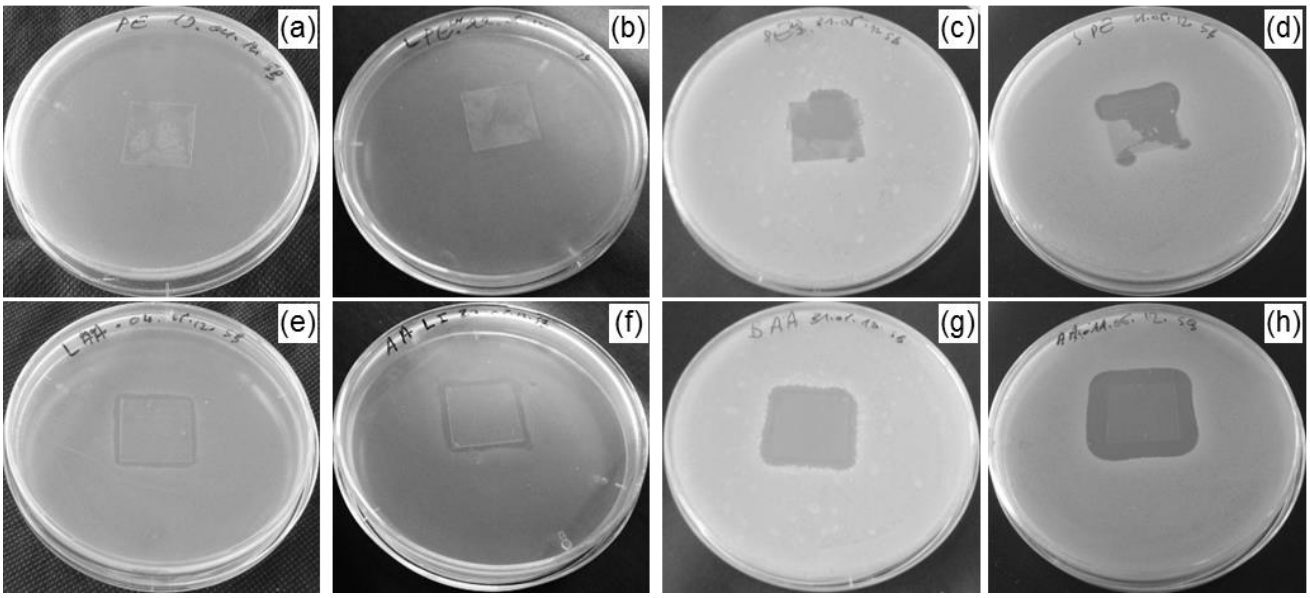

Fig. 3 Antibacterial activity assay of native films + nisin against *L. innocua* (a), *L. monocytogenes* (b), *B. cereus* (c), *S. aureus* (d) and of AA treated films + nisin against *L. innocua* (e), *L. monocytogenes* (f), *B. cereus* (g), *S. aureus* (h)

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Assessment of the antibacterial activity of nisin-functionalized films at 4°C against some food pathogens

In order to evaluate the effectiveness of both types of nisin-functionalized films at refrigeration temperature, the culturability loss test was performed at 4°C and compared to the control one at 20°C and this is against *L. innocua*, *L. monocytogenes* and *B. cereus*. Control experiments were also carried out with no addition of nisin-films and showed a stable viable count over the 30 min of incubation in all cases (data not shown). At 20°C, native and AA treated films induced, respectively, 0.5-0.7 and 1.2-1.6 log reduction of the viable count of *L. innocua*, *L. monocytogenes* and *B. cereus* populations (Fig. 4). At 4°C, a slight but not significant extra reduction of 0.2-0.4 and of 0.1-0.2 log units was recorded respectively on the native and AA treated films for the same bacterial populations (Fig. 4). These results showed also the higher antibacterial activity of the hydrophilic surfaces versus the hydrophobic ones since the AA treated films showed higher bactericidal effect than the native ones at both temperatures and for all the bacterium tested (Fig. 4).

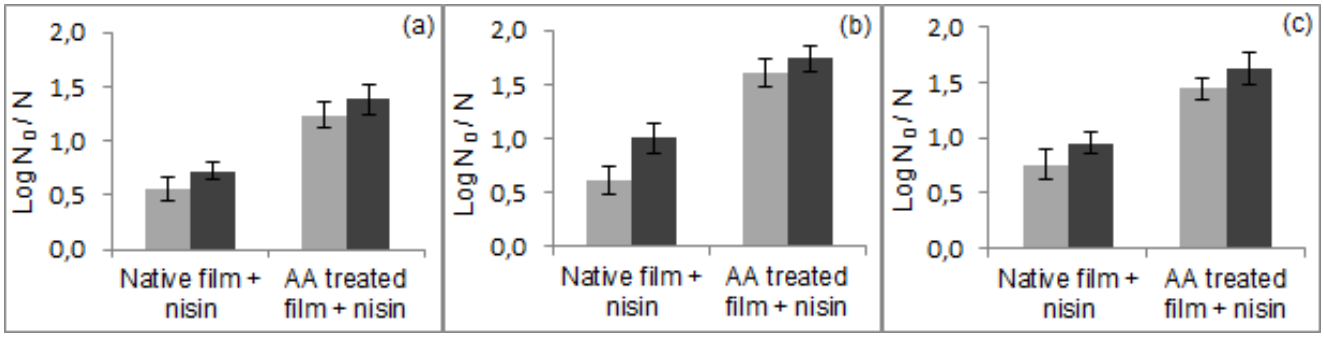

Fig. 4 Effect of nisin-functionalized films on the culturability loss of *L. innocua* (a), *L. monocytogenes* (b) and *B. cereus* (c) at 20 (■) and 4°C (■). N_0 and N are, respectively, the CFU/ml before and after exposure of bacteria culture to nisin-functionalized films for 30 min. Error bars represent the standard deviation of the mean of three experiments

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Discussion

Our findings showed that the activity of the activated films was directly correlated to both the nature of the film and the amount of nisin adsorbed on the surfaces.

It is now established that the nature of the film determines the types of interactions that govern peptide adsorption on the surfaces. On the hydrophilic surfaces, the hydrophilic interactions as electrostatic and hydrogen bonding are predominant (Lakamraju et al. 1996). On the hydrophobic surfaces, and due to the presence of hydrophobic regions in nisin molecule, hydrophobic interactions are the main driving forces for adsorption (Daeschel et al. 1992). Those types of interactions can significantly impact the antimicrobial activity of surfaces (Daeschel and Mcguire 1998). Our results were in disagreement with Leung et al. (2003) who found that the highest antibacterial activity was observed on the most hydrophobic nisin-coated films. However, these results were in accordance with Daeschel and Mcguire (1998); Bower et al. (1998; 1995a and b) who reported that antimicrobial activity of adsorbed nisin depended upon surface hydrophobicity, with surfaces of low hydrophobicity retaining more nisin activity than the more hydrophobic ones. Kim et al. (2002) also found an increase in the antibacterial activity against *L. monocytogenes* when nisin was adsorbed onto a hydrophilic surface. This higher activity on the hydrophilic surfaces can be explained by nisin conformation on surfaces. As with most proteins, nisin undoubtedly experiences some degree of distortion at the molecular level when it adsorbs. Nisin is an amphiphilic molecule with predominantly hydrophobic sections and would possibly experience a larger change in conformation when adsorbing on a hydrophobic surface than when adsorbing on a hydrophilic one (Bower et al. 1995a). In addition, if greater molecular distortion occurred when nisin adsorbed to a hydrophobic surface, then a lower effectiveness of nisin might be expected (Bower et al. 1995b). Therefore, surface-induced changes in conformation may account for the lower activity of nisin exhibited on hydrophobic surfaces.

Moreover, our results showed that the amount of adsorbed nisin was higher on the hydrophilic surface than on the hydrophobic one, as determined by both XPS and BCA methods. These results were in agreement with Lakamraju et al. (1996) and Daeschel et al. (1992) findings. However, Bower et al. (1995a) reported that the low-hydrophobicity surfaces generally displayed more nisin activity than higher-hydrophobicity surfaces,

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

despite the finding that proteins adsorbed in greater amounts on the more hydrophobic surfaces (Bower et al. 1995a; Elwing et al. 1988).

The nisin adsorbed amount on the surface can be related to the nature of the observed interactions between the peptide and the surface and to the overall molecule size. It has been reported that on a hydrophobic support and due to hydrophobic interactions; hydrophobic domain of the peptide may be oriented toward the hydrophobic support and the hydrophilic domain having less contact. The inverse would be observed on hydrophilic surfaces, due to hydrogen bonding or electrostatic interactions (Lakamraju et al. 1996; Karam et al. 2013). The overall dimensions of the molecule, modeled as a cylinder, are about 5 nm in length (side hydrophobic domain) and 2 nm in diameter (end hydrophilic domain) (Goodman et al. 1991). Therefore, a side-on adsorption (adsorption sites of 5 x 2 nm) on the hydrophobic surface (Lakamraju et al. 1996), with an extended structure, covering a relatively large area of the surface (Daeschel and Mcguire 1998), would provide less amount of adsorbed nisin. However, an end-on adsorption (adsorption sites of 2 x 2 nm) on the hydrophilic film (Lakamraju et al. 1996), with a more closely packed arrangement on the surface, would give a larger amount of the peptide on the surface. But how the nisin adsorbed amount can be correlated to the observed antibacterial activity? Nisin mode of action and our data obtained by XPS and BCA release tests can answer this. To exert its antimicrobial effect, the nisin molecule must first desorb from the surface and then cross the cell membrane (Bower et al. 1995a). Therefore the amount of nisin available for antibacterial activity can be related to the amount of nisin able to desorb from the surfaces. Consequently, the higher activity observed on the hydrophilic films can be also associated to the higher amounts of nisin adsorbed and released from those surfaces as compared to the hydrophobic ones.

Our results showed also that nisin-functionalized films were active against *L. innocua* and the three food pathogens *L. monocytogenes*, *B. cereus* and *S. aureus*. Moreover, *S. aureus* showed higher sensitivity than the other test bacteria toward both types of films. This can be related to the lower MIC value recorded for this pathogen as compared to the three other micro-organisms that had similar higher values (Table 1). Moreover, food pathogens like *L. monocytogenes* as well as some strains of *B. cereus* are able to survive and grow at refrigeration temperatures (Farber and Peterkin 1991; Beuchat et al. 1997). And since nisin activity is affected by temperature changes (Abee et al. 1994; Beuchat et al. 1997; Pol and Smid 1999), the films' antibacterial activity was evaluated at

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

refrigeration temperature against *L. monocytogenes* as psychrotrophic bacteria, against *B. cereus* and against *L. innocua* which was used as a target bacterium throughout this study. *S. aureus* was of less concern for this test because it is known that its growth and its enterotoxin production are inhibited at low temperatures (Hennekinne et al. 2012). The present work showed that despite the large inoculum charge used, both types of nisin-activated films were effective at refrigeration temperature after 30 min of incubation time. A general trend of activity increase was observed at this temperature but no significant difference between 20 and 4°C can be established (Fig. 4). Our work preliminary results provide the possibility of combining the antibacterial surfaces with refrigerated storage but will need to be further studied for longer storage period and in the food systems where they are intended to be applied.

Acknowledgements

This work was supported financially by the "Action de Recherche Concertée d'Initiative Régionale (ARCIR) de la région Nord Pas de Calais"(France) and by a PhD fellowship within the Erasmus Mundus Program. The Fonds Européen de Développement Régional (FEDER), CNRS, Région Nord Pas-de-Calais and Ministère de l'Education Nationale de l'Enseignement Supérieur et de la Recherche were also acknowledged for funding XPS spectrometers within the Pôle Régional d'Analyses de Surface.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

References

Abee T, Rombouts FM, Hugenholtz J, et al. (1994) Mode of Action of Nisin Z against *Listeria monocytogenes* Scott A Grown at High and Low Temperatures. Appl Environ Microbiol 60:1962–1968.

Appendini P, Hotchkiss JH (2002) Review of antimicrobial food packaging. Innovative Food Sci Emerging Technol 3:113–126. doi: 10.1016/S1466-8564(02)00012-7

Beuchat LR, Clavero MR, Jaquette CB (1997) Effects of nisin and temperature on survival, growth, and enterotoxin production characteristics of psychrotrophic *Bacillus cereus* in beef gravy. Appl Environ Microbiol 63:1953–1958.

Bower CK, McGuire J, Daeschel MA (1995a) Suppression of *Listeria monocytogenes* colonization following adsorption of nisin onto silica surfaces. Appl Environ Microbiol 61:992–997.

Bower CK, McGuire J, Daeschel MA (1995b) Influences on the antimicrobial activity of surface-adsorbed nisin. J Ind Microbiol 15:227–233. doi: 10.1007/BF01569829

Bower CK, Daeschel MA, McGuire J (1998) Protein Antimicrobial Barriers to Bacterial Adhesion. J Dairy Sci 81:2771–2778. doi: 10.3168/jds.S0022-0302(98)75835-7

Daeschel MA, Mcguire J, Al-Makhlafi H (1992). Antimicrobial activity of nisin adsorbed to hydrophilic and hydrophobic silicon surfaces. J Food Prot 55(9):731-735.

Daeschel MA, Mcguire J (1998) Interrelationships between protein surface adsorption and bacterial adhesion. Biotechnol Genet Eng Rev 15:413–438.

Delves-Broughton J, Blackburn P, Evans RJ, Hugenholtz J (1996) Applications of the bacteriocin, nisin. Antonie van Leeuwenhoek 69:193–202. doi: 10.1007/BF00399424

Elwing H, Welin S, Askendahl A, Lundström I (1988) Adsorption of fibrinogen as a measure of the distribution of methyl groups on silicon surfaces. J Colloid Interface Sci 123:306–308. doi: 10.1016/0021-9797(88)90249-4

Farber JM, Peterkin PI (1991) *Listeria monocytogenes*, a food-borne pathogen. Microbiol Rev 55:476–511.

Goodman M, Palmer DE, Mierke D, Ro S, Nunami K, Wakamiya T, Fukase K, Horimoto S, Kitazawa M, Fujita H, Kubo A, Shiba T (1991) Conformation of nisin and its fragments using synthesis, NMR and computer simulations. In: Jung G, Sahl H-G (eds) Nisin and Novel Lantibiotics. Escom, Leiden, p 59.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Gupta B, Hilborn JG, Bisson I, Frey P (2001) Plasma-induced graft polymerization of acrylic acid onto poly(ethylene terephthalate) films. J Appl Polym Sci 81:2993–3001. doi: 10.1002/app.1749

Hennekinne J-A, De Buyser M-L, Dragacci S (2012) *Staphylococcus aureus* and its food poisoning toxins: characterization and outbreak investigation. FEMS Microbiol Rev 36:815–836. doi: 10.1111/j.1574-6976.2011.00311.x

Jack RW, Tagg JR, Ray B (1995) Bacteriocins of gram-positive bacteria. Microbiol Rev 59:171–200.

Karam L, Jama C, Nuns N, Mamede AS, Dhulster P, Chihib NE (2013) Nisin adsorption on hydrophilic and hydrophobic surfaces: evidence of its interactions and antibacterial activity. J Pept Sci 19:377–385. doi: 10.1002/psc.2512

Kim Y-M, An D-S, Park H-J, et al. (2002) Properties of nisin-incorporated polymer coatings as antimicrobial packaging materials. Packag Technol Sci 15:247–254. doi: 10.1002/pts.594

Lakamraju M, Mcguire J, Daeschel M (1996) Nisin adsorption and exchange with selected milk proteins at silanized silica surfaces. J Colloid Interface Sci 178:495–504.

Leistner L (2000) Basic aspects of food preservation by hurdle technology. Int J Food Microbiol 55:181–186. doi: 10.1016/S0168-1605(00)00161-6

Leung PP, Yousef AE, Shellhammer TH (2003) Antimicrobial Properties of Nisin-Coated Polymeric Films as Influenced by Film Type and Coating Conditions. J Food Saf 23:1–12. doi: 10.1111/j.1745-4565.2003.tb00347.x

Neetoo H, Ye M, Chen H (2008) Potential antimicrobials to control *Listeria monocytogenes* in vacuum-packaged cold-smoked salmon pâté and fillets. Int J Food Microbiol 123:220–227. doi: 10.1016/j.ijfoodmicro.2008.02.001

Periago PM, Moezelaar R (2001) Combined effect of nisin and carvacrol at different pH and temperature levels on the viability of different strains of *Bacillus cereus*. Int J Food Microbiol 68:141–148. doi: 10.1016/S0168-1605(01)00461-5

Pol IE, Smid EJ (1999) Combined action of nisin and carvacrol on *Bacillus cereus* and *Listeria monocytogenes*. Lett Appl Microbiol 29:166–170. doi: 10.1046/j.1365-2672.1999.00606.x

Quintavalla S, Vicini L (2002) Antimicrobial food packaging in meat industry. Meat Sci 62:373–380. doi: 10.1016/S0309-1740(02)00121-3

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Rouxhet PG, Mozes N, Dengis PB, et al. (1994) Application of X-ray photoelectron spectroscopy to microorganisms. Colloids Surf., B 2:347–369. doi: 10.1016/0927-7765(94)80049-9

Scannell AG., Hill C, Ross R., et al. (2000) Development of bioactive food packaging materials using immobilised bacteriocins Lacticin 3147 and Nisaplin®. Int J Food Microbiol 60:241–249. doi: 10.1016/S0168-1605(00)00314-7

Shirley DA (1972) High-Resolution X-Ray Photoemission Spectrum of the Valence Bands of Gold. Phys Rev B 5:4709–4714. doi: 10.1103/PhysRevB.5.4709

Smith PK, Krohn RI, Hermanson GT, et al. (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85. doi: 10.1016/0003-2697(85)90442-7

Song H-J, Richard J (1997) Antilisterial activity of three bacteriocins used at sub minimal inhibitory concentrations and cross-resistance of the survivors. Int J Food Microbiol 36:155–161. doi: 10.1016/S0168-1605(97)01254-3

Thomas LV, Wimpenny JW (1996) Investigation of the effect of combined variations in temperature, pH, and NaCl concentration on nisin inhibition of *Listeria monocytogenes* and *Staphylococcus aureus*. Appl Environ Microbiol 62:2006–2012.

Turgis M, Vu KD, Dupont C, Lacroix M (2012) Combined antimicrobial effect of essential oils and bacteriocins against foodborne pathogens and food spoilage bacteria. Food Res Int 48:696–702. doi: 10.1016/j.foodres.2012.06.016

Wiechelman KJ, Braun RD, Fitzpatrick JD (1988) Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. Anal Biochem 175:231–237. doi: 10.1016/0003-2697(88)90383-1

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

3. Conclusions

This study allowed dealing with the debate remaining in literature on nisin adsorbed amount and antibacterial activity on hydrophilic and hydrophobic surfaces. Hydrophilic surfaces showed higher adsorbed amount of nisin and higher activity than hydrophobic ones and this is against four types of Gram-positive bacteria. On the basis of these observations, the potential applications of nisin may be more suited for the hydrophilic surfaces, providing efficient antibacterial systems. However, this does not exclude the hydrophobic surfaces that can still be used if they are combined with other control measures. One way of improving the antibacterial activity of native films is combining the low nisin concentrations on this film with other factors or antimicrobials for effective food preservation. Synergistic action of such combination (Pol and Smid, 1999; Razavi Rohani et al., 2011; Ter Steeg et al., 1999) enables using lower nisin concentrations, which is justified by economic and technological considerations but presents mainly the advantage of avoiding Gram-positive bacteria resistance to nisin (Singh et al., 2001). Antimicrobials' combinations or multi-preservation systems can also benefit both types of surfaces by increasing the hurdles for killing bacteria (Leistner and Gorris, 1995; Leistner, 2000) and by expanding the range of activity of nisin-treated films not only against Gram-positive bacteria but against Gram-negative ones too (Delves-Broughton et al., 1996). In addition, the pathogen sensitivity can play a significant role in the degree of effectiveness of the films; the same film can be more or less effective according to the choice of targeted microorganism. Consequently, higher or lower amount of nisin could be adsorbed on surfaces according to the sensitivity of bacteria or the type of food applications.

These findings may have important implications for the application of nisin and other bacteriocins, which have comparable mode of action, for setting up antibacterial surfaces.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

CHAPTER IV:

STUDY OF NISIN ADSORPTION ON PLASMA-TREATED SURFACES FOR SETTING UP ANTIMICROBIAL FOOD PACKAGING

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

1. Introduction

Nisin adsorption on hydrophobic and hydrophilic surfaces was studied in details in the previous two chapters. However, is the hydrophobic/hydrophilic character the only factor affecting such adsorption and surfaces' antibacterial activity?

Peptide interactions and adsorption on surfaces are surface phenomena and are governed by many factors among them the surface characteristics. Packaging materials and polymers possess good bulk and mechanical properties justifying their wide applications. Nevertheless, their surface properties need to be studied and tailored to improve their interactions with peptides. The purpose and the challenge are therefore to find a technology capable of changing the outermost surface properties while keeping the bulk material unaffected. Plasma treatments can perfectly match such requirements and can be used in many ways for surface modification (Chan et al., 1996; Gröning et al., 2001). Such treatments present also other advantages and can improve wettability, printability, dye uptake, sealability, peel strength, adhesion, barrier properties and mechanical resistance of materials (Chan et al., 1996; Ozdemir et al., 1999). These particular properties, highly desirable for food packaging applications, will be added to the antimicrobial functions of the packaging if plasma is used as a pre-treatment for peptide adsorption.

This article will address the following two main questions: (1) Can plasma treatments be used to study the factors affecting nisin adsorption and antibacterial activity on surfaces? (2) Can the plasma treatments improve such adsorption and be used thus for setting up antimicrobial packaging?

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

2. Article IV

Study of nisin adsorption on plasma-treated surfaces for setting up antimicrobial food packaging

Layal Karam[a,b], Charafeddine Jama[b], Anne-Sophie Mamede[c], Ahmad Fahs[d], Guy Louarn[d], Pascal Dhulster[a] and Nour-Eddine Chihib[a,1]

[a] Laboratoire ProBioGEM, Polytech'Lille, Université Lille1, Avenue Paul Langevin, 59655 Villeneuve d'Ascq, France

[b] Laboratoire UMET, UMR-CNRS 8207, Ecole Nationale Supérieure de Chimie de Lille, Université Lille 1, Avenue Dimitri Mendeleïev, 59655 Villeneuve d'Ascq, France

[c] Unité de Catalyse et de Chimie du Solide, UMR-CNRS 8181, Ecole Nationale Supérieure de Chimie de Lille, Université Lille 1, Avenue Dimitri Mendeleïev, 59655 Villeneuve d'Ascq, France

[d] Institut des Matériaux Jean Rouxel (IMN), CNRS-Université de Nantes, 2 rue de la Houssinière, 44322 Nantes, France

Abstract

Setting up antimicrobial food packaging by nisin adsorption on surfaces depends mainly on the surface properties and the surface treatments allowing the modification of such properties. In order to investigate the factors affecting such adsorption, the native low density polyethylene (LDPE) was modified using Argon/Oxygen (Ar/O_2) plasma, nitrogen (N_2) plasma and plasma-induced grafting of acrylic acid (AA). The films were studied by various characterization techniques. The chemical surface modification was confirmed by X-ray photoelectron spectroscopy (XPS), the wettability of the surfaces was evaluated by contact angle measurements, the surface charge was determined by the zeta potential measurements, and the changes in surface topography and roughness were revealed by atomic force microscopy (AFM). Nisin was adsorbed on the native and the modified

[1] Corresponding author. Fax: +33 3 28 76 73 81 (N.E. Chihib).
Email address: Nour-Eddine.Chihib@univ-lille1.fr (N.E. Chihib).

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

surfaces. The antibacterial activity, the nisin adsorbed amount, and the peptide distribution were compared for the four nisin-functionalized films. The highest antibacterial activity was recorded on the Ar/O_2 followed by AA then by N_2 treated films and the lowest activity was on the native film. The observed antibacterial activity was correlated to the type of the surface, hydrophobic and hydrophilic interactions, surface charge, surface topography, nisin adsorbed amount, and nisin distribution on the surfaces.

Keywords: Functional materials; surface properties; peptide adsorption; antibacterial properties; plasma functionalization.

1. Introduction

The constant lifestyle changes, internet purchasing, globalization of food trade, product shelf-life extension, and demand for natural, minimally processed, and ready-to-eat "fresh" food products present new major challenges for food safety and quality. Antimicrobial packaging materials can provide innovative and promising solutions to such challenges. They can effectively kill or inhibit the growth of micro-organisms and thus extend the shelf-life and enhance the safety and quality of packaged products [1].

Adsorption of peptides on surfaces can offer a possible way for setting up antimicrobial food packaging systems. Nisin is a peptide produced by *Lactococcus lactis* subsp. *lactis*. It exerts rapid bactericidal effects against a broad spectrum of Gram-positive bacteria and food pathogens, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus,* and *Clostridium botulinum* [2,3]. The bacteriocin has been widely used in the food industry as a safe and natural preservative [4] and has shown stable activity in the adsorbed state [5]. However, limited information is available on the interactions between the bacteriocin and the packaging films and peptide adsorption behavior on surfaces has not yet been sufficiently clarified. This behavior is largely controlled by the surface properties (type, composition, charge, topography, roughness, hydrophobic/hydrophilic character…). Therefore, studying those factors is fundamental to understand, control, and improve the adsorption behavior and the antimicrobial effectiveness of activated films. Plasma treatments have seen rapid growth in the past decade and can be utilized in many ways for modulating and modifying surface properties of materials [6]. They offer a valuable tool for introducing selectively different functionalities onto polymers [7]; which is required for adsorption and interactions studies. In addition, they are environmentally

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

friendly and can improve barrier and functional properties of food packaging materials without changing their desirable bulk properties [8].

The objectives of this study were then to evaluate the use of plasma surface modification to study nisin adsorption and antibacterial activity on the functionalized surfaces. Low density polyethylene (LDPE), a well-known packaging in the food sector, was treated with different types of plasma to generate different types of surfaces. Nitrogen (N_2) plasma, Argon/Oxygen (Ar/O_2) plasma, and plasma-induced grafting were used to introduce N-functionalities, O-functionalities, and acrylic acid (AA) monomers to the polymer surface. Nisin was then adsorbed on the native and the three modified surfaces. The surfaces were characterized by different methods before and after nisin adsorption.

2. Materials & Methods

2.1. Materials

Low density polyethylene (LDPE) was obtained from Polimeri Europa (France SAS). Pure water (HPLC grade) and acrylic acid (AA) monomers (99.5%) were supplied from Acros Organics (Belgium). A pure grade of nisin A was donated by Danisco, Beaminster Dorset (United Kingdom). *Listeria innocua* LMG 11387 was provided by BCCM (Belgium) and QBCA reagent by Sigma (France). Brain Heart broth, Mueller Hinton agar medium, and Luria Bertani agar were all purchased from Biokar Diagnostics (France).

2.2. Film preparation

LDPE films (70 μm thicknesses) were cut into 2 x 2 cm^2 and washed with ethanol in an ultrasonic bath to remove possible dusts, oily compounds or any chemicals and wetting agents absorbed on the film surface. They were then dried in an oven at 55°C for 3 h. Those films were either used directly or treated for nisin adsorption.

2.3. Surface treatments

The native LDPE films were modified using three types of surface treatments: Nitrogen (N_2) plasma, Argon/Oxygen (Ar/O_2) plasma, and plasma-induced grafting of acrylic acid (AA).

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Plasma treatments were performed in a radio-frequency cold plasma reactor of 350 liters capacity (Europlasma CD1200, Belgium) at an excitation frequency of 13.56 MHz. The preselected vacuum working pressure was 30 mTorr. Experimental designs were set to optimize plasma treatment parameters for each type of gaz. The operating conditions used for N_2 plasma were the following: gas flow rate of 500 sccm (standard cubic centimeter per minute), generator power of 300 Watts, and an exposure time of 300 s. The conditions retained for Ar/O_2 (95/5%) were: gas flow rate of 1000 sccm (standard cubic centimeter per minute), generator power of 420 Watts, and an exposure time of 245 s. The plasma-induced grafting of acrylic acid (AA) monomers was subsequent to the Ar/O_2 plasma treatment described above, as detailed in our previous work [9]. The amount of grafting on the AA treated film was determined using Toluidine Blue O dye test method [10].

2.4. Surface characterization

2.4.1. X-ray photoelectron spectroscopy (XPS)

XPS experiments were carried out using a Kratos Analytical AXIS Ultra^{DLD} spectrometer (United Kingdom). A monochromatized aluminium source (Al Kα = 1486.6 eV) was used for excitation. The analyzer was operated in constant pass energy of 40 eV using an analysis area of approximately 700 μm x 300 μm. Charge compensation was applied to compensate for the charging effect occurring during the analysis. The C 1s hydrocarbon (285.0 eV) binding energy (BE) was used as internal reference. The spectrometer BE scale was initially calibrated against the Ag $3d_{5/2}$ (368.2 eV) level. Pressure was in the 10^{-10} Torr range during the experiments. Quantification and simulation of the experimental photopeaks were carried out using CasaXPS software. Quantification took into account a non-linear Shirley background subtraction [11].

2.4.2. Contact angle measurements

Static contact angle measurements of the native and treated samples were carried out at room temperature on a Digidrop goniometer (GBX, France) using pure water. Triplicate tests were performed for the films and at least six different measurements were performed on each sample surface. The average values for contact angles and the standard deviation were then calculated.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

2.4.3. Zeta potential

The zeta potential measurements were performed using SurPASS Electrokinetic Analyzer (Anton Paar, France). The samples were studied inside the measuring cell in contact with the electrolyte (10^{-3} M KCl solution) at the constant value of pH 2, at which nisin adsorption occurred. Before each experiment, an intensive rinsing with the electrolyte solution was done. The zeta potential was calculated from the measured streaming potential using the Helmholtz-Smoluchowski equation and the Fairbrother-Mastin approach [12,13]. An average of at least three individual measurements for each sample was reported.

2.4.4. Atomic force microscopy (AFM)

AFM experiments were carried out using a Bruker Dimension 3100 microscope (USA). Topographical images of the films were realized by intermittent contact mode AFM, in air conditions, and at room temperature. In this mode, during scanning over the surface, the cantilever/tip assembly is sinusoidally vibrated by a piezo mounted above it, and the oscillating tip slightly taps the surface. We have used silicon probes with a rectangular cantilever and a tetrahedral tip. The cantilever used is a NCHV-A provided by Bruker, the lever is typically 125 μm long and the apex curvature radius is in the order of 10 nm. The spring constant of the cantilevers and the resonance frequency are respectively 42 N/m and 320 kHz. All images were collected with a resolution of 512 x 512 pixels and a scan rate of 1 Hz on two different regions of the films. Roughness measurements were performed with the Nanotec WSXM software (Spain). The root-mean-squared roughness (RMS) was measured from the analysis of the images at 1 μm x 1 μm scan size. RMS roughness calculation was based on the standard deviation of the Z values, representing the height value in nm between the lowest and the highest point within the given area.

2.5. Nisin preparation

Pure nisin activity was indicated as 5.2 x 10^7 IU/g. Nisin solutions were prepared by dissolving 1.0 mg/ml of nisin in HCl (0.01 M). Solutions were freshly prepared and filtered (0.22 μm) before each experiment.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

2.6. Nisin adsorption on films

Nisin adsorption was carried out on the native and the three treated films. Each film was immersed in 20 ml of nisin solution (1.0 mg/ml) and it was agitated at 8°C for 16 h. After that, the samples were removed from solution and briefly rinsed in sterile distilled water to remove non adsorbed nisin. All the tests were done after drying the films in sterile Petri dishes at 25°C for 24 h.

2.7. Assessment of the antibacterial activity of nisin-functionalized films

2.7.1. Bacterial culture and growth conditions

The antibacterial tests were carried out against *Listeria innocua*. Pre-cultures were performed by inoculating a single colony in 10 ml of Brain Heart broth. The cultures were made by inoculating 10 ml of Brain Heart broth with 100 µl of the pre-culture. Pre-cultures and cultures were incubated at 37°C for 24 h.

2.7.2. Qualitative assessment of the antibacterial activity

Qualitative antibacterial tests were done using a modified agar diffusion assay [14]. Mueller Hinton agar medium was seeded with the indicator micro-organism, *L. innocua*. The face up of the film to be tested was placed on the agar surface. Bioassay plates containing experimental samples were kept at 4°C for 4 h to initiate nisin diffusion and were then incubated at 37°C for 24 h. Nisin activity was assessed as an inhibition of the indicator bacterium growth under and around the film.

2.7.3. Quantitative assessment of the antibacterial activity

The quantitative inhibitory effect of the four nisin-functionalized films was carried out, at room temperature, by putting each film in 5 ml of *L. innocua* cell suspension of *ca.*10^6 CFU/ml. After 5 and 30 min of contact time, the samples were enumerated by plating onto Luria Bertani agar and incubating for 24 h at 37°C. In each experiment, a control test of *L. innocua* without the test film, was realized under the same conditions. The viable and culturable counts of *L. innocua*, were determined and used to assess the antibacterial activity of the films. Those experiments were made in triplicates and all the values were expressed as a mean value +/- the standard deviation.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

2.8. Determination of nisin adsorption amount and release from the films

The kinetics of peptide release from the films and the nisin adsorbed amount on the surfaces were determined by putting each film in sterile HCl (0.01 M) under slight shaking at room temperature. At time intervals of 0, 0.08, 0.5, 3, 6, 24, 48, and 72 h, nisin concentration was quantified in release solutions using a colorimetric method BCA [15,16]. 500 µl of sample and 500 µl of QBCA reagent were placed in micro-centrifuge tubes, mixed, and incubated for 1 h at 60°C. The absorbance was measured at 562 nm within 10 min using a UV-VIS spectrophotometer (UVmini-1240, Shimadzu, Japan). The percentage of nisin released from the films at different times was calculated from the ratio of nisin amount desorbed at each specific time to the total amount of nisin released after 72 h. The films removed from solutions after 72 h and dried, showed no remaining antibacterial activity (data not shown). Triplicate tests were performed for each sample. The calibration curve used for the determination of nisin concentration had the following equation: Absorbance $_{(562\ nm)}$ = 0.0326 [nisin $_{\mu g/ml}$].

3. Results and discussion

3.1. Surface characterization before nisin adsorption

The surface composition, hydrophobic and hydrophilic character, charge, topography, and roughness of the four types of films were characterized before nisin adsorption.

3.1.1. Surface composition

XPS was used to determine the chemical composition of the films and to confirm surface chemistry modification after plasma treatments. The elemental composition of the films, expressed as atomic concentrations was presented in table 1. The native film contained mainly C and traces of O impurities. After Ar/O_2 plasma and subsequent grafting of AA, an increase in oxygen concentration was observed on the films (Table 1). This can be associated to the created oxygen functional groups [7] and to the grafted acrylic acid chains [17]. Since both Ar/O_2 and AA treatments introduced oxygen to the surfaces, the surface concentration of AA was determined using the dye assay method based on a mole-to-mole complex between the dye and accessible carboxylic acid groups [10]. It was recorded at

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

6.5 ± 0.6 and 1.3 ± 0.1 nmol/cm², respectively, on the AA and the Ar/O_2 treated surfaces. This confirmed the higher surface density of COOH functions on the former film and thus the AA grafting.

Nitrogen plasma produced new N-functionalities but O was detected too (Table 1). The free radicals generated during a plasma treatment can react with residual oxygen in the plasma reactor. In addition, free radicals that remain on the polymer surface after the treatment can react with oxygen when the surface is exposed to the atmosphere. This is the reason why oxygen can be observed during and/or after nitrogen or non-oxygen plasma treatments [7]. Plasma treatments allowed thus to change the surface chemistry and to produce new functional groups on the surfaces.

Table 1

Surface chemical composition determined from XPS analysis of native and treated LDPE films.

LDPE films	**Atomic concentration (%)**		
	C	**O**	**N**
Native	99.4	0.6	–
N_2	83.7	9.8	6.5
AA	85.5	14.5	–
Ar/O_2	87.5	12.5	–

3.1.2. Surface hydrophilicity/hydrophobicity

The degree of hydrophilicity or hydrophobicity of native and treated surfaces can be obtained by water contact angle measurement. The results shown in table 2 indicated that the static water contact angle decreased clearly from 101.8° on the native surface to around 45° on the three modified surfaces. This increase in wettability can be essentially attributed, as observed with XPS, to the introduction of polar groups by oxygen-containing plasma, by nitrogen plasma, and by grafted AA chains that are hydrophilic in nature [8,17,18]. The water contact angles values permitted to illustrate the hydrophobic nature of the native film and the hydrophilic character of the three treated surfaces.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Table 2

Contact angles of native and treated LDPE films. The measurements were expressed as an average value +/- the standard deviation.

LDPE films	Contact Angle (°)
Native	101.8 ± 1.4
N_2	45.1± 2.2
AA	44.2 ± 2.1
Ar/O_2	46.1± 2.3

3.1.3. Surface charge

The magnitude and sign of the zeta potential at a given pH of the electrolyte solution provide important information about the materials' acid-base character. They allow the estimation of both the type and the amount of dissociable functional groups on the surfaces. The zeta potential values of native and treated films are shown in table 3. The native inert film presents no dissociating functional groups. This surface is charged in aqueous solutions by preferential adsorption of electrolyte cations or anions at low or high pH values [19], and recorded then high positive charge at pH 2 (Table 3). After Ar/O_2 and AA treatments, mostly anionic and acidic functional groups are generated [10,20]. At low pH, these functions as carboxylic acid are in their non-ionized form COOH and displayed therefore low positive and close to neutral charges [12,21] (Table 3). In contrast, when submitting the film to nitrogen plasma, cationic basic groups are created on the surface [22]. The introduced nitrogen functional groups are in their protonated form at acidic pH and provided then a highly positively charged surface [12] (Table 3).

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Table 3

Zeta potential of native and treated LDPE films. The measurements were expressed as an average value +/- the standard deviation.

LDPE films	Zeta potential (mV) at pH 2
Native	+ 33.0 ± 3.4
N_2	+ 38.9 ± 2.5
AA	+ 6.7 ± 1.2
Ar/O_2	+ 9.1 ± 4.0

3.1.4. Surface topography and roughness

The native LDPE film showed low surface roughness of 4 nm and clearly revealed a lamellar surface structure (Fig. 1a) indicating the presence of spherulites associated with such types of polymers [23]. A slight decrease in roughness to 3.3 nm and a weak morphology change were observed after nitrogen plasma (Fig. 1b). They can be related to plasma-induced chains scissions that made the lamellar structure less visible but kept the surface smooth and flat with no new defined structure. Nitrogen plasma is less aggressive than oxidizing plasmas [6,24]. After Ar/O_2 plasma treatment (Fig. 1d), the film presented a complete new morphology with granular structure and nano-scale texturing that can be mainly associated to the noble gas plasma Ar treatment [6]. The grain size on this film was in the range of 9-24 nm, which increased the surface roughness to 6 nm. After subsequent AA grafting (Fig. 1c), similar granular structure was observed with higher grain size in the range of 40-55 nm, which further increased the roughness to 8.6 nm. This suggested that the grafted AA chains form their own domains and morphologies at the surface as confirmed by other workers [17].

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

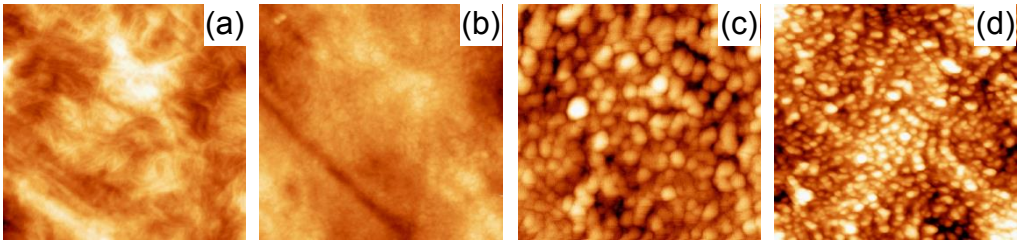

Fig. 1. Representative AFM topographic images of (a) native film (z-scale 30 nm), (b) N_2 treated film (z-scale 20 nm), (c) AA treated film (z-scale 60 nm), and (d) Ar/O_2 treated film (z-scale 30 nm). The scan size was 1 μm x 1 μm.

3.2. Study of nisin adsorption and antibacterial activity on surfaces

After nisin adsorption on surfaces, the antibacterial activity, the nisin adsorbed amount, and the peptide distribution were compared for the four nisin-activated films. An investigation was then carried out to understand the different factors that can affect such activity and nisin adsorption behavior.

3.2.1. Assessment of the antibacterial activity of nisin-functionalized films

3.2.1.1. Qualitative assessment of the antibacterial activity

The antibacterial activity of nisin-functionalized films was assessed qualitatively by the agar diffusion assay against *L. innocua* (Fig. 2). The control films had no antibacterial activity since no inhibition was observed for native, N_2, AA, and Ar/O_2 films (data not shown). However, after nisin adsorption, differences in antibacterial activity were observed among the films. The ranking order of this activity for the four types of surfaces was as following: Ar/O_2 > AA > N_2 > native films, as shown in figure 2. Moreover, the homogeneity of observed activity was evaluated. The native LDPE film displayed a spot-like irregular antibacterial activity in some points of contact between the film and agar plate and this inhibition was strictly confined under the film (Fig. 2a). The N_2 film presented a slight activity spread beyond the film perimeter and an almost complete inhibition under film, discontinued by few colonies growth (Fig. 2b). Both AA and Ar/O_2 films showed uniform activity with clear inhibition area under and around the films (Figs. 2c and d).

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

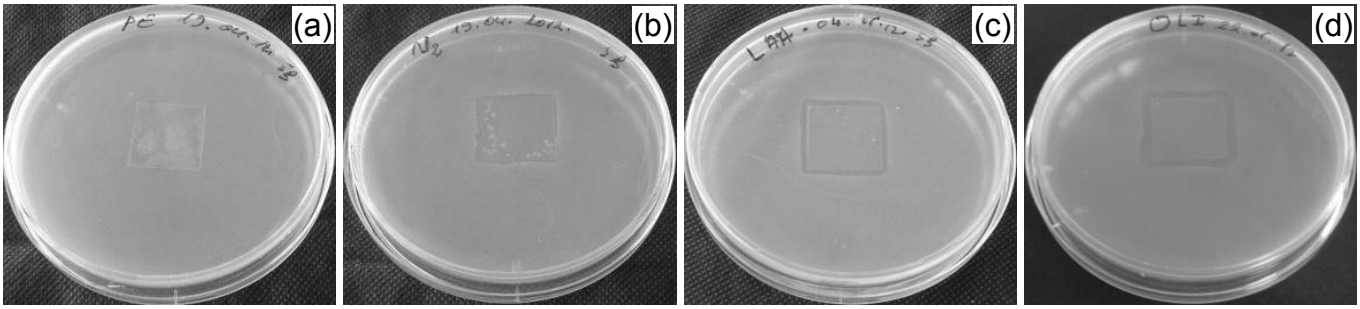

Fig. 2. Antibacterial activity assay against *L. innocua* of native films + nisin (a), N_2 treated film + nisin (b), AA treated film + nisin (c), and Ar/O_2 treated film + nisin (d).

3.2.1.2. Quantitative assessment of the antibacterial activity

The antibacterial activity was also investigated quantitatively by comparing the log reduction of the viable and culturable *L. innocua* cells after being in contact with the different films for 5 and 30 min. A rapid decline in the viable count occurred in the first 5 min of contact between the four tested films and the cell suspension and this inhibitory effect increased further at 30 min of incubation (Fig. 3). The native, N_2, AA, and Ar/O_2 films induced respectively 0.2, 0.5, 0.8, and 1.1 log reduction after 5 min and an increased log reduction of 0.5, 0.9, 1.2, and 1.6 after 30 min of contact time. Control culture with no addition of nisin-films showed a stable viable count along the experiment. As observed with the qualitative tests, the highest inhibitory activity was obtained for Ar/O_2 followed by AA, N_2, and native films. Moreover, both antibacterial tests showed that the three hydrophilic surfaces displayed higher activity than the native hydrophobic film. These results were in agreement with previously reported work that showed that nisin exhibited higher activity on hydrophilic surfaces than on hydrophobic ones [5,9,25-27]. Nisin would experience a larger change in conformation when adsorbing to a hydrophobic surface than when adsorbing to a hydrophilic one and this may affect its functionality [25]. However, this does not explain the difference in activity observed among the hydrophilic films that showed the same hydrophilicity character (Table 2).

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

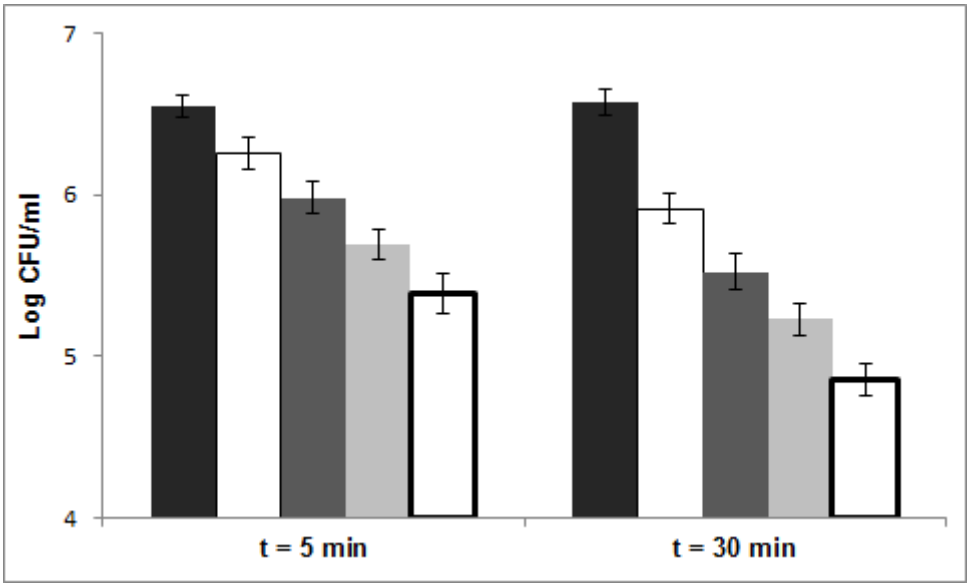

Fig. 3. Survival of *L. innocua* when the cell suspension is in contact with the nisin-functionalized films: control test *L. innocua* cell suspension incubated without the films (■), native film + nisin (□), N_2 treated film + nisin (■), AA treated film + nisin (■), and Ar/O_2 treated film + nisin (◘). Error bars represent the standard deviation of the mean of three experiments.

3.2.2. Nisin adsorption amount and release from the films

The bacteriocin test release was used to determine nisin kinetics of release from the films and its adsorbed amount on the surfaces. The four nisin-functionalized films exhibited similar release kinetics that included three stages as shown in figure 4. Nisin release was fast in the first stage between 5 min and 3 h and then it was slower in the second stage between 6 and 24 h until it leveled off and reached a steady state in the third stage between 48 and 72 h. During the first stage almost 30, 45, and 65% of nisin were released within 5 min, 30 min, and 3 h, respectively. During the second stage, this release was around 80 and 95% after 6 and 24 h, respectively. The third steady stage permitted to determine the total amount of nisin adsorbed on the surfaces. Therefore, the total adsorbed amount of nisin, as well as the amount recorded at each release time can be classified from the highest to the lowest according to the type of the surface, as following: Ar/O_2 > AA > N_2 > native films. This ranking had the same order of observed antibacterial activity on the films. Moreover, the initial burst release of nisin within 5 and 30 min can explain the rapid inhibitory effect of the films after 5 and 30 min of contact with the cell suspension, as shown in the

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

quantitative antibacterial test (Fig. 3). Consequently, the amount of nisin adsorbed on the surfaces was directly correlated to the observed antibacterial activity. This can also be associated to the hydrophilic and hydrophobic interactions since previous reports showed that nisin adsorbed in higher amount on hydrophilic as compared to hydrophobic surfaces [9,28]. But, again, those interactions are not able to explain the different adsorbed amounts recorded on the three hydrophilic surfaces.

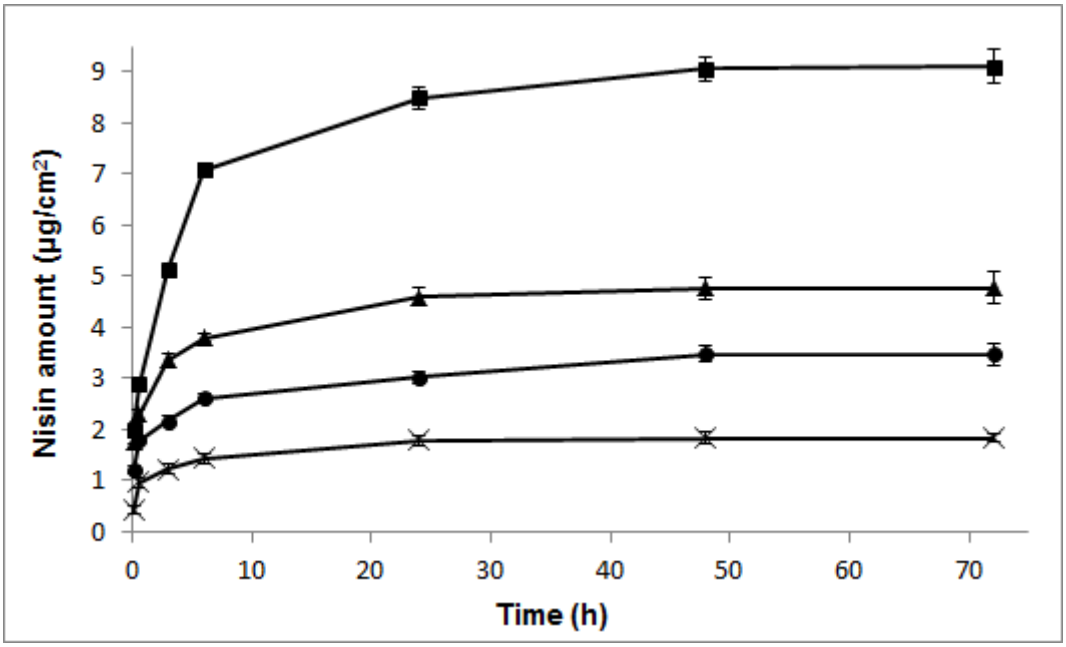

Fig. 4. Amount of nisin released at different times from the nisin-functionalized films: native films + nisin (X), N_2 treated film + nisin (●), AA treated film + nisin (▲), and Ar/O_2 treated film + nisin (■). Error bars represent the standard deviation of the mean of three experiments.

3.2.3. Effect of surface charge on nisin adsorption

Zeta potential measurements can be used to predict the adsorption processes. The surface charge can affect peptide adsorption on the films according to the type of predominant surface interactions. Nisin has an amphiphilic character with hydrophobic and hydrophilic domains [29] and the main types of interactions that govern its adsorption on hydrophobic and hydrophilic surfaces are electrostatic, hydrogen bonding, and hydrophobic ones. On the native hydrophobic film, the hydrophobic interactions are predominant and the

© 2013 Tous droits réservés.
http://doc.univ-lille1.fr

hydrogen bonding and electrostatic ones are neglected [25]. On the hydrophilic surfaces, the hydrophilic interactions as electrostatic and hydrogen bonding are predominant [28]. Subsequently, the surface charge may influence peptide adsorption on the hydrophilic surfaces but it has little effect on the hydrophobic ones. In our work conditions, the peptide adsorption was taking place at pH 2 at which nisin was below its isoelectric point of 8.52 and possessed thus a positive charge. Therefore, nisin and the hydrophilic surfaces had the same charge sign (Table 3). As a result, electrostatic attraction was not the factor supporting nisin binding to the surface and other mechanisms as the hydrogen bonding interaction between the polar groups of both the film and nisin may be driving the adsorption phenomenon [21]. However, the unfavorable contributions from electrostatic repulsion should be considered too. The high positively charged N_2 surfaces can induce higher electrostatic repulsion than the low positively charged Ar/O_2 and AA films (Table 3). This higher repulsion force between nisin and the N_2 film may explain the lower peptide adsorbed amount on this film as compared to the two other hydrophilic surfaces. It cannot though explain the difference observed between Ar/O_2 and AA films because they presented similar surface charges.

3.2.4. Effect of surface topography on nisin adsorption and antibacterial activity

The surface topography and the roughness of the different types of films were investigated by AFM before and after nisin adsorption. AFM allowed to monitor the complete surface topography change after nisin adsorption and to generally observe nisin distribution on surfaces. This distribution can be correlated to the observed antibacterial activity, to the nisin adsorbed amount, and to the types of interactions on surfaces. In addition, the differences in topographies and in roughness before and after nisin adsorption and between the different films can explain the relationships between the surface properties and the nisin adsorbed amount on surfaces.

3.2.4.1. Relationships between nisin distribution, antibacterial activity, nisin adsorbed amount, and the types of interactions on surfaces

Nisin coverage on the native hydrophobic film was clearly different from the one on the three treated hydrophilic films. Separate aggregates or agglomerates distribution was detected on the hydrophobic film (Fig. 5a) versus a uniform and continuous coverage on the hydrophilic films (Figs. 5b, c, and d). Such distribution can be correlated to the

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

localized spot-like antibacterial activity observed on the hydrophobic films as opposed to the regular activity recorded on the hydrophilic films (Fig. 2). Nisin distribution and adsorption amount on hydrophilic and hydrophobic films can be explained by the types of interactions on the surfaces and by the molecule dimensional size. The overall dimensions of the molecule, modeled as a cylinder, are about 50 Å in length (side hydrophobic domain) and 20 Å in diameter (end hydrophilic domain) [28]. On the hydrophobic surface, where hydrophobic interactions are dominant; the point of contact with the surface is from the hydrophobic larger side of the molecule (nisin side-on adsorption and adsorption sites of 50 x 20 Å). On the hydrophilic surface, the hydrophilic side of the nisin oriented to the hydrophilic surface, presents smaller contact points (end-on adsorption and nisin adsorption sites of 20 x 20 Å) [9,28]. Nisin molecules can have an extended structure, occupying larger areas on the hydrophobic surface relative to the hydrophilic one [25] and therefore assembling in large aggregates and lower amount on this surface. However, assembling in smaller contact points on the hydrophilic surface can cover more adsorption sites conferring a continuous distribution or a more closely packed arrangement and a higher adsorbed amount on the surface.

3.2.4.2. Relationships between the surface topography, the surface roughness, and nisin adsorbed amount on surfaces

The changes in the surface features of the same material after different plasma treatments, highly influenced nisin adsorption on surfaces as evidenced by the surface topographies and by the evaluation of roughness parameters. Our findings showed that the surfaces with flat structures (native and N_2 films) (Figs. 1a and b) displayed lower amount of nisin and a slight increase in the roughness from 4 to 4.6 nm and from 3.3 to 4 nm after nisin adsorption on the native and nitrogen films, respectively. Conversely, the surfaces with granular structure (AA and Ar/O_2 films) (Figs. 1c and d) showed higher amount of nisin and a decrease in the roughness from 8.6 to 4 nm and from 6 to 1.3 nm after nisin adsorption on the AA and Ar/O_2 films, respectively. The granular structure may provide more anchoring or filling sites for nisin adsorption and then explain the higher amount of peptides detected on the Ar/O_2 and AA surfaces than on the N_2 and native ones. Moreover, filling up the sites of granular surface can render it smoother while covering a flat surface can render it slightly rougher, as observed by roughness measurements. Between the N_2 and native films, presenting similar topographies and surface charges, the higher amount

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

and activity of nisin can be related to the hydrophobic and hydrophilic interactions on those two surfaces, as explained previously. Among the AA and Ar/O_2 films, presenting similar topographies, hydrophilicity, and surface charges; the higher amount recorded on the latter films can be associated to the size domain of the granula on the surfaces. The bigger domains on the AA surfaces decreased the number or density of grains formed per unit area, providing less interface area or filling holes than the Ar/O_2 surface. In addition, the higher amount of nisin recorded on Ar/O_2 as compared to AA surfaces, was reflected by a higher decrease of roughness. A higher filling up of the valleys between the granular sites of the former films may lead to a more flattened surface relative to the latter ones.

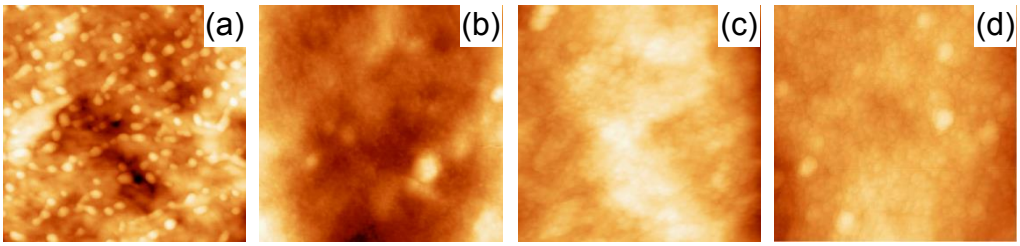

Fig. 5. Representative AFM topographic images of (a) native film + nisin (z-scale 30 nm), (b) N_2 treated film + nisin (z-scale 30 nm), (c) AA treated film + nisin (z-scale 30 nm), and (d) Ar/O_2 treated film + nisin (z-scale 10 nm). The scan size was 1 μm x 1 μm.

4. Conclusion

Nisin adsorption on surfaces and the antibacterial activity of nisin-functionalized films depended on many factors among them the type of the surface, hydrophobic and hydrophilic interactions, surface charge, surface topography, nisin distribution, and nisin adsorbed amount on the surfaces. The correlation between those factors as well as the contribution of each factor to the observed antibacterial activity can be summed up as following:

- The type of the surface highly impacted the films' antibacterial activity that was ranked in the following order: Ar/O_2 > AA > N_2 > native films.
- The hydrophobic and hydrophilic interactions can explain the lower antibacterial activity, the lower nisin adsorbed amount, and the different nisin ditribution on the native hydrophobic surfaces as compared to the hydrophilic ones.

© 2013 Tous droits réservés.
http://doc.univ-lille1.fr

- The surface charge effect is dependent upon which type of interactions predominates on the surfaces and is then important on the hydrophilic surfaces. The higher electrostatic repulsion on the nitrogen film may explain the lower adsorbed amount on this film as compared to the two other hydrophilic surfaces.
- The surface topography can control the amount of nisin adsorbed on the surfaces. The surfaces with granular structure adsorbed higher amount of nisin than the flat ones. The size of the granular domains explained the difference between Ar/O_2 and AA films.
- Nisin distribution on surfaces can influence the homogeneity of the observed antibacterial activity and can be mainly related to the hydrophobic and hydrophilic interactions on those films.
- Nisin adsorbed amount was directly proportional to the observed antibacterial activity. This amount was dependent on the surface topography, the surface charge, the molecule orientation, and the types of interactions on surfaces.

Therefore the effectiveness of the antibacterial surface cannot be interpreted by the effect of one factor solely but by the combined effect of many factors. Plasma treatments provide a promising technology for setting up antimicrobial food packaging and contact surfaces but the studied factors should be taken in consideration for each couple film-bacteriocin.

Acknowledgements

This work was supported financially by the "Action de Recherche Concertée d'Initiative Régionale (ARCIR) de la région Nord Pas de Calais"(France) and by a PhD fellowship within the Erasmus Mundus Program. The Fonds Européen de Développement Régional (FEDER), CNRS, Région Nord Pas-de-Calais and Ministère de l'Education Nationale de l'Enseignement Supérieur et de la Recherche were acknowledged for funding XPS spectrometers within the Pôle Régional d'Analyses de Surface. We are also grateful for Dupont Health and Nutrition (United Kingdom) for donating the pure nisin for our research work.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

References

[1] P. Appendini, J.H. Hotchkiss, Review of antimicrobial food packaging, Innovative Food Sci. Emerging Technol. 3 (2002) 113–126.

[2] R.W. Jack, J.R. Tagg, B. Ray, Bacteriocins of gram-positive bacteria., Microbiol. Rev. 59 (1995) 171–200.

[3] I.E. Pol, E.J. Smid, Combined action of nisin and carvacrol on *Bacillus cereus* and *Listeria monocytogenes*, Lett. Appl. Microbiol. 29 (1999) 166–170.

[4] J. Delves-Broughton, P. Blackburn, R.J. Evans, J. Hugenholtz, Applications of the bacteriocin, nisin, Antonie van Leeuwenhoek. 69 (1996) 193–202.

[5] C.K. Bower, M.A. Daeschel, J. McGuire, Protein Antimicrobial Barriers to Bacterial Adhesion, J. Dairy Sci. 81 (1998) 2771–2778.

[6] P. Gröning, M. Collaud Coen, L. Schlapbach, Polymers and cold plasmas, Chimia. 55 (2001) 171–177.

[7] C.-M. Chan, T.-M. Ko, H. Hiraoka, Polymer surface modification by plasmas and photons, Surf. Sci. Rep. 24 (1996) 1–54.

[8] M. Ozdemir, C.U. Yurteri, H. Sadikoglu, Surface treatment of food packaging polymers by plasmas, Food Technol. 53(4) (1999) 54-58.

[9] L. Karam, C. Jama, N. Nuns, A.-S. Mamede, P. Dhulster, N.-E. Chihib, Nisin adsorption on hydrophilic and hydrophobic surfaces: evidence of its interactions and antibacterial activity, J. Pept. Sci. 19 (2013) 377–385.

[10] B. Gupta, J.G. Hilborn, I. Bisson, P. Frey, Plasma-induced graft polymerization of acrylic acid onto poly(ethylene terephthalate) films, J. Appl. Polym. Sci. 81 (2001) 2993–3001.

[11] D.A. Shirley, High-Resolution X-Ray Photoemission Spectrum of the Valence Bands of Gold, Phys. Rev. B. 5 (1972) 4709–4714.

[12] W.-C. Lin, S.-H. Lee, M. Karakachian, B.-Y. Yu, Y.-Y. Chen, Y.-C. Lin, et al., Tuning the surface potential of gold substrates arbitrarily with self-assembled monolayers with mixed functional groups, Phys. Chem. Chem. Phys. 11 (2009) 6199–6204.

[13] M. Wegmann, B. Michen, T. Luxbacher, J. Fritsch, T. Graule, Modification of ceramic microfilters with colloidal zirconia to promote the adsorption of viruses from water, Water Res. 42 (2008) 1726–1734.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

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

[15] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, et al., Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.

[16] K.J. Wiechelman, R.D. Braun, J.D. Fitzpatrick, Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation, Anal. Biochem. 175 (1988) 231–237.

[17] N. Grover, H. Singh, B. Gupta, Characterization of acrylic acid grafted poly(ethylene terephthalate) fabric, J. Appl. Polym. Sci. 117 (2010) 3498–3505.

[18] P. Slepička, S. Trostová, N. Slepičková Kasálková, Z. Kolská, P. Sajdl, V. Švorčík, Surface Modification of Biopolymers by Argon Plasma and Thermal Treatment, Plasma Processes Polym. 9 (2012) 197–206.

[19] R. Schweiss, P.B. Welzel, C. Werner, W. Knoll, Interfacial charge of organic thin films characterized by streaming potential and streaming current measurements, Colloids Surf., A. 195 (2001) 97–102.

[20] A. Mochizuki, T. Ogawa, K. Okamoto, T. Nakatani, Y. Nitta, Blood compatibility of gas plasma-treated diamond-like carbon surface—Effect of physicochemical properties of DLC surface on blood compatibility, Mater. Sci. Eng., C. 31 (2011) 567–573.

[21] D. Huo, Y. Li, Q. Qian, T. Kobayashi, Temperature–pH sensitivity of bovine serum albumin protein-microgels based on cross-linked poly(N-isopropylacrylamide-co-acrylic acid), Colloids Surf., B. 50 (2006) 36–42.

[22] L. Tušek, M. Nitschke, C. Werner, K. Stana-Kleinschek, V. Ribitsch, Surface characterisation of NH_3 plasma treated polyamide 6 foils, Colloids Surf., A. 195 (2001) 81–95.

[23] J. Song, U. Gunst, H.F. Arlinghaus, G.J. Vancso, Flame treatment of low-density polyethylene: Surface chemistry across the length scales, Applied Surface Science. 253 (2007) 9489–9499.

[24] M. Collaud Coen, G. Dietler, S. Kasas, P. Gröning, AFM measurements of the topography and the roughness of ECR plasma treated polypropylene, Appl. Surf. Sci. 103 (1996) 27–34.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

[25] M.A. Daeschel, J. Mcguire, Interrelationships between protein surface adsorption and bacterial adhesion, Biotechnol. Genet. Eng. Rev. 15 (1998) 413–438.

[26] C.K. Bower, J. McGuire, M.A. Daeschel, Suppression of *Listeria monocytogenes* colonization following adsorption of nisin onto silica surfaces., Appl. Environ. Microbiol. 61 (1995) 992–997.

[27] C.K. Bower, J. McGuire, M.A. Daeschel, Influences on the antimicrobial activity of surface-adsorbed nisin, J. Ind. Microbiol. 15 (1995) 227–233.

[28] M. Lakamraju, J. Mcguire, M. Daeschel, Nisin adsorption and exchange with selected milk proteins at silanized silica surfaces, J. Colloid Interface Sci. 178 (1996) 495–504.

[29] F.J.M. Van De Ven, H.W. Van Den Hooven, R.N.H. Konings, C.W. Hilbers, NMR studies of lantibiotics, Eur. J. Biochem. 202 (1991) 1181–1188.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

3. Conclusions

Plasma surface treatments permitted first to understand the different factors affecting nisin adsorption on surfaces. Although the hydrophilic/hydrophobic character highly affects the adsorption behavior, other surface properties such as surface topography, roughness, and charge can have an influence too. Second, surface modification permitted to improve the peptide adsorption and the antibacterial activity of nisin-functionalized films. Ar/O_2 plasma treatment showed the best results but the other treatments can be also used taking into consideration the discussed findings. The ideal choice may not be maximizing nisin quantity and antibacterial activity but rather optimizing it. Now that we understand how each treatment can affect the surface characteristics and antibacterial activity, we can combine the properties and tailor-made specifications to the requirements of particular applications. Therefore, plasma treatments provide a promising technology for setting up antimicrobial food packaging.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

CONCLUSIONS AND PERSPECTIVES

Conclusions

Active antimicrobial packaging has become one of the major areas of research in food packaging because of its importance for improving the safety, quality, and shelf-life of packaged foods. When the antimicrobial packaging is obtained via peptide adsorption on surfaces, the peptides-materials-bacteria interactions are key factors controlling the success of such applications. This thesis permitted to investigate those interactions and to study the various factors affecting nisin adsorption and antibacterial activity on surfaces. It assessed additionally the possible use of nisin-functionalized films and of plasma treatments for future food applications.

The combination of surface characterization techniques (ToF-SIMS and XPS) permitted to probe nisin interactions and orientation behavior on hydrophobic and hydrophilic surfaces. Nisin was more oriented by its hydrophobic side to the hydrophobic substrate and by its hydrophilic side to the outer layers of the adsorbed peptide, in contrast to what was observed on the hydrophilic substrate. Such confirmed interactions induce higher peptide conformational change on hydrophobic surfaces relative to hydrophilic ones and affect thus nisin functionality and antibacterial activity.

Nisin adsorption on surfaces and the antibacterial activity of nisin-functionalized films depended on many factors among them the type of the surface, hydrophobic and hydrophilic interactions, peptide conformational change, surface charge, surface topography, nisin adsorbed amount, and nisin distribution on the surfaces. Hydrophilic surfaces (Ar/O_2, AA, and N_2 treated films) showed higher and uniform antibacterial activity as compared to hydrophobic native film that showed lower and localized irregular activity. This can be associated to the hydrophobic and hydrophilic interactions, to the peptide conformational change, to the amount of adsorbed nisin and to the distribution of nisin on surfaces. When comparing the hydrophilic films themselves, the highest antibacterial activity was recorded for Ar/O_2, followed by AA and then by N_2 films. The difference in activity between those films can be explained by the amount of adsorbed nisin, the charge and the topography of the surfaces.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

In order to assess possible future food packaging applications, the effectiveness of nisin-functionalized films was evaluated against some food pathogens and at refrigeration temperature. The films were active against *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus*. Moreover, *Staphylococcus aureus* showed the highest sensitivity toward nisin-treated films as compared to the two other food pathogens. This means that the antibacterial activity of the films is bacteria-dependent too and the sensitivity of the targeted bacteria can play a significant role in the degree of effectiveness of the bioactive packaging. The antibacterial films were also effective at refrigeration temperature with no significant difference between 20 and 4°C. Those preliminary encouraging results will need to be further studied for longer storage period and in the food systems where they are intended to be applied.

Plasma treatments provided a multi-purpose technique since they permitted to selectively modify the surface properties for adsorption studies, to improve nisin adsorption on surfaces, and to impart effective antibacterial films. Such processes can provide thus high potential for future food packaging applications keeping in mind the other advantages that can be obtained simultaneously with the eco-friendly plasma treatments as enhancement of barrier and functional properties of the packaging materials and preservation of their desirable bulk properties.

Future development of antimicrobial food packaging relies mainly on the surface modification techniques, on the surface characterization methods, and on the proper antimicrobial-material-bacteria combinations. However, it is important to remember that antimicrobial packaging should be considered as part of hurdle technology and it should not be used as a substitute for good manufacturing practices, effective sanitation, proper hygiene, and control measures with respect to the raw materials, the food plant, the food products, and the food processing personnel.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Perspectives

This study provides a basis and can open a door for many other research developments in this field. Obviously, a lot of fundamental and applied researches are still needed and below are some proposed perspectives:

- Other types of plasma functionalization and of plasma-induced grafting can be used by varying the types of gas and the operating conditions or by varying the type and amount of grafted monomers on the surfaces. This is interesting to produce different surface characteristics for efficient approaches to specific applications and may achieve better antimicrobial surfaces.
- Plasma polymerization processes can be also evaluated for peptide functionalization. Nisin incorporation in the thin plasma-deposited film may provide a matrix retarding the peptides release from the packaging material to the product, allowing thus an extended antimicrobial activity.
- Other surface testing techniques would be combined or used to speculate new surface characteristics or different aspects of the phenomena at the interfaces. For example, AFM can be used to measure the force of interactions between the peptides and the modified surfaces.
- New peptides, enzymes, and proteins with antimicrobial properties can be used to activate the surfaces. Nisin showed a successful model or "case study" encouraging thus the emergence and the applications of other potential bacteriocins. Combinations of different antimicrobials and different treatments can be previewed too.
- Our findings can be also applied for various packaging materials matching various food application needs. Sustainable recyclable materials or packaging made of renewable resources are privileged for their beneficial environmental impact.
- Antibacterial tests in growth media should be followed by tests in targeted food. As the peptide activity will vary according to its interactions with the food matrix and to the sensitivity of the bacteria in the contaminated food, conclusions on how antimicrobial films will perform with a food product must be determined for each food application. Similar studies should also include (1) peptides kinetics of release from the packaging to the food in order to estimate shelf-life extension and (2) simulation experiments to account for the different conditions that may affect the packaging effectiveness during food processing, transport, distribution, and storage.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

- Interactions studies can benefit other types of antimicrobial food packaging and not only peptide adsorption techniques. For instance, the methods based on coating, incorporation in the packaging and covalent immobilization of the antimicrobials, require also a thorough understanding of antibacterial activity, surfaces properties and of peptides interactions and distribution on surfaces.

Finally, the advances in this interdisciplinary field require the effective collaboration among materials scientists, plasma physicists, chemists, biologists, and bioengineers.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

REFERENCES

The listed references below concern only the thesis introduction, objectives, and conclusions, as well as the introduction and conclusions for each research paper.

Abee, T., Rombouts, F.M., Hugenholtz, J., Guihard, G., Letellier, L., 1994. Mode of Action of Nisin Z against Listeria monocytogenes Scott A Grown at High and Low Temperatures. Appl. Environ. Microbiol. 60, 1962–1968.

Appendini, P., Hotchkiss, J.H., 2002. Review of antimicrobial food packaging. Innovative Food Science & Emerging Technologies 3, 113–126.

Beuchat, L.R., Clavero, M.R., Jaquette, C.B., 1997. Effects of nisin and temperature on survival, growth, and enterotoxin production characteristics of psychrotrophic Bacillus cereus in beef gravy. Appl. Environ. Microbiol. 63, 1953–1958.

Bower, C.K., McGuire, J., Daeschel, M.A., 1995. Suppression of Listeria monocytogenes colonization following adsorption of nisin onto silica surfaces. Appl. Environ. Microbiol. 61, 992–997.

Boziaris, I.S., Nychas, G.-J.E., 2006. Effect of nisin on growth boundaries of Listeria monocytogenes Scott A, at various temperatures, pH and water activities. Food Microbiology 23, 779–784.

Chan, C.-M., Ko, T.-M., Hiraoka, H., 1996. Polymer surface modification by plasmas and photons. Surface Science Reports 24, 1–54.

Chihib, N.-E., Ribeiro da Silva, M., Delattre, G., Laroche, M., Federighi, M., 2003. Different cellular fatty acid pattern behaviours of two strains of Listeria monocytogenes Scott A and CNL 895807 under different temperature and salinity conditions. FEMS Microbiology Letters 218, 155–160.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

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

Delves-Broughton, J., Blackburn, P., Evans, R.J., Hugenholtz, J., 1996. Applications of the bacteriocin, nisin. Antonie van Leeuwenhoek 69, 193–202.

Dutta, P.K., Tripathi, S., Mehrotra, G.K., Dutta, J., 2009. Perspectives for chitosan based antimicrobial films in food applications. Food Chemistry 114, 1173–1182.

Farber, J.M., Peterkin, P.I., 1991. Listeria monocytogenes, a food-borne pathogen. Microbiol. Rev. 55, 476–511.

Goddard, J.M., Hotchkiss, J.H., 2007. Polymer surface modification for the attachment of bioactive compounds. Progress in Polymer Science 32, 698–725.

Granum, P.E., Lund, T., 1997. Bacillus cereus and its food poisoning toxins. FEMS Microbiology Letters 157, 223–228.

Gröning, P., Collaud Coen, M., Schlapbach, L., 2001. Polymers and cold plasmas. Chimia 55, 171–177.

Hennekinne, J.-A., De Buyser, M.-L., Dragacci, S., 2012. Staphylococcus aureus and its food poisoning toxins: characterization and outbreak investigation. FEMS Microbiology Reviews 36, 815–836.

Kaur, G., Singh, T.P., Malik, R.K., Bhardwaj, A., 2011. Mechanism of Nisin, Pediocin 34, and Enterocin FH99 Resistance in Listeria monocytogenes. Probiotics and Antimicrobial Proteins 4, 11–20.

Kerry, J.P., O'Grady, M.N., Hogan, S.A., 2006. Past, current and potential utilisation of active and intelligent packaging systems for meat and muscle-based products: A review. Meat Science 74, 113–130.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Kim, M.S., Khang, G., Lee, H.B., 2008. Gradient polymer surfaces for biomedical applications. Progress in Polymer Science 33, 138–164.

Lakamraju, M., Mcguire, J., Daeschel, M., 1996. Nisin adsorption and exchange with selected milk proteins at silanized silica surfaces. Journal of Colloid and Interface Science 178, 495–504.

Leistner, L., Gorris, L.G.M., 1995. Food preservation by hurdle technology. Trends in Food Science & Technology 6, 41–46.

Leistner, L., 2000. Basic aspects of food preservation by hurdle technology. International Journal of Food Microbiology 55, 181–186.

Muramoto, S., Brison, J., Castner, D.G., 2012. Exploring the Surface Sensitivity of TOF-Secondary Ion Mass Spectrometry by Measuring the Implantation and Sampling Depths of Bi_n and C_{60} Ions in Organic Films. Analytical Chemistry 84, 365–372.

Neunlist, M.R., Federighi, M., Laroche, M., Sohier, D., Delattre, G., Jacquet, C., Chihib, N.-E., 2005. Cellular Lipid Fatty Acid Pattern Heterogeneity Between Reference and Recent Food Isolates of Listeria Monocytogenes as a Response to Cold Stress. Antonie Van Leeuwenhoek 88, 199–206.

Ozdemir, M., Yurteri, C.U., Sadikoglu, H., 1999. Physical Polymer Surface Modification Methods and Applications in Food Packaging Polymers. Critical Reviews in Food Science and Nutrition 39, 457–477.

Papagianni, M., 2003. Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. Biotechnology Advances 21, 465–499.

Periago, P.M., Moezelaar, R., 2001. Combined effect of nisin and carvacrol at different pH and temperature levels on the viability of different strains of Bacillus cereus. International Journal of Food Microbiology 68, 141–148.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

Pol, I.E., Smid, E.J., 1999. Combined action of nisin and carvacrol on Bacillus cereus and Listeria monocytogenes. Letters in Applied Microbiology 29, 166–170.

Quintavalla, S., Vicini, L., 2002. Antimicrobial food packaging in meat industry. Meat Science 62, 373–380.

Razavi Rohani, S.M., Moradi, M., Mehdizadeh, T., Saei-Dehkordi, S.S., Griffiths, M.W., 2011. The effect of nisin and garlic (Allium sativum L.) essential oil separately and in combination on the growth of Listeria monocytogenes. LWT - Food Science and Technology 44, 2260–2265.

Sanni, O.D., Wagner, M.S., Briggs, D., Castner, D.G., Vickerman, J.C., 2002. Classification of adsorbed protein static ToF-SIMS spectra by principal component analysis and neural networks. Surface and Interface Analysis 33, 715–728.

Singh, B., Bernadette Falahee, M., Adams, M.R., 2001. Synergistic inhibition of Listeria monocytogenes by nisin and garlic extract. Food Microbiology 18, 133–139.

Solano, R., Lafuente, S., Sabate, S., Tortajada, C., García de Olalla, P., Hernando, A.V., Caylà, J., 2013. Enterotoxin production by Staphylococcus aureus: An outbreak at a Barcelona sports club in July 2011. Food Control 33, 114–118.

Suppakul, P., Miltz, J., Sonneveld, K., Bigger, S. w., 2003. Active Packaging Technologies with an Emphasis on Antimicrobial Packaging and its Applications. Journal of Food Science 68, 408–420.

Ter Steeg, P.F., Hellemons, J.C., Kok, A.E., 1999. Synergistic actions of nisin, sublethal ultrahigh pressure, and reduced temperature on bacteria and yeast. Appl. Environ. Microbiol. 65, 4148–4154.

Wan, J., Coventry, J., Swiergon, P., Sanguansri, P., Versteeg, C., 2009. Advances in innovative processing technologies for microbial inactivation and enhancement of food

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

safety – pulsed electric field and low-temperature plasma. Trends in Food Science & Technology 20, 414–424.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr

RESUME

La mondialisation du commerce alimentaire et les changements des modes de consommation présentent de nouveaux défis majeurs en sécurité alimentaire. La mise au point d'emballages alimentaires actifs, par adsorption de peptides antimicrobiens sur des matériaux, est une approche innovante et proactive pour améliorer la sécurité, la qualité et la durée de vie des produits emballés. L'adsorption de peptides en surface et l'activité antimicrobienne des supports fonctionnalisés dépendent principalement des propriétés de surface, des traitements de surface permettant de modifier ces propriétés et des interactions peptides-matériaux-bactéries. Dans cette thèse, le choix du peptide antimicrobien s'est porté sur la nisine, bactériocine à activité antilisteria, produite par des souches de *Lactococcus lactis* subsp. *lactis*. L'emballage choisi était le polyéthylène à basse densité, un support fréquemment utilisé dans le secteur agro-alimentaire. Plusieurs procédés de traitements plasma froid ont été mis au point pour développer des surfaces présentant des caractéristiques différentes et des fonctionnalités spécifiques nécessaires à l'étude des mécanismes d'adsorption. Des techniques physico-chimiques de caractérisation ont permis d'une part, de mettre en évidence la fonctionnalisation des supports par les traitements de surface et par la nisine et d'autre part, d'étudier les interactions aux interfaces. L'étude antimicrobienne a été menée pour comparer et confirmer l'activité antimicrobienne des différents emballages traités. Ces analyses ont également été effectuées contre des pathogènes alimentaires et à basse température pour évaluer une possible application industrielle de ces emballages.

Mots-clés : Emballage actif, emballage antimicrobien, nisine, traitement plasma, adsorption de peptides, interactions aux interfaces, techniques de caractérisation de surfaces, pathogènes alimentaires.

ABSTRACT

The globalization of food trade and changes in lifestyles present new major challenges for food safety. Setting up active food packaging, via antimicrobial peptide adsorption on materials, is an innovative and proactive approach to improve the safety, quality, and shelf-life of packaged foods. Peptide adsorption on surfaces and the antimicrobial activity of the functionalized materials depend mainly on surface properties, on surface treatments allowing the modification of such properties, and on peptides-materials-bacteria interactions. In this thesis, nisin, an antilisterial bacteriocin, produced by *Lactococcus lactis* subsp. *lactis*, was used as the antimicrobial peptide. The selected packaging was the low density polyethylene, a commonly used packaging in the food sector. Different cold plasma processes were optimized to develop surfaces with various characteristics and specific functionalities needed for the adsorption studies. Physico-chemical surface characterization techniques permitted from one side, to confirm the surface functionalization by surface treatments and by nisin and from another side, to study the surface interactions. The antimicrobial study was undertaken to compare and confirm the antimicrobial activity of the different treated packagings. This work was also carried out against some food pathogens and at refrigeration temperature in order to assess possible future food packaging applications.

Keywords: Active packaging, antimicrobial packaging, nisin, plasma treatment, peptide adsorption; surface interactions, surface characterization techniques, food pathogens.

© 2013 Tous droits réservés.

http://doc.univ-lille1.fr