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Genipin Cross-Linked Nanocomposite Films for the Immobilization of Antimicrobial Agent

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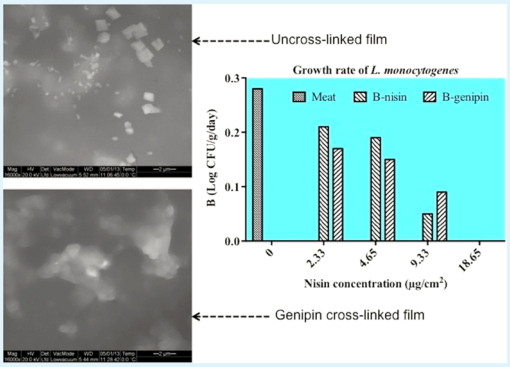
ABSTRACT: Cellulose nanocrystal (CNC) reinforced chitosan based antimicrobial films were prepared by immobilizing nisin on the surface of the films. Nanocomposite films containing 18.65 $\mu g/cm^2$ of nisin reduced the count of *L. monocytogenes* by 6.73 log CFU/g, compared to the control meat samples (8.54 log CFU/g) during storage at 4 °C in a Ready-To-Eat (RTE) meat system. Film formulations containing 9.33 $\mu g/cm^2$ of nisin increased the lag phase of *L. monocytogenes* on meat by more than 21 days, whereas formulations with 18.65 $\mu g/cm^2$ completely inhibited the growth of *L. monocytogenes* during storage. Genipin was used to cross-link and protect the activity of nisin during storage. Nanocomposite films cross-linked with 0.05% w/v genipin exhibited the highest bioactivity (10.89 $\mu g/cm^2$) during the storage experiment, as compared to that of the un-cross-linked films (7.23 $\mu g/cm^2$). Genipin cross-linked films were able to reduce the growth rate of *L. monocytogenes* on ham samples by 21% as compared to the un-cross-linked films. Spectroscopic analysis confirmed the formation of genipin-nisin-chitosan heterocyclic cross-linked network. Genipin cross-linked films also improved the swelling, water solubility, and mechanical properties of the nanocomposite films.



KEYWORDS: *chitosan, nisin, genipin, surface modification, Listeria monocytogenes, antimicrobial films*

INTRODUCTION

The purpose of food packaging is to preserve the quality and safety of the packaged food. In the context of a constantly growing population and globalization of markets, prevention of food contamination by microorganisms or pathogens is becoming increasingly important. In the United States, foodborne diseases cause 9.4 million illnesses, 55 961 hospitalization, and 1391 deaths each year.[1] Antimicrobial packaging is gaining interest from researchers and industries due to its potential to prevent the growth of pathogenic bacteria in food products.[2] Antimicrobial packaging can be defined as the incorporation of antimicrobial agent into packaging in order to prevent surface growth of microorganisms and pathogenic bacteria in foods, thus ensuring the quality and safety of food products during storage.[3] Direct incorporation of antimicrobials into food may lead to drastic loss of antimicrobial efficacy due to the inactivation of the antimicrobials by food components or dilution below active concentration due to migration into the bulk food matrix. Antimicrobial films provide an innovative alternative and can reduce the addition of larger quantities of antimicrobials that are usually incorporated directly into the food bulk. These films can also allow a better efficiency, stability, and controlled release of the antimicrobials to the food surface.[4,5]

Cellulose nanocrystal (CNC), which is also known as nanocrystalline cellulose or cellulose nanowhisker, is made up of highly crystalline, nanosized, rodlike particles that are extracted from softwood bleached kraft pulp by a controlled acid hydrolysis process.[6] CNC extracted from wood was found to exhibit an average length of 110 nm long for a 5−10 nm width.[7] Natural nanocrystals such as CNC have been used in great effects to enhance the mechanical and barrier properties of chitosan films.[8,9] Chitosan is a natural linear polysaccharide made up with of 1,4-linked 2-amino-deoxy-β-D-glucan. Chitosan has been found nontoxic, biodegradable, biofunctional, and biocompatible.[10] While several researchers have reported strong antimicrobial and antifungal activities of chitosan suspension,[11−14] films made from chitosan did not demonstrate any antimicrobial efficacy.[15] The use of natural antimicrobials such as bacteriocins in combination with natural biopolymeric films represent an interesting and highly potential field in the development of environmentally friendly, active packaging materials.

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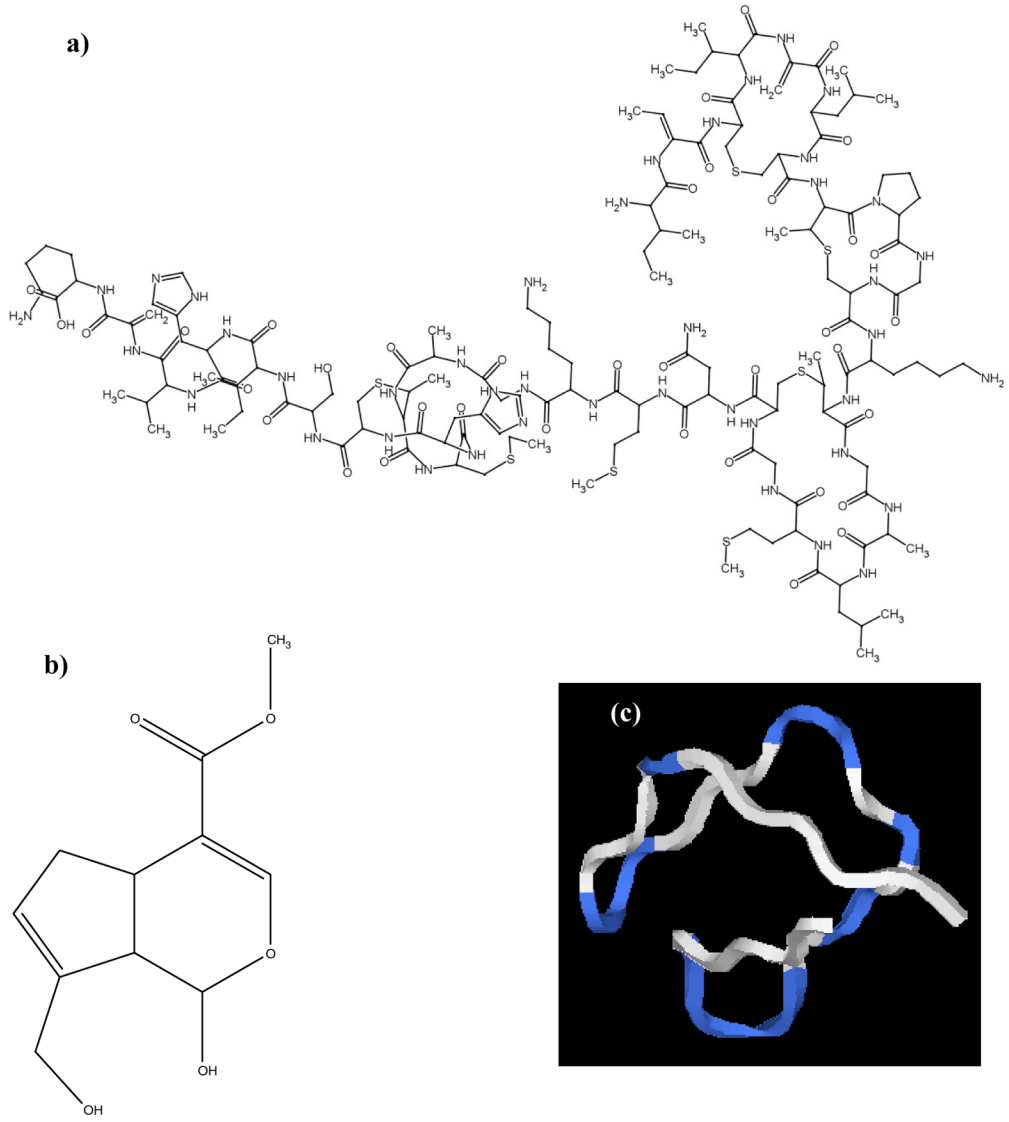

Figure 1. Chemical structure of (a) nisin and (b) genipin (drawn using MarvinSketch 6.2.0, 2014). The secondary structure of (c) nisin was generated from the nisin peptide sequence using I-TASSER server.[16,17]

Nisin is a bacteriocin (Figure 1a,c) produced by the lactic acid bacterium, *Lactococcus lactis*, subsp. *lactis* is one of the most effective antimicrobial agents when it comes to food packaging.[18] It is mostly effective against lactic acid bacteria and other Gram-positive organisms.[19] Nisin is the only bacteriocin that has GRAS (generally considered as safe) status by both the Food and Drug Administration (FDA) and World Health Organization (WHO).[20] The activity of nisin may be lost during storage in food products due to enzymatic degradation and interaction with food components such as protein and fats.[21] Genipin, which is a naturally occurring cross-linking agent, can be used to protect the activity of nisin during storage. Genipin (Figure 1b) is derived from the fruits Genipa Americana and Gardenia jasminoides Ellis.[22] It has the ability to covalently cross-link with amino acids or proteins and has been reported to be 5000−10000 times less cytotoxic than other commonly used cross-linking agent such as glutaraldehyde.[23] Due to its biocompatibility and low toxicity, genipin is recently being used in biomedical applications[23−25] and for controlled drug release.[26−28] Genipin cross-linked biopolymeric films should have high potential in food packaging application.

The objective of this study was to develop an antimicrobial nanocomposite film by immobilizing nisin onto the polymer surface. The antimicrobial efficacy of the nanocomposite films were evaluated *in situ* on Ready-To-Eat (RTE) ham samples during storage. Then the optimal concentration of nisin was used to be cross-linked with different concentration of genipin and the bioactivity of the films was tested *in vitro*. The antimicrobial efficacy of the cross-linked films were also tested *in situ* and compared with that of the un-cross-linked films. Spectroscopic analysis was performed in order to characterize and explain the mechanism of genipin as cross-linking agent. Also, the effect of genipin cross-linking on the mechanical properties, water resistance, and surface morphology of the films was evaluated.

MATERIALS AND METHODS

Preparation of the Nanocomposite Films. In our previous study, CNC improved the mechanical strength of the chitosan films and the optimum CNC concentration was 5% (w/w of chitosan).[8] The nanocomposite films, in the current study, were prepared following a modified method as described in Khan et al.[8] At first, a dilute (0.1% w/v) CNC suspension was prepared by dispersing spray-dried CNC powder (FPInnovations, Pointe-Claire, QC, Canada) in deionized water under

magnetic stirring. Then a 2% (w/v) solution of aqueous acetic acid (Laboratoire Mat, Beauport, Quebec, Canada) was incorporated into the CNC suspension. The CNC suspension was then subjected to ultrasonication (QSonica Q-500, Misonix, Qsonica, LLC, Newtown, CT, USA) at 1000 J/g of CNC. Then a 0.5% ethylene glycol (Laboratoire Mat) and 2% w/v high MW chitosan (DD: 85−90%, viscosity: 2251−2270 mPas, Heppe-medical GmbH, Germany) were incorporated in the suspension. The suspension was then magnetically stirred overnight followed by homogenization with IKA RW-20 mechanical homogenizer at 1500 rpm for 3 h. After homogenization, a paraffin film was wrapped on top of the beaker and kept overnight at 4 °C. The films were made by casting 15 mL aliquots of the chitosan/CNC nanocomposite suspension on Petri dishes which were allowed to dry at room temperature and 30−35% RH. Films were then treated with 1 M NaOH (Laboratoire Mat) for 2 min, washed several times with deionized water, and allowed to dry.

Preparation of Nisin Solution. A stock solution of nisin (Niprosin, purity 2.5%, 77.5% salt, and 20% vegetable protein, Profood, IL, USA) was prepared by dispersing 1 g of the Niprosin powder in 100 mL of deionized water under magnetic stirring. The pH of the suspension was adjusted to 3.0 with diluted lactic acid (3% w/w in water, Laboratoire Mat). It has been reported that nisin is most stable and efficient at pH 3.[29,30] The suspension was stirred for 5 h and kept overnight at 4 °C. Then the suspension was centrifuged for 15 min at 2500 rpm at 4 °C to remove the insoluble fractions; the supernatant was collected and stored at 4 °C.

Immobilization of Nisin onto the Surface of the Films. From the stock solution five different concentrations of nisin solution were prepared by dilution with deionized water and pH of each solution was again adjusted to 3 with lactic acid. Aliquots (15 mL) of nisin solution from each different concentration were applied on the surface of the insoluble chitosan films and allowed to dry for 2 days. The surface area of the films was 50.26 cm^2 (8 cm diameter). The nisin content on each film was calculated according to the amount of pure nisin (in μg) per surface area of the films (in cm^2). The nisin content on the five different formulations was calculated to be 37.30, 18.65, 9.33, 4.66, and 2.33 $\mu g/cm^2$, which corresponds to 1874.8, 937.45, 468.72, 234.36, 117.18, and 58.6 IU of nisin in each film formulation, respectively. The films containing 37.30 $\mu g/cm^2$ of nisin were found to be very brittle and inhomogeneous and were not used for any further experiments.

In Situ Evaluation of the Antimicrobial Activity against *L. monocytogenes*. RTE ham was chosen as a food model to test the antimicrobial activity of the films *in situ* and to optimize the nisin concentration in the films. The *L. monocytogenes* HPB (five strains: 2569, 2558, 2371, 2812, and 1043) used in this experiment were obtained from Health products and Foods Branch of Health Canada (Ottawa, ON, Canada). The microorganisms were kept frozen at −80 °C in tryptic soy broth (Alpha Biosciences, ML, USA) containing glycerol (10% v/v). Before use, the stock cultures were propagated through two consecutive 24 h growth in TSB at 37 °C to obtain the working cultures containing approximately 10^9 CFU/mL. Lean ground pork was purchased from a local grocery store (IGA, Laval, Quebec, Canada). Ground pork was cooked with a salt mixture containing sodium chloride (1.5%), tripolyphosphate (0.43%), sodium erythorbate (750 ppm), and sodium nitrite (100 ppm) (BSA Food Ingredients, St-Leonard, Quebec, Canada) for about 1 h at 162 ± 3 °C in a cooking oven. Following cooking the ham was removed from the oven and placed at 4 °C for 24 h. Then the ham was sliced to 10 g portions. Aliquots (1 mL) of each culture of five strains of *L. monocytogenes* were mixed together to prepare a cocktail mixture. Appropriate dilutions were made in peptone water (0.1%; BD, Sparks, MD, USA) to obtain an inoculation solution containing approximately 10^4 to 10^5 CFU/mL of *L. monocytogenes*. After that, a volume of 500 μL of the inoculation solution was spread on the surface of the ham samples to achieve approximately 10^3 CFU/g of ham. Then the ham samples were sandwiched between two films, vacuum packaged, and stored at 4 °C up to 35 days.

Enumeration of Bacteria. The meat samples were analyzed for the growth of *L. monocytogenes* after 1, 7, 14, 21, 28, and 35 days of storage. On each day of analysis, the nanocomposite films were removed and 3 meat samples from each formulation were put in a sterile filter sample bag (Whirl-Pak; Nasco, Fort Atkinson, WI, USA), diluted 5-fold with peptone water (0.1%; BD) and homogenized in a Lab-blender 400 stomacher (Seward Medical, London, UK) for 1 min at 200 rpm. From this homogenate, appropriate serial dilutions were then plated on PALCAM agar plates (Alpha Biosciences, ML, USA), for the selective enumeration of *L. monocytogenes*. The plates were incubated for 48 h at 37 °C. Following incubation the colony forming units (CFU) were counted using a magnifier and the bacterial counts were expressed as log CFU/g of meat.

Preparation of Genipin-Nisin Cross-Linked Films. Following the *in situ* experiment, the concentration of nisin (18.65 $\mu g/cm^2$) providing the complete inhibition of *L. monocytogenes* was chosen to be cross-linked with genipin (Challenge Bioproducts, Yun-Lin Hsien, Taiwan). Different concentrations of genipin (0.05, 0.1, 0.2, and 0.4% w/v) were mixed with the optimized nisin solution at pH 3 and the reaction was carried out for 24 h at room temperature. Then, 15 mL of the genipin cross-linked nisin solution was applied on the surface of the insoluble chitosan films and allowed to dry for 2 days. The films with nisin only (no cross-linking) were termed as G0 whereas the films with 0.05%, 0.1%, 0.2%, and 0.4% of genipin were termed as G0.05, G0.1, G0.2, and G0.4, respectively. All the films were stored at 4 °C in a desiccator filled with deionized water to obtain 90−100% RH.

Fourier Transform Infrared Spectroscopy (FTIR). The FTIR spectra of the films were recorded using a Spectrum One spectrophotometer (PerkinElmer, Woodbridge, ON, Canada) equipped with an attenuated total reflectance (ATR) device for solids analysis and a high-linearity lithium tantalate detector. Spectra were analyzed using Spectrum 6.3.5 software. Films were stored at 4 °C in a desiccator containing distilled water to ensure a stabilized atmosphere of 90−100% RH. Films were then placed onto a zinc selenide crystal, and the analysis was performed within the spectral region of 850−1750 cm^{-1} with 64 scans recorded at a 4 cm^{-1} resolution. After attenuation of total reflectance and baseline correction, spectra were normalized with a limit ordinate of 1.5 absorbance units.

In Vitro Evaluation of the Bioactivity of the Cross-Linked Films. The bioactivity of the films was tested *in vitro* according to a modified agar diffusion assay against the bacterium *Lactobacillus sakei* ATCC 15521 (American Type Culture Collection, Rockville, MD, USA) in order to optimize the concentration of genipin. *L. sakei* is used as an indicator strain or a standard microorganism to quantify the activity of nisin.[19] All the films (un-cross-linked nisin and genipin cross-linked nisin) were cut into square shapes (1.44 cm^2) and were sterilized using γ-irradiation at 2.5 kGy at the Canadian Irradiation Centre (CIC, Laval, Quebec, Canada) at room temperature. A 20 mL of De-Man Rogosa, Sharp (MRS, Alpha Biosciences, Maryland, USA) agar plates were inoculated with bacterial cultures to obtain colony count of approximately 10^8 CFU/mL. The films were then put onto the inoculated MRS agar plates and were incubated at 37 °C for 72 h. The plates were examined for the "zone of inhibition" and the diameter of the zone was measured with a caliper (mm). From the inhibition diameter the area of the whole zone was calculated and reported as the surface area of inhibition. A standard curve was generated by plotting the inhibition area (cm^2) of the different concentrations of un-cross-linked nisin films in the *X*-axis against the nisin concentration ($\mu g/cm^2$) in the films (*Y*-axis). Regression analysis was performed to determine the standard equation. The inhibition areas of the films (at 4 °C and 90−100% RH) were measured (as described before) during 1, 7, 14, 21, 28, and 35 days of storage.

Swelling Ratio and Water Solubility of the Films. Swelling ratio (SR) of the films was determined gravimetrically according to a modified procedure as described by Jin et al.[31] Preweighed films (1.44 cm^2) were immersed in a beaker containing 100 mL of distilled water at room temperature. Films were then removed at specified time intervals (1, 2, 4, 8, and 24 h) and weighed immediately after removing the surface adsorbed water with a filter paper. The SR was calculated according to the following equation:

$$SR = [(W_t - W_0)/W_0] \times 100 \quad (1)$$

where W_0 and W_t are the weight of the films before and after immersion, respectively.

Water solubility (WS) of the films was tested in order to determine the resistance of the films in water. At first films (1.44 cm^2) were dried at 105 °C for 24 h to determine initial dry matter. Then they were immersed in 100 mL of distilled water in a beaker at room temperature, covered with paraffin film, and kept for 24 h. Then films were removed from the beaker and dried again at 105 °C for 24 h to determine final dry matter. The WS was calculated by the following equation:

$$WS = [(W_i - W_f)/W_f] \times 100 \quad (2)$$

where W_i and W_f are the weight of the initial and final dry matter of the films, respectively.

Mechanical Properties of the Films. Tensile strength (TS), tensile modulus (TM), and elongation at break (Eb) of the films were measured by using a universal testing machine (model H5KT, with a 1 kN load cell, Tinius-Olsen, Horsham, USA) according to a method described in Huq et al.[32] The film thickness was measured using a Mitutoyo Digimatic Indicator (Type ID-110E; Mitutoyo Manufacturing Co. Ltd., Tokyo, Japan) at five random positions around the film. All the films were equilibrated at 4 °C and 90−100% RH prior to mechanical analysis.

Antimicrobial Activity of the Cross-Linked Films against *L. monocytogenes*. Following the *in vitro* and physicochemical experiment, the concentration of genipin (0.05%) providing the maximum bioactivity at the end of the storage and mechanical properties was used to prepare films with the previously presented nisin concentrations (18.65 to 2.33 $\mu g/cm^2$). The films were tested *in situ* (as described before) during storage of ham against *L. monocytogenes*.

Bacterial Growth Rate Calculation. The growth rate (B) of *L. monocytogenes* on ham samples can be described according to the Gompertz equation,[33] over duration of 35 days.

$$N_t = A + C \exp[-\exp\{-B(t - M)\}] \quad (3)$$

where N_t = microbial count (Log CFU/g at t); A = lower asymptotic line of the growth curve (initial bacterial count); C = difference between upper asymptotic line (N_{max} = maximum population level) and lower asymptotic line; B = relative maximum growth rate at time M; M = time at which maximum growth rate is obtained.

The bacterial growth data were analyzed using the program DMFit 3.0 for Microsoft Excel based on Baranyi and Roberts.[34] The B values of *L. monocytogenes* in control ham, ham covered with un-cross-linked films (no genipin), and ham covered with cross-linked films in the presence of 0.05% genipin were presented as B-ham, B-nisin, and B-genipin, respectively.

Scanning Electron Microscopy (SEM). The SEM investigation of the film samples (5 × 5 mm^2) were performed on an Environmental SEM (ESEM, Quanta 200 FEG, FEI Company Hillsboro, OR, USA) under low vacuum mode with an accelerating voltage of 20.0 kV and at 0 °C temperature. ESEM eliminates the need for any conducting coating thus preventing any potential damage of the samples due to coating. The microscope was equipped with an energy dispersive X-ray (EDX) spectrometer (Genesis 2000, XMS System 60 with a Sapphire Si/Li detector from EDAX Inc., Mahwah, NJ, USA).

Statistical Analysis. All the *in situ* analysis was performed with three different meat samples (per day) for each film specimens and plated in three different Petri dishes. The swelling ratio, water solubility, and *in vitro* assay were performed with three different film specimens and the average value was reported. The mechanical analysis was performed with six different film specimens and the average value was reported. An analysis of variance (ANOVA) and multiple comparison tests of Turkey's-b were used to compare all the results for each analysis together. Differences between means were considered significant when the confidence interval is smaller than 5% ($P \leq 0.05$). The analysis was performed by the PASW Statistics 18 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Antimicrobial Activity in Situ. This experiment was designed to evaluate the antimicrobial efficacy of the films in a RTE ham system during storage. The growth of *L. monocytogenes* was exponential up to 21 days in the ham samples wrapped with control films (no nisin) and then the bacterial growth reached the stationary phase with limited growth rate. After 35 days of storage there was no significant ($P > 0.05$) difference in the bacterial count between the control ham (ham without film) (8.42 log CFU/g) and control film (film without nisin) (8.54 log CFU/g) samples (Figure 2). The films containing 2.33 $\mu g/cm^2$ of nisin

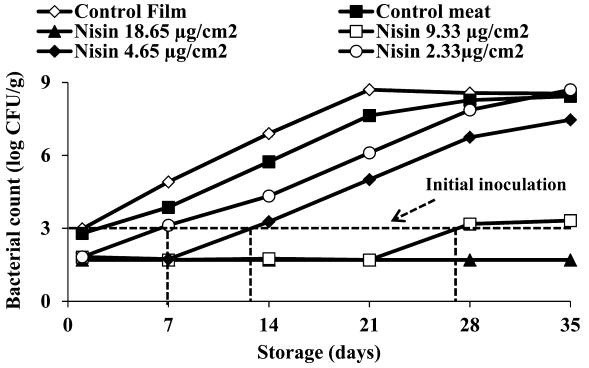

Figure 2. Antimicrobial activity of nisin films *in situ* against *L. monocytogenes* during storage at 4 °C.

reduced the count of *L. monocytogenes* by 1.2 log CFU/g at day 1 but this film was not able to prevent the growth of the *L. monocytogenes* during storage. After 35 days of storage the level of *L. monocytogenes* on ham covered with the films containing 2.33, 4.65, 9.33, and 18.65 $\mu g/cm^2$ was 8.7, 7.47, 3.32, and 1.7 log CFU/g, respectively. As a result, the count of *L. monocytogenes* was reduced by almost 0.95, 5.10, and 6.72 log CFU/g, respectively, by the films containing 4.65, 9.33, and 18.65 $\mu g/cm^2$ of nisin after 35 days of storage, compared to the control meat samples. Also, the films containing 18.65 $\mu g/cm^2$ completely inhibited the growth of *L. monocytogenes* during storage and the bacterial count was below the detection limit (1.7 log CFU/g). The antimicrobial films also increased the lag phase of *L. monocytogenes* on ham surface. According to Robinson et al.[35] the lag phase of bacterial growth can be defined as the time interval between the inoculation of a bacterial culture and the time its growth rate is maximum. The lag phase can be measured as the point (days) at which the slope of the exponential phase of growth intercepts a horizontal line drawn from the initial cell concentration. The lag phase of *L. monocytogenes* increased from 7 to more than 35 days with the increase of nisin concentration from 2.33 to 18.65 $\mu g/cm^2$. Other researchers have also reported antimicrobial activity of nisin against *L. monocytogenes*.[18,36,37] Nguyen et al.[38] prepared bacterial cellulose films with 2500 IU/mL of absorbed nisin and tested the efficacy of the films on sausage meat against *L. monocytogenes*. The authors reported that the antimicrobial films reduced the population of *L. monocytogenes* by 2 log CFU/g, under refrigerated condition after 14 days of storage. Marcos et al.[39] reported the antimicrobial activity of alginate films containing 2000 AU/cm^2 of enteriocin (a bacteriocin) against *L. monocytogenes* on vacuum packed RTE ham samples. The antimicrobial films initially (up to day 15) inhibited the growth of *L. monocytogenes* but could not prevent the growth during long-term storage. After 29 days of storage, the count of *L. monocytogenes* on RTE ham samples was 1.7 log CFU/g lower with antimicrobial films than that with the control films without nisin.

Chitosan based films are also believed to have antimicrobial activity but, in this experiment, the control films could not

prevent the growth of *L. monocytogenes*. The antimicrobial activity of chitosan is related to its molecular weight, degree of deacetylation, viscosity, pH, concentration of the solution, and so forth, and the activity varies against different microorganisms.[12,14,40] Also, the cationic amino groups of chitosan play an important role in it is antimicrobial activity. The positively charged amines interact with the negatively charged bacterial cell membrane, causing a leakage of intracellular constituents.[41] However, in this experiment the chitosan-based nanocomposite films were treated with NaOH in order to make them insoluble. As a result, the positively charged amine groups were neutralized, thus diminishing their antimicrobial activity. Ouattara et al.[42] (2004) also reported that neutralized chitosan films could not prevent bacterial growth when applied on the surface of processed meats. The authors suggested that dispersion of the chitosan molecules within the meat matrix is a prerequisite for the antimicrobial activity of chitosan. Foster amd Butt[15] reported strong bactericidal activity of chitosan solution against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Escherichia coli*; on the contrary the chitosan films made from the same solution did not exhibit any antimicrobial activity. The authors postulated that the antimicrobial activity of chitosan is due to surface−surface interaction between the chitosan chains and microbial cell walls.

The antimicrobial efficacy of the films containing nisin, observed in this study, can be in part explained by the mechanism of nisin's surface adsorption. Nisin is an amphiphilic molecule containing hydrophobic and hydrophilic residues. The N-terminal part of nisin contains a large number of hydrophobic residues and the C-terminal part is considered to be hydrophilic due to the presence of positively charged lysine and histidine residues.[43] During adsorption of nisin onto a hydrophilic surface like chitosan films, the hydrophilic part of nisin is oriented toward the film surface, thus establishing enhanced surface−protein hydrophilic association, and the hydrophobic part is oriented outward.[44] Considering the orientation of nisin on the surface of the chitosan films, the "Barrel-stave" mechanism can be used to describe the antimicrobial activity of nisin against *L. monocytogenes*. The "Barrel-stave" mechanism describes the bactericidal activity of individual peptide molecules oriented in such a way so that the hydrophilic amino acid residues are on the inside and their hydrophobic residues are facing outward. According to this mechanism, the N-terminal (hydrophobic) residues of nisin disrupt the cytoplasmic membrane of susceptible bacteria through the formation of pores in the membrane.[45−49] So, the orientation of nisin on the surface of chitosan films plays a major role in the antimicrobial activity of the films and the "Barrel-stave" model helps to explain the mechanism of bactericidal action.

Spectroscopic Analysis of the Films. ATR-FTIR analysis was performed to characterize and determine changes in the infrared bands related to the nisin immobilization and genipin cross-linking of the film. The FTIR spectra of the nanocomposite films with surface immobilized nisin (un-cross-linked) are presented in Figure 4. The spectra revealed characteristic changes in the region of 1750−850 cm^{-1} due to the incorporation of nisin, as compared to the chitosan based control (no nisin) films. The spectra of the control film (Figure 3a) revealed characteristics band at 1640 cm^{-1} (corresponding to Amide I, C═O stretch combined with N−H deformation in amides), 1560 cm^{-1} (corresponding to Amide II, N−H deformation in amides combined with $-NH_3^+$ deformation), 1402 cm^{-1} (corresponding to Amide III band, C−N stretch in

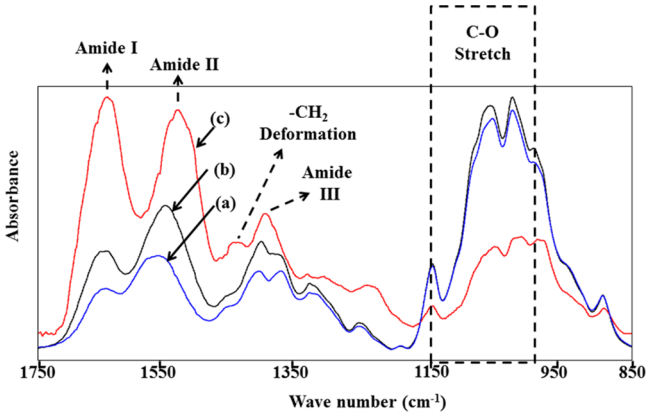

Figure 3. FT-IR spectra of the (a) control nanocomposite (no nisin) films and nanocomposite films containing (b) 2.33 and (c) 18.65 $\mu g/cm^2$ nisin.

primary amides combined with COO− symmetric stretch in carboxylic acid salts), 1335 cm^{-1} (corresponding to $-CH_3$ symmetric deformation), 1150 cm^{-1} (corresponding to anti-symmetric CH−O−H stretch in secondary alcohols), 1060 cm^{-1} (corresponding to CH_2-O-H stretch in primary and cyclic alcohols), and at 1030 cm^{-1} (corresponding to carbon ring in cyclic compounds).[8,50,51] The absorbance of the Amide I, II, and III bands increased with increase in nisin concentration (Figure 3b and c), indicating immobilization of nisin onto film surface.[52] A shift of the Amide II band toward lower wavenumber (1560 to 1535 cm^{-1}) was also observed. This shift suggested possible hydrogen bonding-induced stabilization of the films in the presence of nisin.[53] The appearance of a new band at 1442 cm^{-1} (correspond to $-CH_2$ scissors deformation) for the films with 18.65 $\mu g/cm^2$ of nisin (Figure 3c) could be due to the amino acid residues in nisin. The decrease in the absorbance of the characteristic saccharide bands (such as 1150, 1060, 1025 cm^{-1}) at high nisin concentration (18.65 $\mu g/cm^2$), possibly due to the masking of the resonance, provides further evidence of surface immobilization of nisin.

The spectra of the genipin−nisin cross-linked films was compared with those of the un-cross-linked films (Figure 4) and revealed some interesting changes that were used to explain the reaction mechanism of genipin cross-linking. The absorbance of the Amide II (1535 cm^{-1}) and III (1402 cm^{-1}) bands decreased

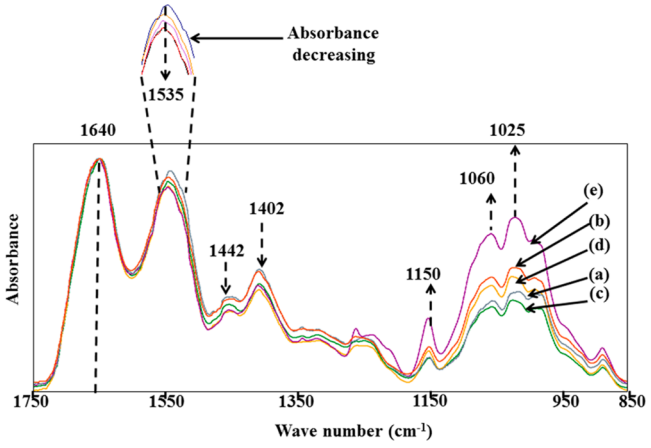

Figure 4. FT-IR spectra of the (a) un-cross-linked, (b) 0.05, (c) 0.1, (d) 0.2, and (e) 0.4% genipin cross-linked films.

with the increase in genipin concentration, whereas the absorbance of the Amide I band (1640 cm^{-1}) remained constant for all samples. As a result, the ratio of Amide I/Amide II band absorbance increased with the increase in genipin concentration. The absorbance of the $-CH_2$ band (1442 cm^{-1}) decreased whereas, the absorbance of bands at 1060 and 1025 cm^{-1} increased with genipin cross-linking.

According to the literature,[26,54] genipin undergoes two different cross-linking reactions involving two different sites on its structure. One of the reaction mechanisms is the nucleophilic substitution of the ester group of genipin by primary amine group leading to the formation of secondary amide linkage (mechanism I). The decrease in the absorbance of the Amide II band suggested formation of secondary amide linkage between genipin and surface immobilized nisin,[54] while the other reaction mechanism is the ring opening reaction of the genipin molecule at C-3 position leading to the formation of a heterocyclic cross-linked compound (mechanism II). The ring opening reaction begins with a nucleophilic attack by the amino group on the C-3 carbon atom of genipin and formation of an intermediate aldehyde group. It proceeds further through the opening of the dehyropyran ring of genipin, followed by attack on the intermediate aldehyde group by the secondary amine group leading to the formation of a bifunctional heterocyclic compound.[25] The increase in the absorbance ratio of Amide I/Amide II band indicated formation of tertiary amide (heterocyclic pyridine derivative) linkage between genipin and the surface immobilized nisin.[22] The decrease of Amide III band correlated with genipin cross-linking could be due to a decrease of primary amides into secondary amides and tertiary amides. The increase in absorbance at 1060 and 1025 cm^{-1} could be related to the presence of genipin functional groups. Thus, the decrease in Amide II and III band coupled by the increase in the absorbance of Amide I/Amide II band allowed confirming the cross-linking of genipin via primary amino groups of surface immobilized nisin to form secondary amide linkage and the formation of bifunctional heterocyclic compounds after ring opening reaction.

Effect of Genipin Cross-Linking on the Bioactivity of Nisin during Storage. The nanocomposite film containing 18.65 $\mu g/cm^2$ was considered an optimized nisin concentration as it completely inhibited the growth of *L. monocytogenes* during storage and was chosen for the characterization of genipin cross-linked films. The *in vitro* bioactivity of the films is presented in Figure 5a. At day 1, the inhibiton area of the film formulations G0, G0.05, G0.1, G0.2 and G0.4 were 11.43, 4.89, 5.08, 5.17, and 2.35 cm^2, respectively. Initially, the bioactivity of the genipin cross-liked films was significantly lower ($P \leq 0.05$), compared to the un-cross-linked films regardless of the genipin concentration. The bioactivity of the films (un-cross-linked and cross-linked) revealed an intriguing outcome during further storage days. The bioactivity of the un-cross-linked films was stable up to 7 days but it started to decline after that and continued to decline in later days. At day 35, the inhibition area obtained from the un-cross-linked films was 8.04 cm^2, which was significantly lower ($P \leq 0.05$) than that of the films cross-linked with 0.05% genipin (10.84 cm^2).

The inhibition area of the films was correlated with the standard curve (Figure 5b) to calculate the available nisin remaining in all film formulations (G0, G0.05, G0.1, G0.2, and G0.4). The standard curve fitted the experimental data well, as the regression coefficient (R^2) of the curve was found to be 0.99. The estimated available nisin content in the films during storage

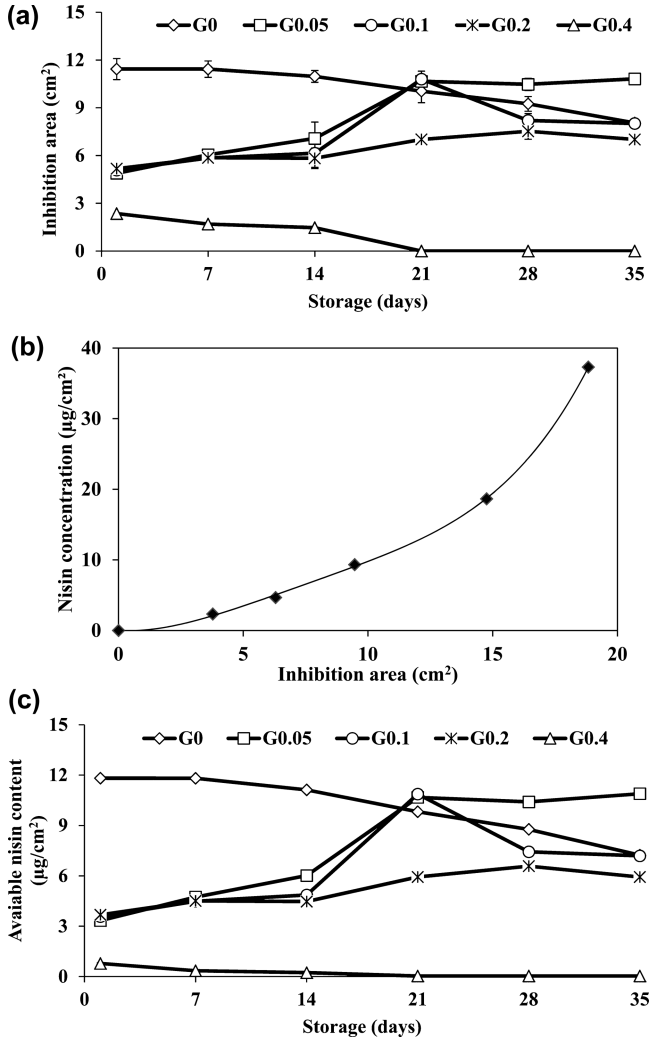

Figure 5. (a) *In vitro* bioactivity of the films during storage at 4 °C and 90−100% RH. (b) A 4th order polynomial "standard curve" of inhibition area (cm^2) versus nisin concentration ($\mu g/cm^2$). (c) Available nisin content of the films during storage at 4 °C and 90−100% RH.

was calculated from the fourth-order polynomial equation (eq 4) and is presented in Figure 5c.

$$Y = 0.0009X^4 - 0.0264X^3 + 0.2956X^2 - 0.243X + 0.0335 \quad (4)$$

where Y is the available nisin content in the films and X is the inhibition area.

Similar to the inhibition zones, the initial nisin content in the cross-linked films seemed to be lower than that of the un-cross-linked films. At day 1, the nisin content of the un-cross-linked films was 11.81 $\mu g/cm^2$, whereas the nisin of the films cross-linked with 0.05%, 0.1%, 0.2%, and 0.4% of genipin were 3.34, 3.57, 3.68, and 0.78 $\mu g/cm^2$, respectively. However, the nisin content of the un-cross-linked films decreased during storage and after 35 days of storage the nisin content was 7.23 $\mu g/cm^2$. So, the nisin content, after 35 days, was almost 40% lower than its initial content. It is very interesting to note that contrary to the un-cross-linked films, the bioactivity of the films cross-linked with 0.05%, 0.1%, and 0.2% of genipin increased during storage. Despite having low initial bioactivity, films cross-linked with 0.05% genipin exhibited the highest bioactivity (10.89 $\mu g/cm^2$) at the end of the storage experiment. At day 35, the nisin content

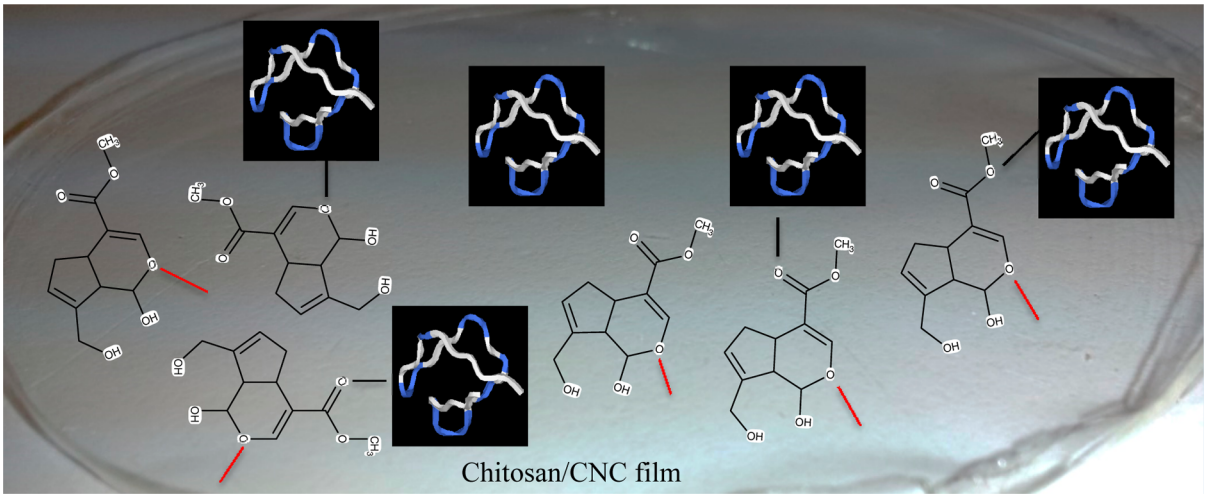

Figure 6. Schematic representation of the immobilization of nisin on the surface of the chitosan/CNC films due to genipin cross-linking. Black lines indicate possible linkage between nisin and genipin; red lines indicate possible linkage between genipin and chitosan.

of the 0.05% genipin films were significantly higher ($P \leq 0.05$) than both the un-cross-linked and 0.1% genipin cross-linked films.

The reason behind the initial low activity of the cross-linked films could be due to the immobilization of the nisin on to the surface of the films as a result of cross-linking. Sebti et al.[52] also reported low activity of the cross-linked nisin-hydroxypropylmethylcellulose (HPMC) films compared to the un-cross-linked nisin-HPMC films. Genipin undergo pH dependent cross-liking reaction with primary amine groups. At pH 3, genipin will have weak interaction with the protonated lysine and histidine residues present at the C-terminal position of nisin. When the genipin−nisin formulation was applied on the surface of the chitosan films, the neutralized chitosan surface favored the ring opening polymerization reaction of genipin.[55,56] So, it can be hypothesized that one of the sites of genipin reacted with the chitosan backbone, while the other sites reacted with the nisin, thus immobilizing nisin on the surface of the films (Figure 6). FTIR spectra also confirmed the formation of heterocyclic genipin-surface immobilized nisin cross-linked network. The increase in the activity of the films cross-linked with low genipin concentration can be attributed to the storage condition (in 90−100% RH) of the films. The high RH caused chain-relaxation of the genipin-chitosan cross-linked network; as a result more nisin was available on the surface of the films.[54] Balaguer et al.[57] have also reported that RH induced release of nisin from cross-linked giladin films. Films cross-linked with 0.4% genipin showed very negligible bioactivity throughout experiment. The reason could be due to the use of high concentration of cross-linking agent. With the increase of cross-linking agent, more active sites are available to link with the chitosan surface and nisin; as a result the cross-linked network irreversibly immobilized nisin. From this study, it could be suggested that low concentration of genipin can be used to cross-link and protect the activity of nisin during storage. Thus, films prepared or cross-linked with a natural cross-linking agent like genipin can offer effective inhibition of microorganisms in food by slow diffusion of the antimicrobial agents.

Swelling Ratio and Water Solubility of the Films. The swelling behavior of the films as a function of time is presented in Figure 7a. The swelling ratio (SR) of the un-cross-linked films increased up to 8 h and reached equilibrium thereafter. Genipin

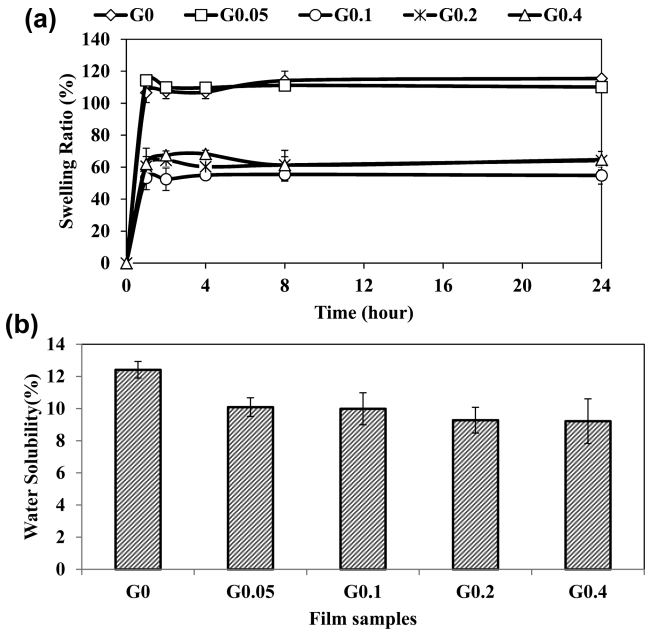

Figure 7. (a) Effect of the genipin concentration on the swelling ratio of the films. (b) Effect of the genipin concentration on the water solubility of the films.

cross-linking decreased the SR of the surface films, and there were significant differences ($P \leq 0.05$) in the SR between the un-cross-linked and films cross-linked with 0.1% to 0.4% genipin. After 24 h the SR of the un-cross-linked films was 114%, whereas the SR of the 0.1%, 0.2%, and 0.4% genipin cross-linked films were 54%, 64%, and 64% ($P \leq 0.05$), respectively. These results showed that incorporation of 0.1% genipin was enough to reduce the SR of films by more than half of its original value. The decrease in the SR of the genipin cross-linked films could be due to the formation of genipin-chitosan-nisin cross-linked network, as described previously. However, higher concentration of genipin (0.2% and 0.4%) could not decrease the SR more, because genipin was applied on the surface of the films, so it could react and form a cross-link network only with the outer surface. Yuan et al.[58] also reported significant decrease in the SR of the chitosan microspheres due to genipin cross-linking.

The water solubility (WS) of the films is presented in Figure 7b. WS in a very important parameter in the context of food packaging as it makes it possible to predict the stability of the films requiring high water resistance. The higher WS of the un-cross-linked nisin films could be due to the depletion of surface adsorbed nisin when kept under water for 24 h. As the nisin is depleted from the surface, the films lost weight, hence the higher WS values. The WS of the surface immobilized nisin films decreased significantly ($P \leq 0.05$) with the incorporation of only 0.05% of genipin; as a result, genipin cross-linking improved the stability of the films. However, increase of genipin concentration did not reduce the WS further. Jin et al.[18] reported improvement in the stability of chitosan/PEO blends films due to genipin cross-linking.

Mechanical Properties of the Films. Tensile strength is related to the mechanical strength of films, whereas the tensile modulus and elongation at break is related to the rigidity and flexibility of the films, respectively. The tensile strength, tensile modulus, and elongation at break (Eb%) of the un-cross-linked (nisin control) and cross-linked with 0.05%, 0.1%, 0.2%, and 0.4% of genipin are presented in Table 1. The tensile strength and modulus of the films increased significantly ($P \leq 0.05$) with the incorporation of only 0.05% of genipin. These values are in accordance with Jin et al.[18] where the authors have reported improvement of the mechanical strength of the films due to genipin cross-linking. Other authors such as Nunes et al.[59] have reported no significant improvement of the mechanical properties of the chitosan−caffeic acid films due to genipin cross-linking. Qiu et al.[60] reported a significant ($P < 0.05$) increase in the compressive elastic modulus of the porcine acellular dermal matrix due to cross-linking with different genipin concentration (0.025−0.5%). It is interesting to note that in the current study, both the tensile strength and modulus of the films decreased with high genipin concentration (0.1−0.4%). The decrease in the mechanical properties of the films at high genipin concentration could be attributed to the higher heterogeneity on the surface of the films as a result of cross-linking.[61] Films cross-linked with high concentration of genipin also exhibited very negligible bioactivity during the storage experiment. High genipin cross-linking had a positive effect on the flexibility as films cross-linked with 0.2% and 0.4% of genipin significantly ($P \leq 0.05$) improved the flexibility (Eb%) of the films.

Table 1. Effect of Genipin Concentration on the Mechanical Properties of Films[a]

film samples	tensile strength (MPa)	tensile modulus (GPa)	elongation at break (%)
G0	110.0 ± 5.1^{b}	2.99 ± 0.26^{c}	22.5 ± 2.0^{a}
G0.05	117.5 ± 3.5^{c}	3.61 ± 0.15^{d}	22.4 ± 4.0^{a}
G0.1	108.0 ± 5.3^{b}	2.48 ± 0.29^{b}	21.1 ± 4.0^{a}
G0.2	93.6 ± 5.1^{a}	1.91 ± 0.35^{a}	30.7 ± 3.8^{b}
G0.4	99.1 ± 5.5^{a}	2.15 ± 0.12^{a}	30.6 ± 7.0^{b}

[a]Values are means ± standard deviations. Within each column, means with the same letter are not significantly different ($P > 0.05$).

Antimicrobial Activity of the Cross-Linked Films against *L. monocytogenes*. The bacterial growth rate (B) calculation was performed in order to evaluate the effect on genipin cross-linking against *L. monocytogenes* and the results are presented in Figure 8. It was observed that regardless of the concentration of the nisin, all the antimicrobial films (both the un-cross-linked and cross-linked) reduced the B of *L. monocytogenes*, as compared to the control meat samples. The B decreased linearly with the increase in nisin concentration. The B for the control meat samples (B-meat) was 0.28 log CFU/g/day. The growth rate for the films with 18.65 $\mu g/cm^2$ of nisin (both the un-cross-linked and cross-linked) were 0 log CFU/g/day as they completely inhibited the growth of *L. monocytogenes*. It was interesting to compare the B of the un-cross-linked films (B-nisin) with that of cross-linked (B-genipin) films. The B of *L. monocytogenes* was lower for the cross-linked films (with 4.65 and 2.44 $\mu g/cm^2$ of nisin) than that of the un-cross-linked films at the same nisin concentration. For example, the B of the cross-linked films containing 4.65 $\mu g/cm^2$ of nisin (0.15 log CFU/g/day) was 21% lower than that of the un-cross-linked films (0.19 log CFU/g/day). The low activity of the un-cross-linked films may result from the enzymatic degradation of nisin due to the interaction with meat components such as, glutathione.[62] The improved antimicrobial activity of the cross-linked films could be attributed to the better retention of the bioactivity of nisin as a result of cross-linking.

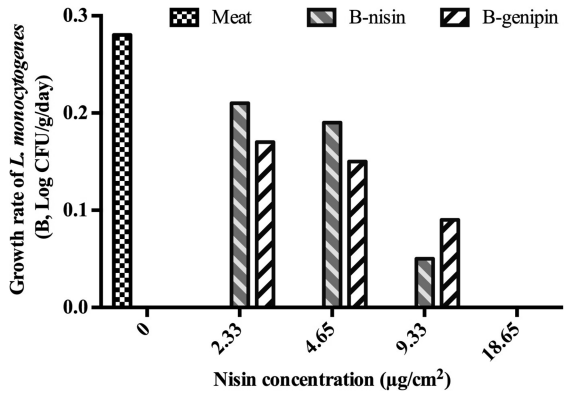

Figure 8. Effect of the genipin cross-linking on the growth rate of *L. monocytogenes*.

Surface Morphology of the Films. The surface morphology of the films as analyzed by scanning electron microscopy (SEM). The surface of the control nanocomposite films (no nisin) revealed a smooth and homogeneous morphology with few white dots (Figure 9a). The white dots could correspond to the presence of CNC in the films.[8] An alternation in the surface morphology of films was observed with the increase in nisin concentration from 2.33 to 18.65 $\mu g/cm^2$. As the nisin concentration increased the film surfaces became rougher (Figure 9b and c). The increased roughness could be due to the deposition of salt crystals (present in nisin) on the surface of the films. Also, an increase in the quantity of nisin granules adsorbed on the surface of the films was observed.

Santiago-Silva et al.[63] have also reported similar changes in the surface morphology of the biopolymeric films due to bacteriocin incorporation. The micrograph of genipin cross-linked films revealed interesting changes in the surface morphology (Figure 9d). The cross-linked films appeared have fewer granules and seemed to be linked by a continuous surface layer, which could arise due to the formation of heterocyclic cross-linked network between genipin and the surface immobilized nisin. The improvement in the WS (observed previously) of the films could be attributed to this cross-linked layer.

CONCLUSION

This study has demonstrated the effectiveness of the antimicrobial nanocomposite films to inhibit the growth of

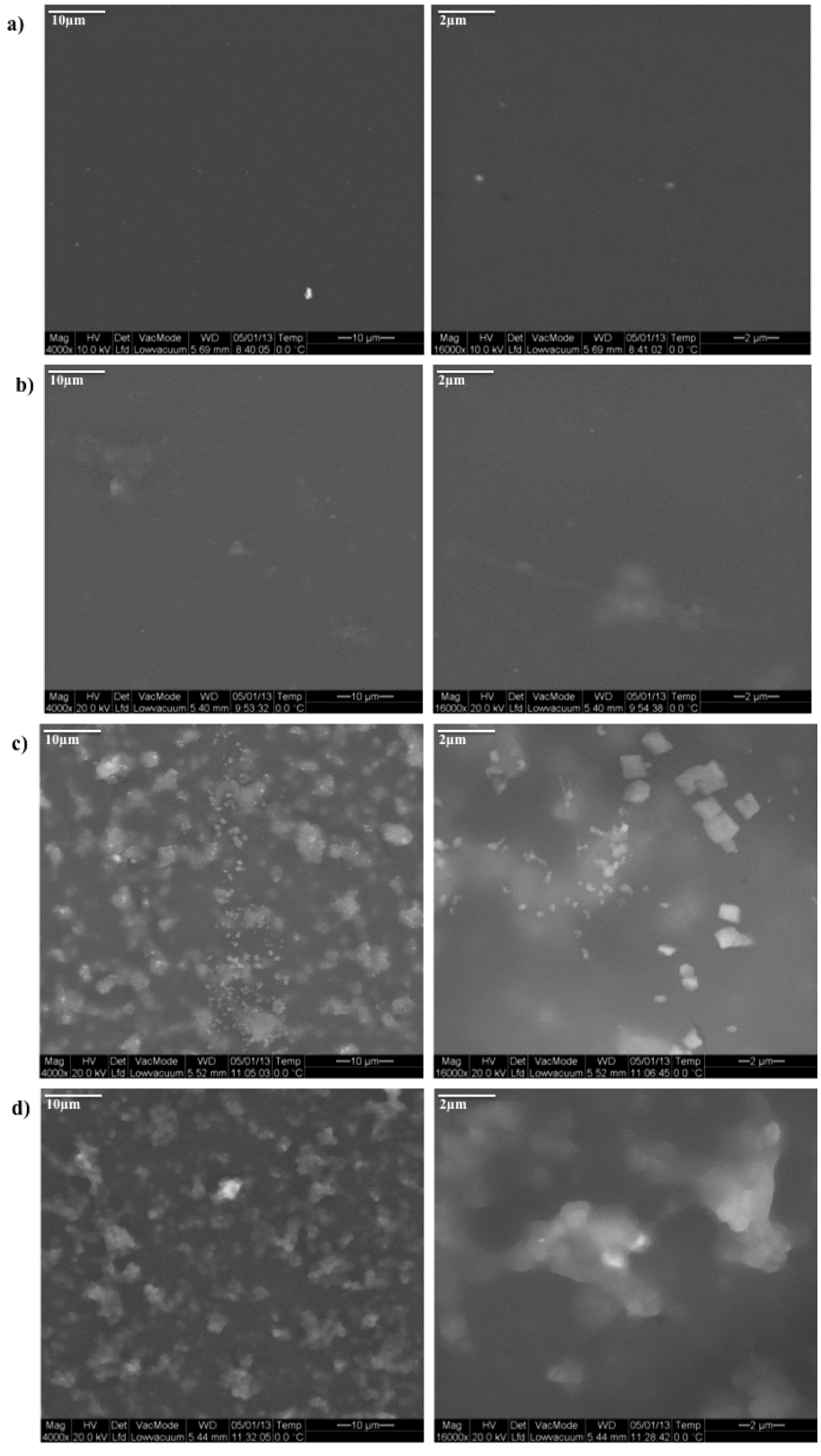

Figure 9. SEM micrographs of the surface of (a) control (no nisin), (b) 2.33 $\mu g/cm^2$ of nisin, (c) 18.65 $\mu g/cm^2$ of nisin, and (d) 18.65 $\mu g/cm^2$ of nisin films cross-linked with 0.05% genipin.

pathogenic bacteria in meat products. The antimicrobial activity was attributed to the orientation and adsorption of nisin on the surface of the nanocomposite films. Cross-linking with low genipin concentration (0.05%) allowed a slow diffusion of nisin from the films and protected its bioactivity during extreme storage condition. Genipin cross-linking improved the water resistance and mechanical strength of the films. The cross-linked films also demonstrated better antimicrobial activity against *L.*

monocytogenes compared to the un-cross-linked films, by reducing the growth rate of the bacteria in meat samples. This naturally cross-linked nanocomposite films should have high potential to ensure the safety of vacuum packaged RTE deli meat during extended exposure. Further work is necessary to determine if such antimicrobial nanocomposite films could be produced on a larger scale.

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Notes

The authors declare no competing financial interest.

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