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Physicochemical Properties and Bioactivity of Nisin-Containing Cross-Linked Hydroxypropylmethylcellulose Films

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Cross-linked hydroxypropylmethylcellulose (HPMC) cast films with citric acid as polycarboxylic cross-linker were elaborated to study the effect of cross-linking level on various properties. Increased amounts of cross-linking agent were not connected to statistically different tensile strength and Young's modulus. Whatever the cross-linking level of the film was, the ultimate elongation parameter decreased by ∼60% compared to the HMPC control film. Moisture sorption isotherms and water contact angle meter showed that the effect of cross-linking degree tends to reduce the hygroscopic and hydrophilic characteristics of films. In addition, to control bacteria growth on food surfaces, the antimicrobial activity of both 98% cross-linked HPMC−nisin and control HPMC−nisin films was tested on *Micrococcus luteus*. Despite the incorporation of a significant content of nisin, cross-linked HPMC−nisin films were completely inactive on the microbial strain compared to the HPMC−nisin control films. Cross-linking conditions likely either denatured the nisin or irreversibly bound nisin to the cross-linked HPMC. However, nisin adsorbed into films made from previously cross-linked HPMC maintained its activity.

KEYWORDS: Antimicrobial packaging; hydroxypropylmethylcellulose; hygroscopic properties; tensile properties; nisin

INTRODUCTION

Renewed interest in food packaging based on natural macromolecules in recent years is due to concerns about the environment and a need to reduce the amount of disposable packaging materials. As mentioned by Yang and Paulson (*1*), films made from polysaccharides are expected to be excellent oxygen barriers due to their highly packed and ordered hydrogen-bonded network structure. However, hygroscopic characteristics of natural polymers such as cellulose derivatives show that swelling by water leads to a loss of the gas and vapor barrier properties. Cellulose-based biopackagings, because of their hydrophilic nature, are poor moisture barriers and are frequently soluble in water, reducing the potential use in food applications. Cross-linking by a polycarboxylic acid seems to be an interesting way to decrease the hydrophilic characteristic and water solubility of cellulosic polymer. Consisting of the formation of a covalent bond between the chains of cellulose, this chemical modification could lead to a decrease in the availability of hydroxyl groups, limiting polysaccharide−water interactions by hydrogen bonding. Cross-linking agents, such as polycarboxylic acids, could, however, partially compensate for the loss of available hydroxyl groups by giving, especially, highly hydrophilic carbonyl groups. According to a previous study (*2*), cross-linking with citric acid of hydroxypropylmethylcellulose (HPMC) films resulted in a decrease of the affinity of the natural polymer toward water and showed a non water solubility of films associated with an improvement of water vapor barrier properties. To determine potential food applications of biopackaging based on cellulose derivatives with the chemically bonded citric acid, further hygroscopic and mechanical properties were thus investigated.

In addition, to control undesirable microorganisms on food, active packaging is one of the innovative concepts that have been introduced as a response to improve safety and to delay spoilage. Listeriosis is one of the most severe foodborne infections with high mortality (*3*). The use of active biopackaging films containing antilisterial substances could be efficient in reducing the growth rate, extending the lag-phase, or inactivating pathogenic microorganisms by contact. Several compounds have been proposed and tested for antimicrobial activity (*4*) such as bacteriocins and especially nisin for *Listeria monocytogenes* inhibition (*5*−*7*). Generally, bacteriocins are low molecular weight cationic amphiphilic peptides, which tend to aggregate and are benign to the producing organism. The mode

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of action of nisin on sensitive bacteria is based on, first, interaction between the peptide and the cell membrane (*8*). Nisin has been accepted as a food additive by both the FDA and WHO (*9*), and its incorporation into a film matrix could combine the preservative functions of antibacterials with the protective functions of preexisting biopackaging concepts. In a previous study, nisin was included in an HPMC−stearic acid matrix. The lipid compound, due to its hydrophobic nature, was used to limit the affinity of the natural polymer toward water. Previous results showed a dramatic decrease of film biocide activity by reducing nisin desorption from the film. Electrostatic interactions between the two entities were supposed to maintain nisin into the film matrix (*10*). Cross-linking of cellulosic ether based films associated with a direct incorporation of preservative into the film-forming solution would be an economical method for manufacturing antimicrobial water vapor barrier biopackaging. However, the film-forming procedure and especially the cross-linking process may affect the antimicrobial activity and the release of nisin used as preservative. The objective of this paper was first to determine the influence of the cross-linking level of HPMC on the mechanical properties of films from especially elongation methods and hygroscopic properties such as moisture sorption isotherm. The cross-linking agent was citric acid, a triacid of natural origin, selected to obtain potential biodegradable films. The compatibility of the cross-linking process and antibacterial activity from nisin incorporation was also assessed by taking into account the high temperatures used to conduct the chemical modification. Results previously obtained from HPMC−nisin associated films indicated a significant antibacterial activity against *L. monocytogenes* and *Micrococcus luteus* (*6*). In this paper, *M. luteus*, an undesirable bacteria responsible of food organoleptic deterioration, was used as the microbial reference strain because of its high nisin sensitivity.

MATERIALS AND METHODS

Materials. *Chemicals.* HPMC (Culminal 50, Aqualon, Rueil Malmaison, France) and polyethylene glycol 400 (PEG 400, Merck) were used for film formation. Citric acid, NaH_2PO_4, ethanol, and phosphate buffer were supplied by Sigma (St Quentin Fallavier, France). Pure nisin (Aplin and Barrett Ltd., Beaminster, Dorset, U.K.) was dissolved in 0.05 M phosphate buffer, pH 6.1, and stored at 4 °C. Nisin concentration is expressed in international units per milliliter (IU mL^{-1}); 1 μg corresponds to 40 IU.

Organisms and Maintenance. M. luteus 270 (Institut Pasteur, Lille, France) was grown at 30 °C in nutritive broth (Difco 3178) in shaken flasks and agitated at 140−160 rpm for 18−24 h.

Methods. *Film Formation. 1. HPMC Control Film Preparation.* Films were prepared using a modified procedure described by Kamper and Fennema (*12*, *13*), by dissolving 9 parts of HPMC polymer in 200 parts of distilled water, 100 parts of absolute ethanol, and 1 part of PEG 400. PEG 400 was used as plasticizer. The solution was then homogenized for 15 min, degassed for 30 s using a vacuum pump, and poured onto a polypropylene support at a thickness of 1 mm. The films were dried at 60 °C for 120 min at ambient relative humidity and then peeled. Films were stored at 50 ± 5% relative humidity (RH) and 23 ± 1 °C for 5 days before use.

2. Antimicrobial HPMC Control Film Preparation by Nisin Incorporation. Nisin was presolubilized in phosphate buffer, pH 6.1, and the solution was added to 200 parts of distilled water, 100 parts of absolute ethanol, and 1 part of PEG 400 prior to incorporation of 9 parts of HPMC. The nisin concentration was adjusted by taking into account the dilution effect to obtain film-forming solutions at 10^3 and 10^5 IU mL^{-1} nisin concentrations. The part of nisin solution volume added to the film-forming solution was ∼1% (v/v). Then, the solution was homogenized, degassed, poured, and dried as described before for the HPMC control film preparation. Films were stored at 50 ± 5% RH and 23 ± 1 °C for 5 days before use. Film samples were then analyzed for antibacterial activity using the disk diffusion assays as described below.

3. Cross-Linked HPMC Film Preparation. Cross-linking corresponds to an ester bond reaction between the hydroxyl groups of HPMC and the carboxylic groups of citric acid. From the preliminary study (*2*), 10 min at 190 °C was the lowest time/temperature necessary to produce colorless and non-water-soluble films. It was also determined that 0, 5, 10, and 15% of citric acid (w/w HPMC) and 50% NaH_2PO_4 (w/w citric acid) induced 0, 45, 79, and 98% cross-linking levels.

Citric acid (0, 5, 10, or 15% w/w HPMC) and NaH_2PO_4 as catalyst (50% w/w citric acid) were added to 200 parts of distilled water, 100 parts of absolute ethanol, and 1 part of PEG 400 prior to incorporation of 9 parts of HPMC. The pH of the film-forming solution was ∼2.65. The film-forming solution was then poured onto a propylene support as before. After the first thermal proceeding (60 °C for 120 min), commonly used to dry all test films, films were peeled and heated again at 190 °C for 15 min (cross-linking necessary conditions). Films were stored at 50 ± 5% RH and 23 ± 1 °C for 5 days before use.

4. Nisin Incorporation in HPMC Matrix before Cross-Linking Process. Nisin (10^3−10^5 IU mL^{-1} final concentrations), citric acid (15% w/w HPMC), and NaH_2PO_4 (50% w/w citric acid) were added to 200 parts of distilled water, 100 parts of absolute ethanol, and 1 part of PEG 400 prior to incorporation of 9 parts of HPMC. The mixture was then homogenized and poured onto a propylene support as before. After the first thermal proceeding (60 °C for 120 min), films were peeled and heated again at 190 °C for 15 min. Film samples were then analyzed for antibacterial activity using the disk diffusion assays as described below.

5. Antimicrobial Cross-Linked HPMC Film Preparation by Nisin Adsorption. Nisin adsorption on a cross-linked film surface was obtained using a modified procedure described by Scannell et al. (*9*). Films were cut into 5-cm squares, placed in sterile Petri dishes, and covered with 30 mL of a nisin solution (10^4 IU mL^{-1}). The dishes were shaken for 2−40 h at 4 °C to allow nisin adsorption on the film surface. Films were then rinsed with sterile distilled water and dried at 50 °C for 20 min. Nisin solution (used in the adsorption procedure) and film samples were then analyzed for antibacterial activity using the agar well diffusion or disk diffusion assay, respectively, as described below.

6. Nisin Desorption Procedure. This procedure was used to evaluate the amount of nisin adsorbed on cross-linked HPMC films. The desorption assay was performed by first placing 2.5 cm^2 sections of films in 2 mL of 2% NaCl solutions. Samples were shaken at 30 °C for 24 h and rinsed with distilled water before drying at 50 °C for 20 min. Films were assayed for antimicrobial activity by the disk diffusion method as described below. The concentration of nisin in the desorption solution was also determined by the agar well diffusion method, as below.

Film Characterization. 1. Film Thickness. Film thickness was measured to the nearest 1 μm (Mitutoyo electronic micrometer). Ten measurements were performed at random positions.

2. Mechanical Properties. The mechanical resistance of films was performed at 23 °C and 50% RH. It included tensile strength (TS, MPa), ultimate elongation (UE, percent at break point), and Young's modulus (Y, MPa). Maximum tensile strength is the largest stress that a film is able to sustain. Ultimate elongation is the maximum percentage change in the length of a film before breaking. Young's modulus, calculated from the slope of the initial linear region of the stress−strain curves, reflects the film stiffness The tests were performed using the Amadel Lhomargy instrument according to AFNOR NF ISO 527-3 (1995) on 10 specimens previously stored for 7 days at 23 ± 1 °C and 50 ± 5% RH. Sample films of approximately 25 mm × 60 mm were uniaxially stretched at a constant velocity of 3 mm/min. The stress−strain curves were computer-recorded. Mechanical characterization was performed at controlled temperature and RH, 23 ± 1 °C and 50 ± 5%, respectively.

3. Scanning Electron Microscopy. Film samples were fractured in liquid nitrogen and placed onto specimen supports to obtain surface and side views (transversal view) of each film. After the gold and palladium coating procedure, samples were viewed using a JEOL 840 A microscope.

4. Fourier Transform Infrared (FTIR) Analysis. Infrared spectra were recorded with FTIR (Nicolet 210), in the 500−4000 cm^{-1} zone, using

200 scans and with a resolution equal to 4. It has been used in a previous study (*2*) to determine the cross-linking level from the increase in absorption intensity of the ester band (1735 cm^{-1}) using the absorbance of the 1371 cm^{-1} band (CH bending vibration of methyl group) as internal standard. The standard band does not vary with cross-linking, allowing the effect of film thickness to be eliminated. The films were directly analyzed in transmittance or attenuated total reflectance (ATR). The cross-linking level (COOH → COOR) was calculated from the ratio between these two bands and from a triethylcitrate (esterified citric acid) calibration curve (*2*). FTIR analyses were used to compare HPMC film structure and to determine potential bonding between nisin and the different compounds of packaging.

5. Film Water Affinity. a. Sorption Isotherms. Moisture sorption isotherms [equilibrium moisture content (EMC) versus water activity, a_w] of the films were determined at 25 ± 1 °C for 1 month as previously described (*6*). Films were conditioned in hermetically sealed glass jars containing different saturated salt solutions giving different a_w values. Aluminum dishes were weighed to the nearest 0.0001 g, and ~0.20 g of film was added to each dish. Film EMC was determined after sample drying at 103 °C for 2 h. The experiment was triplicated. Film sorption isotherms were averaged and fitted by the Guggenhein−Anderson−DeBoer (GAB) model, using the Matlab v6 software (Mathworks)

$$x = \frac{x_m c k a_w}{(1 - k a_w)(1 - k a_w + c k a_w)}$$

with x = EMC (g of water/g of dry films), a_w = water activity, x_m = monolayer moisture content (g of water/g of dry films), c = constant related to thermal effect, and k = GAB coefficient.

Equation parameters (x_m, c, and k) were estimated by a nonlinear regression based on the least-squares method.

b. Water Contact Angle Meter. A water drop is deposited on the surface of different films (size 65 mm × 25 mm). The θ angle in the interface water/film is measured (Krüss instrument) at the end of 5 s at the nearest 1°. θ varies from 0 to 90° from hydrophilic (0°) or hydrophobic (90°) film nature. Five measurements on each film were performed at random positions.

Antimicrobial Effectiveness of Pure Nisin Solution and Films. 1.Agar Well Diffusion Method. A growth inhibition zone assay was conducted by inoculating the nutritive agar broth (12 g/L) with 0.1% (v/v) of an 18 h culture of *M. luteus*. For tests of nisin in solution, 70 μL of the nisin solution was then pipetted into wells (5−6 mm diameter) previously cut into agar medium. Dishes were refrigerated at 4 °C for 3 h to allow diffusion of the bacteriocin and then incubated at 30 °C for 24−48 h. Data were expressed as growth inhibitory zone diameter (mm) and measured at the nearest 1 mm.

2. Disk Diffusion Method. To determine the antimicrobial activity of films, 5 mm diameter disks were cut from different films and placed on nutritive agar broth. The method was previously standardized by adjusting the microbial inoculation rate (0.1% v/v of *M. luteus*) and the thickness of the agar medium layer (7 mm). Dishes were refrigerated at 4 °C for 3−4 h to allow diffusion of the bacteriocin and then incubated at 30 °C for 24−48 h. Data are expressed as growth inhibitory zone diameter (mm) and measured at the nearest 1 mm.

Statistical Treatment. All experiments were replicated at least three times. Treatment means were separated using Student's t test at 95% probability ($p < 0.05$) (StatGraphics v4, Sigma).

RESULTS AND DISCUSSION

As previously studied (*2*), the cross-linking level illustrated the ester bond rate from the cross-linking reaction between the hydroxyl groups of HPMC and the carboxylic groups of citric acid. These percentages were calculated according to an FTIR method based on the ester bond vibration (1740 cm^{-1}) related to the 1371 cm^{-1} bond as internal standard. The calculation of the amount of ester bonds in the membrane led to the determination of the theoretical cross-linking percentage. These percentages of approximately 0, 45, 79, and 98% correspond to 0, 5, 10, and 15% of citric acid (w/w HPMC) and 50% NaH_2PO_4 (w/w citric acid). From the preliminary study (*2*), 10 min at 190 °C was the lowest time/temperature necessary to produce colorless and non-water-soluble films. Whereas the reference films were transparent, films with chemical modification developed a yellow color, the more marked because the thermal processing was carried out over a long duration (>15 min) and the acid content was high. This phenomenon may be attributable to the degradation of the citric acid into aconitic acid (1-propene-1,2,3-tricarboxylic acid) during the heat treatment due to a dehydration of a hydroxyl group in the α-position of an acid function (*14*).

Table 1. Mechanical Properties and Water Contact Angle of HPMC Films with Different Cross-Linking Levels (from 0 to 98%) and HPMC Control Film[a]

	HPMC films after thermal processing (190 °C, 15 min) at cross-linking level of (%)				HPMC control film
	0%[b]	45%	79%	98%	
Y (MPa)	21 ± 3b	21 ± 3b	18 ± 3b	14 ± 3b	19 ± 6b
TS (MPa)	32 ± 6b	31 ± 6b	31± 4b	27 ± 3b	34 ± 6b
UE (%)	2.8 ± 0.1c	2.0 ± 0.1d	2.4 ± 0.2e	2.7 ± 0.2b	6.6 ± 1.3f
water angle	44 ± 4b	51 ± 1b	53 ± 5b	55 ± 5b	49 ± 7b

[a] Values, followed by their standard deviations, are means of at least three experiments. Treatment means were separated using Student's t test ($p < 0.05$). Values followed by the letter b are not statistically different ($p < 0.05$). Values followed by the letter c, d, e, or f are statistically different ($p < 0.05$). [b] 0% cross-linking level = HPMC film without citric acid and NaH_2PO_4, heated at 190 °C for 15 min.

Film Thickness. The film thicknesses were not statistically different. They varied from 26 ± 12 μm for HPMC control films to 29 ± 8 μm for HPMC film at different cross-linking levels. Neither nisin incorporation into the film or nisin adsorption on the film surface induced any film thickness variation.

Mechanical Properties. The domains of elastic and plastic strain as well as the breaking strength are significant characteristics for the biopackaging, which have a protection function of the integrity of food.

The shapes of typical stress−strain curves for chemically modified films were similar, with a plateau area for longer film deformation and a sharp break for shorter deformation. The ultimate elongation (UE), Young's modulus (Y), and tensile strength (TS) were measured from films with different cross-linking levels and were compared to the HPMC control films (**Table 1**). Film with a 0% cross-linking level corresponded to an HPMC film that did not contain any cross-linking agent (citric acid) but that was heated (190 °C, 15 min) according to cross-linking time/temperature conditions. This film was used as reference, allowing the study of the impact of time/temperature on film properties.

The mechanical resistance of films to elongation was slightly different depending on the cross-linking level (**Table 1**). Whatever the cross-linking level, the UE parameter decreased from 58 to 70% compared to the control HPMC film, showing a decrease of the plastic deformation when chemical modification occurred. For TS and Y values, strong standard deviations have been found. Generally, as the film structure softened, TS decreased and UE increased, similar to results obtained by Park et al. (*15*), who showed that trends for UE values of film containing fatty acid were opposite to those for TS. These properties are largely associated with the distribution and the intensity of the inter- and intramolecular interactions in the matrix. Extensive interchain bonding may contribute to the low

flexibility of films. However, it is necessary to know that the mechanical properties of biopackaging were difficult to determine experimentally because of difficulties in sample preparation and sensitivity to fluctuations in environmental factors. Moreover, the slight influence of chemical modification level on film deformation was possibly due to the high brittleness of HPMC films. No surfactant was used to plasticize the film solution prior to casting. Their addition aimed to increase film resistance and decrease film stiffness.

The slight reduction in Young's modulus with the percentage of cross-linking was not expected, indicating a decrease in the rigidity of films, whereas cross-linking, by the formation of ester bonds, would improve film hardness. The influence of the interactions depends on the probability and energies brought into play, and the cross-linked films would be more rigid because direct bonds between the molecular chains would have to decrease the chain segmental mobility, causing the mechanical strength of the films to be increased, which was not observed. However, results were in accordance with those of Rioux et al. (*16*) showing a decrease in yield strength and stress at break at increasing cross-linking degree on starch. These authors mentioned that this phenomenon could be due to the effect of higher heterogeneity of the spacing between cross-links. The heterogeneity will distribute most of the applied stress on the chains, which are not stabilized by H-bonding. These chains breaking first transfer the stress to other chains, forcing them to either break or slip to relieve that stress. Nevertheless, results from Pérez-Gago et al. (*17*) suggested that covalent cross-linking due to heat denaturation of the whey protein was accountable for higher tensile properties.

To study the influence of the chemical modification on the microstructure of films, microscopic assays were conducted on 98% cross-linked and HPMC control films. HPMC control films showed a homogeneous spongy aspect (**Figure 1**, photo 1). Whereas 98% cross-linked films presented cracks and tears, which would be apparently related to the ester bond rate (**Figure 1**, photo 2), the same observation was made on 79% cross-linked film, in contrast, of 0, 5 and 10% of cross-linking levels (data not shown). This phenomenon would be due to numerous esters bonds with 10−15% of citric acid compared to lower polyacid content, leading to tension in the film matrix and to the formation of cracks, which conforms with the results obtained for the mechanical properties of cross-linked films, especially the decrease in the Young's modulus.

Film Water Affinity. *Moisture Sorption Isotherms.* Kim and Ustunol (*11*) mentioned that the moisture sorption isotherm represents the combined hygroscopic properties of its individual components. Any modification in the composition or structure of the food or the material may, in turn, influence its sorption isotherm. Polar groups led to a water absorption, for instance, from the surrounding air, which led to an increase in the moisture permeation rate by the water plasticizer role into the film matrix. Moreover, these authors (*11*) mentioned that experimentally derived moisture sorption isotherms are a useful way to assess the equilibrium moisture content of hydrophilic films at a given relative humidity to estimate their barrier and other properties. HPMC film moisture sorption increased rapidly at high a_w from 0.75 to 0.85 while increasing slowly or moderately at intermediate and low a_w, suggesting a swelling phenomenon as water activity increased (*18*) (**Figure 2**). Films of 45 and 79% cross-linking levels did not show any reduction of water sorption. In the domain of high water activities, the percentage of sorbed water was slightly lower for the 98% cross-linked membrane than for the control HPMC film, respectively,

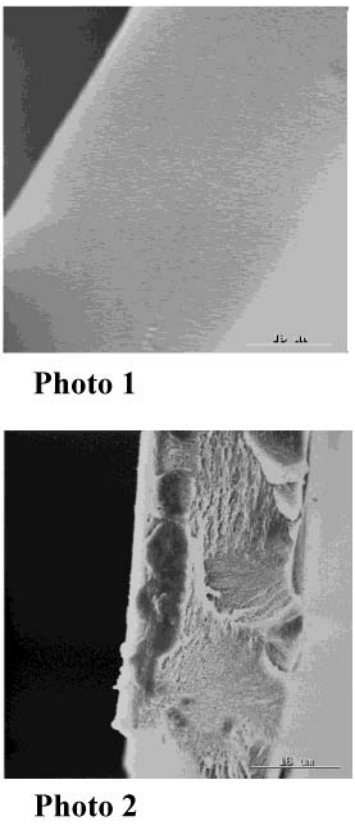

Figure 1. Scanning electron microscopic views of transversal cryofracture of HPMC control film (photo 1, top) and 98% cross-linked HPMC film (photo 2, bottom) (magnification ×1500).

4.5 and 7.7% (a_w = 0.85). This is attributable to the higher hygroscopic nature of water-soluble HPMC films compared to that of the cross-linked matrix. However, the sorbed water in the cross-linked film remained strong, and it was hypothesized to be a result of the additional hydrophilic groups (−OH, −CO, and unreacted −COOH) introduced by citric acid. Results from thermically untreated HPMC films associated with polyacid and catalyst showed that the modification of film composition weakly increased EMC from 7 to 7.7% [a_w = 0.85, 15% citric acid (w/w HPMC) and 50% NaH_2PO_4 catalyst (w/w citric acid)]. As a result, unreacted carboxylic acid groups of citric acid did not significantly influence moisture sorption and the cross-linking process only slightly limited water sorption of such hydrophilic biopolymers.

According to Fringant et al. (*19*), the first step of water sorption mechanisms corresponds to the fixation of water molecules on the specific hydrophilic groups of the amorphous phase; afterward, the amount of sorbed water depends on the swelling capacity of the polymer. The cross-linking by citric acid modified the water solubility in the polymer only at high a_w. Moreover, as is already known, at some critical temperature, a three-dimensional gel structure will be formed from cellulosic polymers. This phenomenon is attributable to the increase of intermolecular hydrophobic interactions caused by thermal disruption of hydration shells surrounding polymer chains (*20*). Experiments were therefore conducted on thermically treated free citric acid HPMC films. A thermal treatment of ∼190 °C for 15 min led to a weak decrease in water absorption, from 7.7 to 5.8% (a_w = 0.85), possibly due to a hysteresis phenomenon.

Results were in accordance with previous studies conducted on the moisture transfer through cross-linked films, which

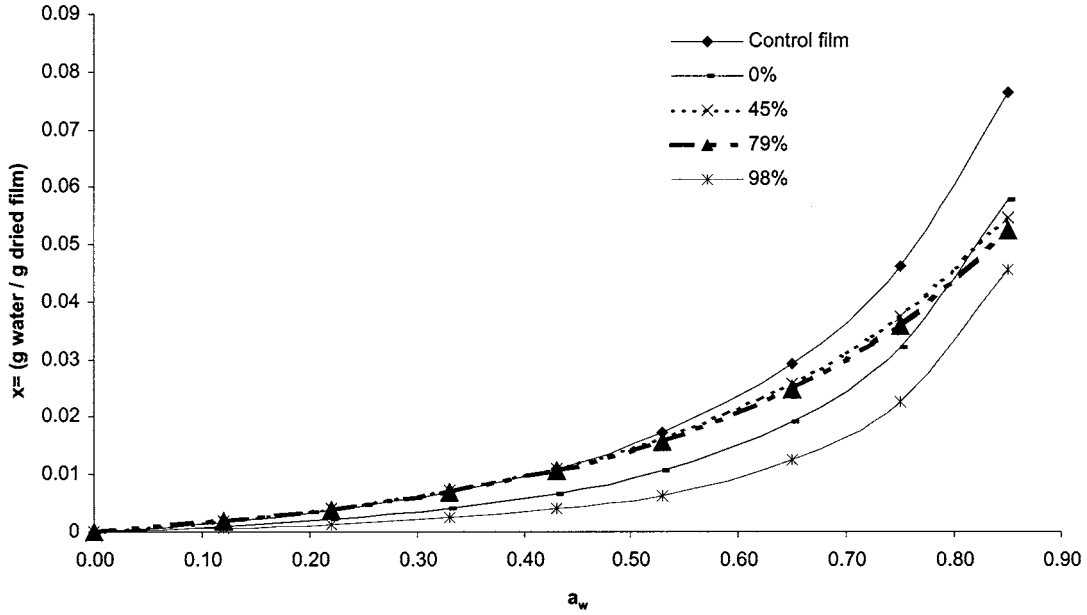

Figure 2. Water sorption isotherms of HPMC films with different cross-linking levels (from 0 to 98%) and control HPMC film. Experimental moisture sorption isotherm values (means of three experiments) were averaged and fitted by the GAB model.

Table 2. Influence of the Thermal Processing on the Residual Inhibitory Activity[a]

	film incorporating nisin and contents of citric acid and catalyst corresponding to 98% cross-linked matrix		nisin (powder)		control HPMC–nisin film	
duration of thermal processing at 190 °C (min)	0	15	0	15	0	15
residual antimicrobial activity (%)	100	0	100	100	100	55

[a] Residual activity against *M. luteus* strain of different films (containing nisin, citric acid, and catalyst and heated or not at 190 °C for 15 min and HPMC control film just containing nisin and heated or not at 190 °C for 15 min) and nisin powder. 100% corresponded to 14 ± 1 mm growth inhibition diameter.

suggested a weak improvement of moisture barrier properties with the chemical modification extend (*2*).

Water Contact Angle Meter. A complementary method of surface tension measurement was used to investigate the influence of cross-linking level on film liquid water affinity. The water angle was measured by depositing a water drop on the film surface (**Table 1**). The angle could vary between 0 and 90°, respectively, from high water drop surface contact to very low water drop surface contact. The chemical modification did not influence significantly the matrix affinity for liquid water, despite a tendency showing an increase in the contact angle with the percentage of cross-linking. In accordance with results previously obtained, cross-linking slightly increased film hydrophobic character and decreased absorbed water by decreasing water solubility in the film matrix (*2*).

Antibacterial Effectiveness of Films. To study the nisin stability in cross-linked HPMC films, the bioactivities of nisin in different HPMC films were compared. The produced films were based on a solution of HPMC with 15% citric acid (w/w HPMC) and 50% NaH_2PO_4 (w/w citric acid). If the prepared films were treated at 190 °C for 15 min, the matrix was chemically cross-linked (cross-linking level of 98%); without the thermal processing, the matrix was not cross-linked. To incorporate the bacteriocin in the film, 10^3 IU mL^{-1} of nisin was added into the film-forming solution. The thermal processing performed the films. Nisin activity between 98 and 0% cross-linked films was compared against *M. luteus*.

No residual antibacterial activity was observed from 98% cross-linked films with 10^3 IU mL^{-1} of nisin (data not shown). The nisin concentration was then increased to 10^5 IU mL^{-1}. For clarity, the antibacterial activity of films was expressed as a percentage compared to control HPMC films and was determined on *M. luteus* as reference strain. Despite the incorporation of a significant content of nisin, the cross-linked films were completely inactive (**Table 2**). Two assumptions can be proposed.

The high temperature and citric acid incorporation necessary for cross-linking reaction would denature the peptide. To verify this, powdered nisin was heated at 190 °C for 15 min. It was then suspended in 0.05 M phosphate buffer, pH 6.1, before its activity against *M. luteus* was tested. Nisin was also suspended in 0.05 M phosphate buffer, supplemented with citric acid at 15% (w/v) level (**Table 2**). One hundred percent of the residual antibacterial activity was obtained after thermal processing, showing that nisin was not denatured by the time/temperature conditions of the cross-linking process (190 °C, 15 min) and that citric acid did not influence nisin stability. This result was predicted because of the thermostability of the bacteriocin nisin.

Chemical bonds could appear between nisin and other components of film during the heat treatment. The primary amine group from the N -terminal position and from the lysine residues could react on the carboxylic function available on citric acid to form amide bonds. In addition, HPMC could potentially graft nisin via esters bonds from the nisin C-terminal carboxylic acid group and cellulosic hydroxyl group. In both cases, nisin desorption would be strongly limited, involving a weak bioactivity. Thermically treated nisin films, without citric acid and catalyst, were elaborated, and the antibacterial activity was determined (**Table 2**). A residual activity of ∼55% was obtained. As a result, the loss of film bioactivity could not be

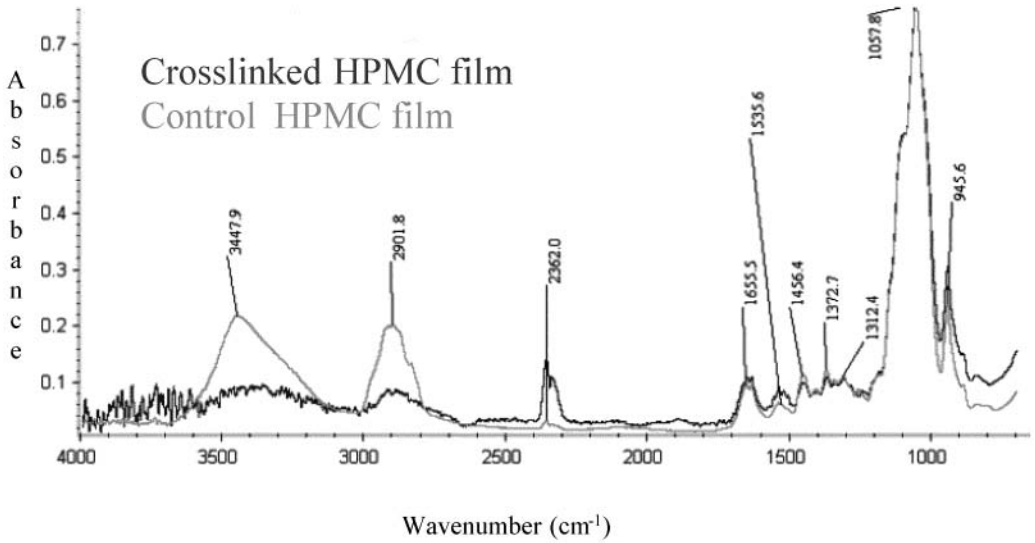

Figure 3. FTIR spectra of cross-linked HPMC films with different cross-linking levels (from 0 to 98%) and control HPMC film.

due to the thermosensitivity as observed before, but probably to a nisin retention in the film matrix via chemical bonds with HPMC or with citric acid. ATR infrared spectra were recorded (**Figure 3**). Spectra showed specific bands of nisin (1200−1700 cm^{-1}) with particularly the increase in absorption intensity of peptide bonds (CO−NH bending vibration around 1655 cm^{-1}) and the contribution of nisin in the hydrocarbon chains of approximately 1460 and 1370 cm^{-1}, the CH bending vibration of the methyl group. In addition, there was no significant influence of the cross-linking thermal treatment on the absorption intensity of the ester bonds (1735 cm^{-1}). Potential amide bonds from nisin and other film components could not be determined from FTIR analysis because of peptide bonds. Ester bonds between nisin and HPMC were not highlighted, but the sensitivity of IR could not allow ester band visualization, taking into account the low nisin concentrations in HPMC films (0.1% w/w dry mass).

In conclusion, cross-linking appears to be unsuited to keep the antimicrobial activity of this homogeneous formulation and one-stage-produced films. Another method, based on the adsorption of bacteriocin to cross-linked films, was conducted, and the activity of the bioactive film was determined qualitatively according to the disk diffusion assay on *M. luteus*. The size of the clear zone of cross-linked films was taken to be equal to the bioactivity of non-cross-linked film produced from 10^4 IU mL^{-1} of nisin (data not shown). Preliminary assays were conducted to decrease the adsorption process duration used by Scannell et al. (*9*), 40 h, and to facilitate an industrial application. The inhibition zones of films versus adsorption duration are presented **Figure 4**. As a result, 1.5 h of bacteriocin adsorption was sufficient to produce the bioactive films. According to Appendini et al. (*21*), proteins have a strong capacity of adsorption on biopackaging due to their amphiphilic character and the antimicrobial activity of films increases with the hydrophilic nature of the polymeric matrix.

The FTIR analysis of bioactive films allowed verification of the nisin adsorption on the material surface (data not given). The specific band of the bacteriocin (1655 cm^{-1}, CO−NH bending vibration) and the band related to the CH bending vibration of the methyl group (1460 and 1370 cm^{-1}) increased in adsorption intensity compared to control film without nisin.

The activity against *M. luteus* growth of cross-linked films charged with adsorbed nisin from a 10^4 IU mL^{-1} nisin solution is presented **Table 3**. A 15 mm growth inhibition diameter was

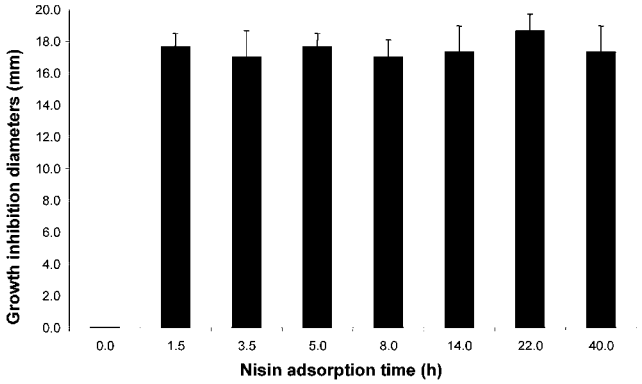

Figure 4. Influence of the adsorption time on cross-linked HPMC film bioactivity against *M. luteus*. Films were charged with adsorbed nisin from a 10^4 IU mL^{-1} nisin solution. Each point of the graph is the mean of three experiments, and the standard deviations are vertical lines calculated using Student's *t* test ($p < 0.05$).

Table 3. Antibacterial Activity against *M. luteus* from the 98% Cross-Linked HPMC Film after 2 h of Nisin Adsorption from a 10^4 IU mL^{-1} Nisin Solution, from the Desorbed Cross-Linked HPMC Film, and of the Desorption Solution[a]

	cross-linked film after nisin adsorption	cross-linked film after nisin desorption	solution of desorption
antibacterial activity as growth inhibitor diameter (mm)	15 ± 2	7 ± 1	10 ± 1

[a] Values are expressed in growth inhibition diameter (mm). Data, the mean of three experiments, are followed by their standard deviations.

observed. After the desorption procedure, there was >67% of residual antibacterial activity. Although the nisin concentration was not linearly correlated to the growth inhibition zones, we conclude that a sufficient rate of nisin can still desorb from film to give a potentially effective antibacterial packaging. This is particularly interesting to food applications, because the film would thus protect the foodstuff surface, which is very sensitive to bacterial development.

Conclusions. Cross-linked HPMC is a promising active support, which could be potentially useful in active food packaging elaborated from renewable resources to control bacterial development. The cellulose polymer network of the

film was resistant to water solubilization, and the cross-linking level tends to slightly reduce its hygroscopic properties. By cross-linking HPMC with citric acid, films showed brittle stress strain behavior. Flexibility has to be improved in order to produce biopackaging for food applications by increasing plasticizer content. From nisin adsorption experiments, it was concluded that bioactive cross-linked film could minimize the microbial contamination of food product surfaces. The release of nisin into model food products will result in an immediate reduction of bacterial Gram-positive population associated with a surface antimicrobial activity during storage, transportation, and handling. Moreover, the biodegradability of films must be evaluated to verify that the development of cross-linked films would reduce environmental pollution.

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