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ARTICLE

Preparation of Antimicrobial Membranes: Coextrusion of Poly(lactic acid) and Nisaplin in the Presence of Plasticizers

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Nisin is a naturally occurring antimicrobial polypeptide and is popularly used in the food and food-packaging industries. Nisin is deactivated at temperatures higher than 120 °C and, therefore, cannot be directly incorporated into poly(L-lactic acid) (PLA), a biomass-derived biodegradable polymer, by coextrusion because PLA melts at temperatures around 160 °C or above. However, PLA can remain in a melt state at temperatures below the T_m in the presence of lactic acid or other plasticizers. In the present study, PLA was coextruded with lactic acid, or lactide, or glycerol triacetate at 160 °C. After the PLA was melted, the temperature of the barrels was reduced to 120 °C, and then Nisaplin, the commercial formulation of nisin, was added and the extrusion was continued. The resultant extrudates possess the capability to suppress the growth of the pathogenic bacterial *Listeria monocytogenes*, demonstrating a significant antimicrobial activity. The present study provides a simple method to produce PLA-based antimicrobial membranes. The method can also be used for the coextrusion of other heat-sensitive substances and thermoplastics with high melting temperature.

KEYWORDS: Poly(lactic acid) (PLA); nisin (Nisaplin); extrusion; antimicrobial; packaging; membranes

INTRODUCTION

For food preservation and for high-quality foods, the improvement of food packaging technology and materials is of importance. The research and development of packaging materials/technologies have shown a clear and irreversible trend toward "green" and "active" packaging. The concept "green" reflects the need for sustainable materials and for environmental protection. The principle of "active packaging" includes the use of chemical, biological, or biochemical means to interfere with bacterial growth in the headspaces or on the surfaces of foods and the application of various biopreservatives onto packaging materials, such as antioxidants, bacteriocins, and other antimicrobials. Nisin is a bacteriocin that has been approved by the U.S. FDA for use in food preservation (*1*) and has been tested for active packaging purposes for decades (*2*) by incorporation into packaging membranes such as from proteins (*3*), polysaccharides (*4*−*6*), thermoplastics (*7*−*11*), and their combinations (*12*−*15*). The resultant nisin−polymer materials showed antimicrobial activity by suppressing the growth of Gram-positive bacteria such as *Listeria monocytogenes* and *Salmonella typhimurium* and thus were able to extend the shelf life of foods and preserve the food quality. Packaging materials prepared from proteins or polysaccharides are weak in mechanical properties and have poor water resistance. Most packaging materials are currently developed from petroleum-derived, nonbiodegradable thermoplastics; however, serious environmental problems are a concern with the use of this type of plastic. The biobased thermoplastic poly(lactic acid) (PLA) may be the exception. This commercially available polymer is produced from biobased precursors, lactic acid or lactide. PLA is easily processable and water resistant. Thin PLA membranes are good water vapor barriers and have relatively low gas transmittance (*16*−*21*). Furthermore, as the progress in fermentation technology advances, the cost of PLA production decreases, and it may be able to beat the petroleum-derived thermoplastics some day.

As previously reported, nisin (in the formulation with salts and milk proteins under the trade name Nisaplin) could be incorporated into PLA by methods of diffusion or coextrusion with the polyester following its complexation with pectin (*22*, *23*) or by mechanical mixing in a PLA/CH_2Cl_2 solution, followed by film casting (*24*). However, the processing was somewhat complicated. Furthermore, a large volume of organic solvent was also used in some cases (*24*). Nisin also can be incorporated into PLA nanoparticles by CO_2 antisolvent precipitation (*25*), but the method cannot be applied to membrane preparation. An ideal approach to prepare antimicrobial PLA membranes incorporating Nisaplin is to coextrude the two in one step, which would be simple and efficient and could be easily handled for quality control and quality assurance. However, PLA melts at around 160 °C, whereas the maximal temperature at which nisin can retain its bioactivity is 120 °C. Hence, although nisin/PLA films could be prepared by extrusion, the required temperatures for melting the PLA during the process would result in films with little or no antimicrobial activity.

In the present work we blended Nisaplin into plasticized PLA using a benchtop microextruder for mixing and processing. Lactic acid (LA) and lactide (LD), the monomer and dimer of PLA, or glycerol triacetate (GTA), was used as plasticizer and blended with PLA. The plasticized PLA can be coextruded with Nisaplin

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at 120 °C. The antimicrobial activity of the resultant PLA/Nisaplin membranes was examined by incubating the membranes with pathogenic bacteria *L. monocytogenes*. In this study, we also evaluated the potential of the membranes for use as an active inner layer of beverage containers by investigating the duration and activity of the incorporated Nisaplin released from the membrane to various media.

EXPERIMENTAL PROCEDURES

Materials. PLA for membrane preparation was obtained from Cargill Dow (Minneapolis, MN) and stored at room temperature in sealed double-layered PVC bags. The weight-average molecular weight (M_w) and number-average molecular weight (M_n) of the PLA were 148,000 and 110,000 respectively. The PLA possesses a melting temperature (T_m) of 155−160 °C and a glass transition temperature (T_g) of 55−60 °C, according to the provider. Lactic acid (LA) and lactide (LD) were obtained from Fisher Scientific (Fair Lawn, NJ). Glycerol triacetate (GTA), sodium chloride, vitamin C, and tetrahydrofuran (THF) were purchased from Sigma-Aldrich (St. Louis, MO). Nisaplin (containing 2.5% nisin) and pectin were from Danisco Cultor USA (New Century, KS). Sugar of sugar cane, fat-free milk, and 100% orange juice were purchased from a local grocery store. Deionized water (DI water) was prepared using a Barnstead E pure water system (Dubuque, IA) and used as the medium for controlled release studies.

PLA/Nisaplin Membrane Preparation. PLA/Nisaplin complexes in the form of strands or thin membranes were prepared using a HAAKE MiniLab II Rheomex CTW5 benchtop twin-screw extruder (ThermoFisher Scientific, Newington, NH). The extruder employs two corotating conical screws (109.5 mm in length and 5 and 14 mm in diameter at the die and rear ends, respectively) and has a bypass channel that allows materials to be either recycled or directly extruded through the use of a "cycle/flush" valve. The back channel is constructed as a rheological slit capillary with two pressure sensors, which provide rheological information about the flow behavior of samples. The extruder can be controlled either by an attached workstation or with a computer and operation/analysis software (version 4.17, Polylab Monitor Software).

PLA and a designated amount of plasticizer were mixed mechanically in a glass vial. A fraction of the mixtures was first added to the extruder using a pneumatic feeder. The extrusion was operated at 160 °C at the beginning, with the unit set to recycle mode. After the PLA was melted, the barrel temperature was then set to 120 °C. When this temperature was reached, the rest of the mixture with Nisaplin (5 wt % of total mass) was added and the extrusion was continued. Membranes were prepared using a slit die that is 5 mm in width and 0.5 mm in thickness.

For the samples prepared for controlled release studies, particles of sodium chloride, pectin, sugar, or vitamin C, used as pore-forming reagents, were added to the formulations together with PLA and the plasticizers in the first loading.

Measurement of PLA Molecular Weight (MW). The MW of PLA before and after extrusion was determined by gel permeation chromatography (GPC) using a Shimadzu chromatograph (LC-10AD, Kyoto, Japan) as described previously (*26*). Prior to chromatography, specimens of each sample, about 1 g, were placed in a gastight glass vial containing 10 mL of THF. The vials were shaken at room temperature for at least 32 h to completely dissolve the PLA. The supernatant was pipetted off and analyzed. The chromatograph was equipped with a Phenogel column (GP/4446; 300 mm × 7.8 mm) and a Phenogel guard column (22824G; 50 mm × 7.8 mm) from Phenomenex (Torrace, CA), a refractive index detector (RID-10A), and an SCL-10A datastation. THF was used as the mobile phase. The measurements were conducted at the flow rate of 1.0 mL/min at room temperature. For the calibration curve and MW calculation, the Mark−Houwink constants for PLA of $K = 5.450020 \times 10^{-3}$ and $a = 0.73$ were used. A set of polystyrene samples was used as standards.

Differential Scanning Calorimetry (DSC) Analysis. DSC was performed on the extruded specimens using a Perkin-Elmer Pyris 1 (Norwalk, CT). The samples, 5−20 mg, were crimp sealed in 40 μL stainless steel pans. The instrument was purged with nitrogen. All samples were equilibrated at 0 °C for 2 min, heated to 180 °C (or 240 °C) at 10 °C/min, and then rapidly cooled to 0 °C. It was then equilibrated for 2−5 min, depending on the maximum temperature used, and again heated to the maximum temperature at 10 °C/min. Peak temperatures and peak areas were determined using the instrument software.

Mechanical Testing. Mechanical properties of the resultant membranes were measured using an upgraded Instron mechanical property tester, model 1122, equipped with Testworks 4 data acquisition software (MTS Systems Corp., Minneapolis, MN). Specimens with the size of 85 × 5 × 0.5 mm (length × width × thickness) were tested at 21 °C and 65% relative humidity at the following settings: gauge length, 102 mm; strain rate, 50 mm/min. The properties measured included tensile strength (MPa), tensile modulus (MPa), maximal elongation (%), and toughness (J/cm^3). All tests were run five times for each sample, and the average and standard deviation were calculated.

Scanning Electron Microscopy (SEM) Examination. For SEM examination, samples were coated with a thin layer of gold and characterized on a Quanta 200 KeV FEG SEM (FEI, Hillsboro, OR). Images were collected with high-vacuum/secondary electron-imaging mode at magnifications of 250× and 2500×. For samples containing pore-forming reagents and tested after immersion in aqueous solutions for 2−6 weeks, SEM examination was conducted following dehydration and freeze−fracture. Images of fracture surfaces were collected.

Antimicrobial Assay. The agar diffusion assay was used to determine the capability of Nisaplin (both incorporated and in the free form) in suppressing/inhibiting the growth of a pathogenic bacteria, *L. monocytogenes* Scott A 724. The stock cultures were obtained from our in-house culture collection. Stock cultures were maintained at −80 °C in brain−heart infusion (BHI) broth (Difco Laboratory, Detroit, MI). Working cultures of *L. monocytogenes* were maintained on BHI agar at 4 °C and were subcultured biweekly and grown aerobically at 37 °C in BHI broth. Prior to inoculation of product, cultures were grown in BHI broth at 37 °C for 16−18 h.

A glass test tube containing testing specimens (0.18−0.20 g for each) was filled with 9 mL of BHI broth and inoculated with 1 mL of an overnight culture of *L. monocytogenes* (approximately 1×10^3 cells). The test tubes were shaken at 150 rpm at 22 °C. At two time points, one at the beginning of the experiment and another at 24 h after the experiment, aliquots containing 1 mL of incubated sample were serially diluted with sterile phosphate buffer (Hardy Diagnostics, Santa Maria, CA) and then pour plated onto BHI agar. Plates were incubated at 37 °C for 24 h before the colony-forming units (CFU) were counted.

To determine the time-dependent antimicrobial activity of membranes after their contact with aqueous phase, the membranes containing pore-forming reagents were immersed in release media (200 mg in 10 mL, solid/liquid) at room temperature with frequent and gentle shaking. After 4 weeks of incubation, the membranes were taken out, rinsed with 5 mL of fresh DI water 3 times, vacuum-dried for 24 h, and then investigated for antimicrobial activity. The release media used in the present study were DI water for sodium chloride and sugar powder, fat-free milk for pectin, and 100% orange juice for vitamin C.

Experiments were run in triplicate. Each data point was expressed as the mean ± SD. All data were analyzed by analysis of variance using SAS version 9.1 software (SAS Institute, Cary, NC). Duncan's multiple-range tests were used to determine the significant difference of mean values. Unless stated otherwise, significance is expressed at the 5% level.

RESULTS AND DISCUSSION

Membrane Preparations. Table 1 shows the effect of the inclusion of lactic acid on the rheological behavior of PLA preparations under processing conditions. The values of torque and ΔP are measures of the resistance of the polymers to screw rotation and the pressure drop along the slit capillary, respectively. The values reflect the viscoelastic properties of the samples. The higher the value, the more elastic the samples are. These values are a function of both composition and operating temperature. Without the addition of LA, the PLA samples exist as a melt at 160 °C. However, when the temperature was reduced to 120 °C, the PLA samples were no longer in the melt state. The values of torque increased from 50 to >200 Ncm, and ΔP

Table 1. Effect of LA on the Rheological Behavior of PLA/LA Blends

	stage I, at 160 °C		stage II, at 120 °C	
PLA/LA (w/w)	torque (Ncm)	ΔP (bar)	torque (Ncm)	ΔP (bar)
100:0	49	81	>200	>100
95:5	10	9	49	45
92.5:7.5	6	6	36	38
90:10	14	11	24	27
80:20	1	1	3	2
95:5 (+ 5% NaCl)	10	9	47	46

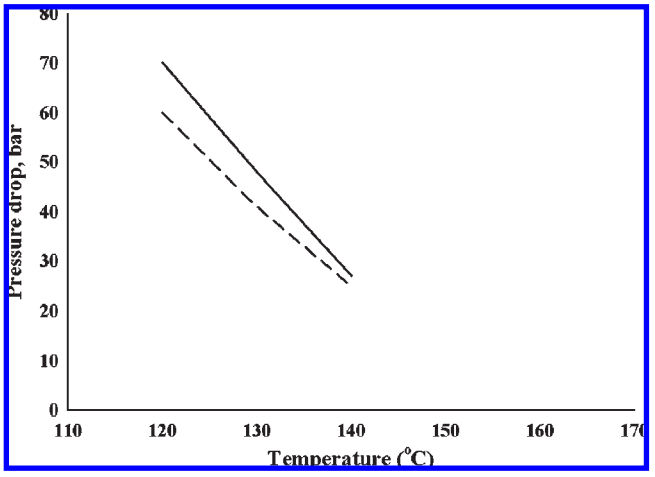

Figure 1. Effect of temperature on the reological properties of PLA blends containing 10% LD (solid line) or 20% GTA (broken line).

increased from 80 to >100 bar. At this temperature, the samples were too elastic to be extruded. With the addition of LA (5−10% of total mass), the PLA samples exhibited lower values of torque and ΔP at both 160 and 120 °C. Although these values increased as the temperature decreased, the samples were still viscous rather than elastic (ΔP value is < 100 bar) at 120 °C and, therefore, could be extruded. However, when the LA content was too high, for example, at about 20%, the samples had too low of a viscosity to be easily handled. Appropriate plasticizer contents could be further optimized for successful extrusion. Furthermore, the addition of the inorganic compound NaCl has no discernible effect on the values of torque and pressure drop of the extruded PLA/LA blends under the experimental conditions used (**Table 1**). Similar results were observed for other pore-forming agents, such as pectin, vitamin C, and sugar powder (data not shown).

The coextrusion of LD or GTA with PLA also reduced the viscosity of the samples. For the PLA blended with LD (10 w%) or GTA (20 w%), the values of torque and ΔP decreased as the temperature increased, and the values of torque and ΔP at 120 °C were about 50 Ncm and 30−50 bar, respectively (**Figure 1**).

It was also noted that the mixtures of PLA and plasticizers in the barrel could only maintain the lower values of torque and ΔP at 120 °C for a limited period, even when the temperature was maintained at this constant value. The length of the period was dependent on the nature and amount of the plasticizers used. In the case of LA, it was found to be about 7, 10, and 15 min for polymers with LA contents of 5, 10, and 20%, respectively. It was observed that the mixtures became elastic at time periods longer than these. For the PLA blends with 10% LD or 20% GTA content, the time length was about 3−5 min. Therefore, Nisaplin was fed into the extruder as soon as the barrel reached 120 °C, before the PLA/LA became too elastic to process.

Changes in PLA Molecular Weight. As shown in **Table 2**, slightly lower values of M_w and M_n were recorded for PLA after extrusion. Significant decreases in values of M_w and M_n were recorded for PLA/LA blends. These results were anticipated, as PLA is sensitive to thermal degradation and the α-hydroxyl ester linkages are water labile. The decrease in PLA molecular weight in the PLA/LA blends after melt processing could be further accelerated by the acid microenvironment that was created by the inclusion of LA monomers. A similar result from the coextrusion of PLA and sugar beet pulp, which contains a large amount of carboxylic groups, has been reported previously (*26*). The increase in M_w/M_n of PLA indicated there were more short-chain PLA molecules in the blends than in the neat PLA samples. This may explain why there was a significant decrease in the values of torque and pressure drop, ΔP, during the coextrusion of PLA and its monomer. On the other hand, the inclusion of LD or GTA was not as effective as the use of LA in altering the values of M_w, M_n, and M_w/M_n of PLA in the blends. Probably, this could be attributed to transesterifications occurring in the extrusion processing. The inclusion of sodium chloride particles dramatically reduced the M_w and M_n and increased the M_w/M_n of PLA in the PLA/LA blends due to the residual moisture in the salts (3.4 ± 0.6 wt % as measured by weight loss after placing in a vacuum oven at 105 ± 3 °C for 24 h).

Table 2. Effect of Plasticizer on PLA Molecular Weight

plasticizer	M_w	M_n	M_w/M_n
PLA[a]	135000	98000	1.38
PLA	121000	83000	1.46
PLA/LA (90:10)	87500	35000	2.50
PLA/LD (90:10)	97500	62500	1.56
PLA/GTA (90:10)	108000	76800	1.41
PLA/GTA (80:20)	105500	79000	1.34
PLA/LA (95:5) + 5% NaCl	75000	31050	2.41

[a] Determined before extrusion.

Thermal Properties Analysis. The DSC thermograms of the extruded PLA and its blends with LA and NaCl/Nisaplin are shown in **Figure 2**. The glass transition and the crystalline status of test specimens are summarized in **Table 3**. The glass transition temperature (T_g) and melting temperature (T_m) of PLA were found to be about 58 and 152 °C, respectively. The addition of LA reduced the T_m of the resultant blends to about 130 °C. The inclusion of LD or GTA also showed a similar effect on the T_m of the blends. The peak of each sample broadened toward lower temperatures, and the onset of the transition also moved toward lower temperature ranges. Correspondingly, the T_g for each of the blends was also shifted to lower temperatures. In comparison of GTA with LA, more GTA than LA was needed to reduce the T_g and T_m of the blends to a similar level. The lowering of the T_g and T_m of the blends could be attributed to the presence of short-chain molecules (**Table 2**), which reduced the macromolecular interactions. These results are consistent with the rheological properties of the blends measured during the extrusion experiments (**Table 1**). As the macromolecular interactions were weakened, more time was required for melted PLA chains to coalesce during extrusion (changing from elastic to viscous at 120 °C), and the "associated" macromolecules were more easily disrupted during the DSC test (earlier onset time). Cold crystallization was observed for the extruded PLA and all blends. For the extruded neat PLA specimens, a slightly higher value of ΔH_m than ΔH_{cc} indicated that only minor crystallization might occur during the cooling following extrusion. The presence of plasticizers seems to slightly alter the crystalline structures of the blends. For the PLA/LD blends, the very similar values of ΔH_m and ΔH_{cc} suggest that the blends were primarily amorphous. The PLA/LA blends were able to crystallize during the cooling process, as suggested by the

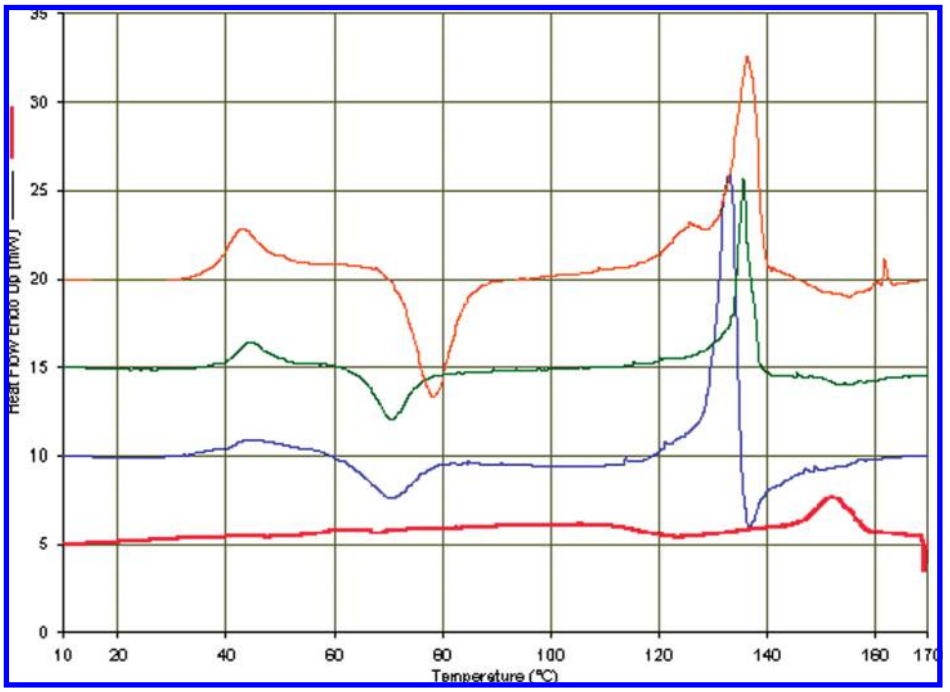

Figure 2. DSC thermograms of extruded PLA and PLA blends: (from bottom to top) neat PLA, PLA/LA, PLA/NaCl/Nisaplin, and PLA/NaCl. The LA content was 10%; the contents of NaCl or NaCl/Nisaplin were 5%.

Table 3. Effect of Plasticizers on the Thermal Properties of PLA Membranes

		exotherm		endotherm	
composition[a]	T_g (°C)	T_{cc} (°C)	ΔH_{cc} (J/g)	T_m (°C)	ΔH_m (J/g)
100% PLA	58	110	−16.9	152	21.9
90% PLA, 10% LA	41	71	-9.3	133	48.6
90% PLA, 10% LD	45	87	−17.3	132	19.6
90% PLA, 10% GTA	55	102	−14.2	146	27.6
85% PLA, 15% GTA	49	94	−12.4	134	30.2
80% PLA, 20% GTA	43	82	−10.1	128	29.5
85% PLA, 10% LA + 5% NaCl	41	71	−16.1	136	35.3

[a] LA, lactic acid; LD, lactide; GTA, glycerol triacetate.

Table 4. Effect of Plasticizers and Sodium Chloride on the Mechanical Properties of Blends of PLA Containing LA or LD

plasticizer and salt	tensile strength (MPa)	elongation at break (%)	Young's modulus (MPa)	toughness (J/cm^3)
PLA, 100%	70.2 ± 9.3	4.3 ± 1.2	1588 ± 80	2.8 ± 1.0
+ LA, 10%	40.6 ± 2.4	4.1 ± 1.0	1115 ± 78	1.8 ± 0.9
+ LD, 10%	53.3 ± 4.5	4.4 ± 1.1	1237 ± 99	2.1 ± 0.4
+ LA, 10%, and NaCl, 5%	29.6 ± 7.1	2.1 ± 0.2	1762 ± 94	1.3 ± 0.4

relatively large differences between ΔH_m and ΔH_{cc} and also for the PLA/GTA blends.

The inclusion of small particles, such as sodium chloride (**Table 3**), pectin, vitamin C, and sugar powder (data not shown), also reduced the values of T_g and T_m.

Measurements of Mechanical Properties. Adequate mechanical properties are very important for polymeric membranes designed for packaging purposes. Packaging materials are often subjected to mechanical stretching; the membranes must be able to resist a considerable stress without fracture. The mechanical properties of resultant PLA membranes containing 10% LA or LD are shown in **Table 4**. The inclusion of LA and LD reduced the tensile strength and Young's modulus of the PLA blends. The blends had less strength and less stiffness, in comparison with membranes from neat PLA. Although blending PLA with LA or LD seemed to have no effect on the value of maximal elongation, the fractural energy of the blends was smaller than that of neat PLA membranes. The lower mechanical properties could be attributed to decreased intramolecular interactions of the PLA macromolecules caused by insertion of the small molecules. For the same reason, the mechanical properties of the PLA/LA and PLA/LD blends were further reduced by the inclusion of pore-forming reagents.

Figure 3 shows the effect of GTA amount on the mechanical properties of PLA blends. GTA is a plasticizer often used in plastic industries. GTA also functions as a lubricant. The inclusion of GTA into PLA altered the brittle nature of PLA by increasing its flexibility, and thus the PLA/GTA blends were tougher than neat PLA specimens, which was expected because of the softening effect. The more GTA that was added, the more flexible and less strong were the blends. Although the inclusion of LA, LD, or GTA into PLA reduced the tensile strength of the resultant blends, the mechanical properties of the blends were quite comparable with some petroleum-derived packaging materials, such as PVC (35 MPa) and PS (55 MPa), and PLA/pectin blends developed in our laboratory (40 MPa) (*27*).

Morphology. The SEM images of freshly extruded membranes are shown in **Figure 4**. A homogeneous, smooth morphology was observed for neat PLA and the blends of PLA/LA, PLA/LD, and PLA/GTA, indicating that the plasticizers and the PLA were highly compatible. The coextrusion of pore-forming reagents, such as sodium chloride, introduced a heterogeneous, craggy fractured surface. Pores could be clearly observed on the surfaces of the salt-containing composites. The pores were possibly created by the air that adhered on the irregular surfaces of the salt particles. The air was separated from the carriers during extrusion, concentrated to form bubbles under pressure by rotation of the twin-screw, and then escaped, leaving void spaces. For the blends containing sodium chloride and Nisaplin, the microparticles were evenly embedded in the continuous phase.

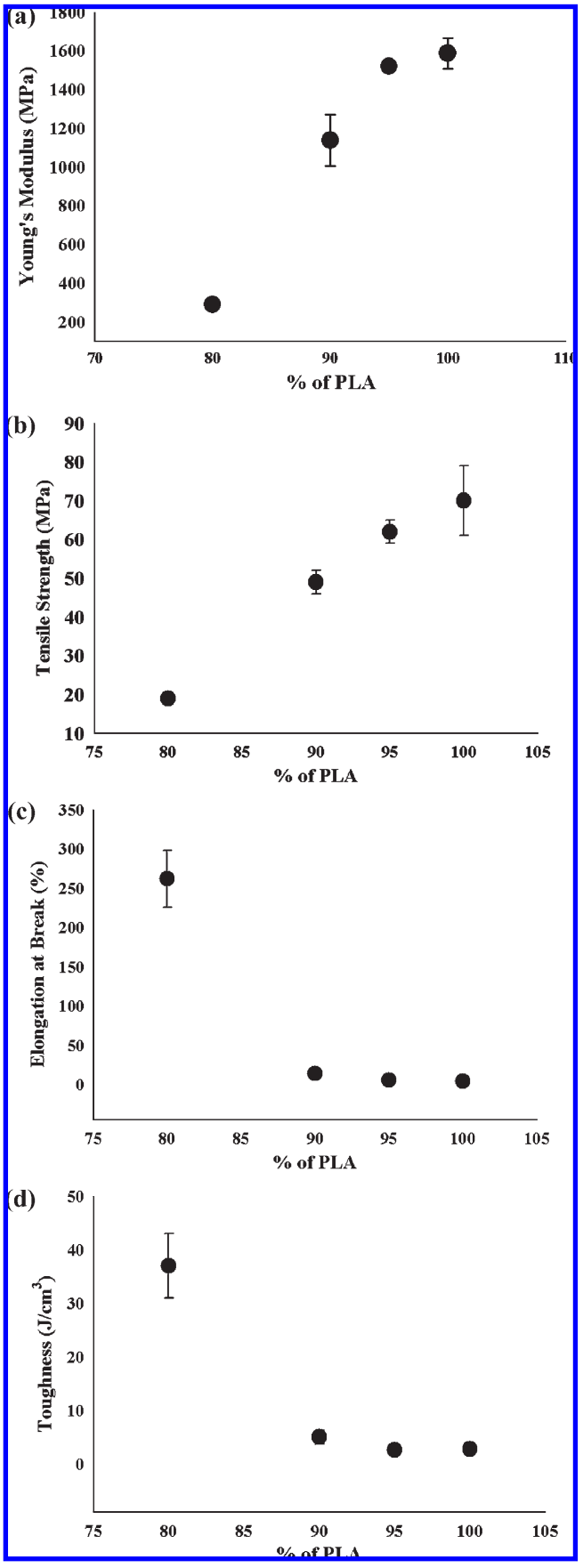

Figure 3. Effect of GTA content on mechanical properties of PLA blends: (**a**) Young's modulus; (**b**) tensile strength; (**c**) maximal elongation; (**d**) toughness.

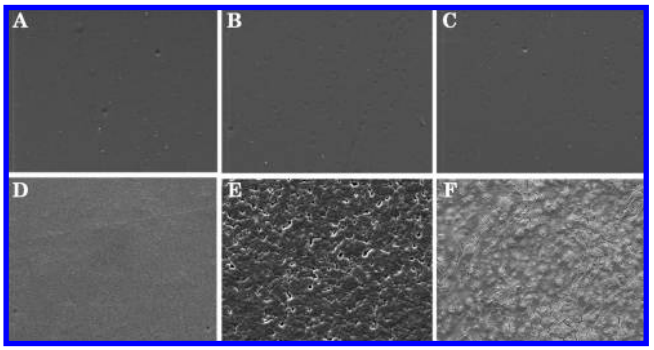

Figure 4. SEM topographic images of extruded membranes of neat PLA (**A**) and PLA blends with LA (**B**), LD (**C**), and GTA (**D**), as well as PLA/LA blends containing NaCl (**E**) and containing the mixture of NaCl and Nisaplin (**F**). Field width = 136 μm.

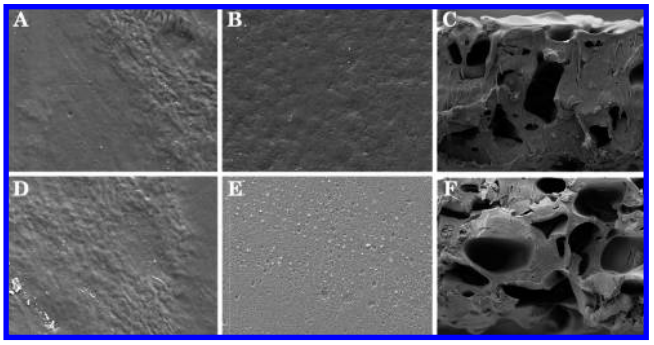

Figure 5. SEM images of membranes from neat PLA (**A**, **D**) and the blends of PLA with LA (**B**, **E**) and LA/NaCl (**C**, **F**) after incubation in DI water for 4 weeks (**A**−**C**) or 6 weeks (**D**−**F**) at room temperature. Field width = 54 μm (**A**, **B**, **D**, **E**) or 544 μm (**C**, **F**).

The introduction of Nisaplin also created pores of small sizes. Some heat-sensitive components in the Nisaplin might have degraded and "evaporated" at higher temperature and under higher pressure during extrusion; therefore, pores were created and particles were segregated. This result is consistent with our previous study, in which microparticles of pectin−Nisaplin particles were extruded together with PLA, resulting in a heterogeneous, porous structure (*23*).

Degradation of PLA in aqueous solutions has been well studied. Bulk degradation has been suggested as the mechanism by which the degradation occurred randomly along the macromolecular chains at the edges or defects of the membrane surfaces. As a result, a rough morphology was created as the incubation time increased (**Figure 5A**,**D**). The inclusion of LA accelerated the PLA degradation (**Figure 5B**,**E**). At week 6, many small pores were created, which were randomly distributed across the whole surface. For the specimens containing salts that are soluble in water and can diffuse from the solid phase into the liquid phase, the fractured surfaces were investigated to obtain more information on structural changes. As seen in **Figure 5C**,**F**, pores were formed, and some were connected to form channels due to the release of embedded salt particles as the water migrated. With the increase in incubation time, the size and number of the pores increased. The membranes, under higher magnification, "turned" to a thin scaffold in appearance. For practical reasons, the effect of other pore-forming reagents on membrane degradation was also examined. These pore-forming reagents were pectin (used in milk as a stabilizer), vitamin C

(a component of orange juice), and sugar from sugar cane (a common component in various beverages). The release media used for incubation with the membranes containing pectin, vitamin C, or sugar powders were fat-free milk, orange juice, and DI water, respectively. Membranes containing these pore-forming reagents also showed morphology before and after incubation similar to that observed for sodium chloride (data not shown).

As the membranes degraded and pores were created, one can reasonably believe that the incorporated Nisaplin was leached out; consequently, the antimicrobial activity of the membrane should be simultaneously decreased. To clarify this presumption, the capability of the blend membrane in suppressing *L. monocytogenes* growth was investigated after incubation with release media.

Antimicrobial Activity. Figure 6 shows antimicrobial activity of membranes prepared from neat PLA and the coextrusion of PLA and Nisaplin at 160 °C, as well as the blends prepared in the presence of LD or GTA. Bacterial growth in BHI was recorded for all Nisaplin-free membranes (samples B−D). No difference could be seen in bacterial growth in BHI between the neat PLA (sample B) and PLA/Nisaplin (sample A) prepared at 160 °C, indicating the deactivation of Nisaplin in the absence of plasticizers. In contrast, bacterial growth was prevented in BHI broth inoculated with *L. monocytogenes* in the presence of PLA/LD (sample E) and PLA/GTA (sample F) blend membranes containing Nisaplin. Furthermore, no bacterial recovery could be detected after culture for 24 and 48 h, demonstrating significant antimicrobial activity of the Nisaplin coextruded with PLA in the presence of LD or GTA under the present experimental conditions. Membranes prepared by coextrusion of PLA, LA, and Nisaplin also showed excellent antimicrobial activity by suppressing the growth of *L. monocytogenes* (**Figure 7**). After being stored at room temperature for 4 weeks, the membranes maintained the antimicrobial activity. The results also indicate that the antimicrobial activity is independent of the influence of storage temperature in the range studied.

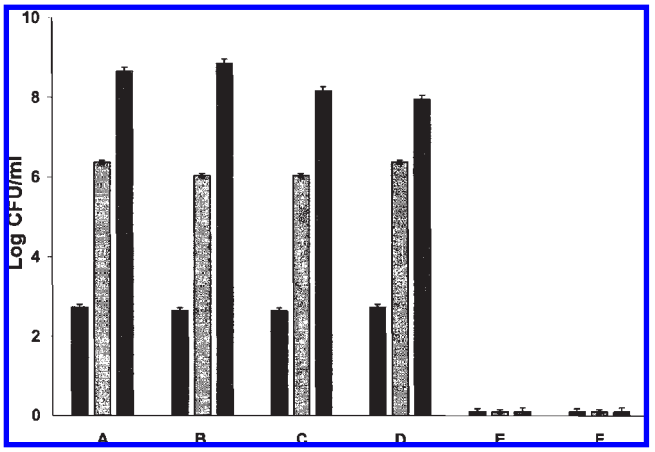

Figure 6. Growth of *Listeria monocytogenes* in BHI broth in the presence of (A) PLA/Nisaplin, (B) PLA, (C) PLA/LD, (D) PLA/GTA, (E) PLA/LD/Nisaplin, or (F) PLA/GTA/Nisaplin at room temperature. For each type of membrane, the column (from left to right) stands for value of bacterial growth at the time when transferred to the agar and after incubation for 24 and 48 h, respectively.

We then investigated the antimicrobial activity of PLA/LA/Nisaplin membranes after incubation in DI water for 4 weeks. The membranes were able to maintain the activity as *L. monocytogenes* growth was suppressed (compare the C group to the control). The inclusion of a pore-forming reagent, sodium chloride, enhanced the antimicrobial activity of the blend membrane as one can see by comparison of samples C2 and C1 or of the C group to the B group. Sample C1 showed statistically significant less activity than samples B1 and B2, whereas sample C2 was almost as active as B1 and B2, but not statistically different. Possibly, this is because the Nisaplin incorporated on the C1 surface area might be washed out by water incubation, whereas that incorporated beneath the surfaces of C1 membranes would take time to diffuse to the liquid phase. After water incubation, less Nisaplin in C1 than in B1 and B2 was available to inhibit bacterial growth. When a pore-forming reagent was incorporated (sample C2), the pores and channels facilitated the release of the incorporated Nisaplin; therefore, more Nisaplin would be available on C2 surfaces in the BHI solution.

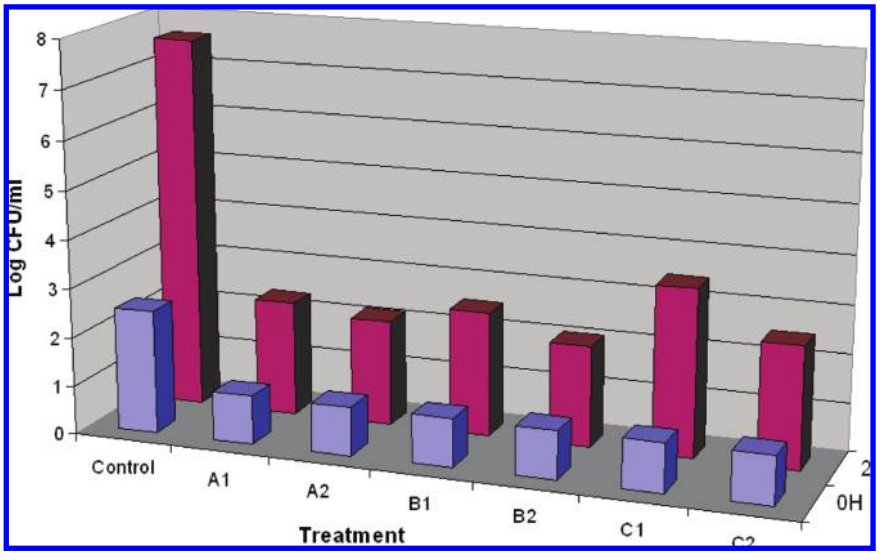

Figure 7. Growth of *Listeria monocytogenes* in BHI broth in the presence of neat PLA and blends of PLA, LA, Nisaplin, and the pore-forming reagent, sodium chloride, at room temperature at the time when transferred to culture liquids (front row) and 24 h after incubation (back row). Control, PLA; A1, B1, and C1, PLA/LA/Nisaplin blends; A2, B2, and C2, PLA/LA/Nisaplin blends containing 5% NaCl; A1 and A2, stored at 4 °C for 4 weeks; B1 and B2, stored at room temperature for 4 weeks; C1 and C2, preincubated in DI water at room temperature for 4 weeks. Compared to the A1, A2, B1, B2, and C2 treatments, the C1 treatment shows significantly ($p < 0.05$) less inhibitory effect against *Listeria*.

Table 5. Survival of *L. monocytogenes* in Brain Heart Infusion Broth at 22°C

		PLA membrane containing Nisaplin and		
incubation time (h)	neat PLA membrane	pectin	vitamin C	sugar
0 (initial)	2.45[a]	ND[b]	ND	ND
24 after initial	7.59	ND	ND	ND
48 after initial	8.09	ND	ND	ND

[a] Log colony-forming units per ml. [b] Not detected (<1 colony-forming unit per mL).

Finally, we investigated the antimicrobial activity of the PLA/LA/Nisaplin membranes containing other pore-forming reagents, such as powders of pectin, vitamin C, and sugar, after being incubated in various media for 4 weeks. As shown in **Table 5**, all testing membranes showed antibacterial activity.

Results from the present research suggest that antimicrobial membranes of PLA and Nisaplin could be prepared simply by coextrusion of the two in the presence of plasticizers, such as LA, LD, or GTA, without losing bioactivity. The resultant membranes possess mechanical properties that match those of some commercially available petroleum-derived plastics (*27*). The inclusion of pore-forming reagents accelerated the Nisaplin release and improved the antimicrobial activity of the membranes under the present experimental conditions. How to control the release rate of the incorporated Nisaplin, and thus to optimize the polypeptide's activity, is currently under investigation in our laboratory.

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LITERATURE CITED

(1) Benkerroum, N.; Sandine, W. E. Inhibitory action of nisin against *Listeria monocytogenes*. *J. Dairy Sci.* **1988**, *71*, 3237–3245.
(2) Sobrino-López, A.; Martín-Belloso, O. Use of nisin and other bacteriocins for preservation of dairy products. *Int. Dairy J.* **2008**, *18*, 329–343.
(3) Dawson, P. L.; Hirt, D. E.; Rieck, J. R.; Acton, J. C.; Sotthibandhu, A. Nisin release from films is affected by both protein type and film-forming method. *Food Res. Int.* **2003**, *36*, 959–968.
(4) Luchansky, J. B.; Call, J. E. Evaluation of nisin-coated cellulose casings for the control of *Listeria monocytogenes* inoculated onto the surface of commercially prepared frankfurters. *J. Food Prot.* **2004**, *67* (5), 1017–1021.
(5) Franklin, N. B.; Cooksey, K. D.; Getty, K. J. K. Inhibition of *Listeria monocytogenes* on the surface of individually packaged hot dog with a packaging film coating containing nisin. *J. Food Prot.* **2004**, *67* (3), 480–486.
(6) Nguyen, V. T.; Gidley, M. J.; Dykes, G. A. Potential of nisin-containing bacterial cellulose film to inhibit *Listeria monocytogenes* on processed meats. *Food Microbiol.* **2008**, *25*, 471–478.
(7) Natrajan, N.; Sheldon, B. Efficacy of nisin-coated polymer films to inactivate *Salmonella typhimurium* on fresh broiler skin. *J. Food Prot.* **2000**, *63* (9), 1189–1196.
(8) Mauriello, G.; De Luca, E.; La Storia, A.; Villani, F.; Ercolini, D. Antimicrobial activity of nisin-activated plastic film for food packaging. *Lett. Appl. Microbiol.* **2005**, *41*, 464–469.
(9) Grower, J. L.; Cooksey, K.; Getty, K. J. L. Development and characterization of antimicrobial packaging film coating containing nisin for inhibition of *Listeria monocytogenes*. *J. Food Prot.* **2004**, *67* (3), 475–479.
(10) Chollet, E.; Swesi, Y.; Degraeve, P.; Sebti, I. Monitoring nisin desorption from a multi-layer polyethylene-based film coated with nisin loaded HPMC film and diffusion in agarose gel by an immunoassay (ELISA) method and numerical modeling. *Innovative Food Sci. Emerging Technol.* **2009**, *10*, 208–214.
(11) Siragusa, G. R.; Cutter, C. N.; Willett, J. L. Incorporation of bacteriocin in plastic retains activity anf inhibits surface growth of bacteria on meat. *Food Microbiol.* **1999**, *16*, 229–235.
(12) Natrajan, N.; Sheldon, B. Inhibition of *Listeria monocytogenes* on poultry skin using protein- and polysaccharide-based films containing a nisin formulation. *J. Food Prot.* **2000**, *63* (9), 1268–1272.
(13) Scannell, A. G. M.; Hill, C.; Ross, R. P.; Marx, S.; Hartmeier, W.; Arendt, E. K. Development of bioactive food packaging materials using immobilized bacteriocins Lacticin 3147 and Nisaplin®. *Int. J. Food Microbiol.* **2000**, *60*, 241–249.
(14) Liu, L. S.; Jin, T.; Liu, C.-K.; Hicks, K. B.; Mohanty, A. K.; Bhardwaj, R.; Misra, M. A preliminary study on edible, antimicrobial extruded films made from pectin and other food hydrocolloids. *J. Nat. Fibers* **2008**, *5* (4), 366–382.
(15) Del Nobile, M. A.; Conte, A.; Buonocore, G. G.; Incoronato, A. L.; Massaro, A.; Panza, O. Active packaging by extrusion processing of recyclable and biodegradable polymers. *J. Food Eng.* **2009**, *93*, 1–6.
(16) Cutter, C. N. Opportunities for bio-based packaging technologies to improve the quality and safety of fresh and further processed muscle foods. *Meat Science* **2006**, *74*, 131–142.
(17) Suyama, N. E.; Copinet, A.; Tighzert, L.; Coma, V. Mechanical and barrier properties of biodegradable films made from chitosan and poly(lactic acid) blends. *J. Polym. Environ.* **2004**, *12*, 1–6.
(18) Calo-Mata, P.; Arlindo, S.; Boehma, K.; Miguel, T.; Pascoal, A.; Barros-Velazquez, J. Current applications and future trends of lactic acid bacteria and their bacteriocins for the biopreservation of aquatic food products. In *Food Bioprocess Technology*; Springer: New York, 2007; Vol. *1*, pp 43−63.
(19) Delves-Broughton, J. Nisin as a food preservative. *Food Aust.* **2005**, *57*, 525–520.
(20) Stoyanova, L. G.; Egorov, N. S.; Fedorova, G. B.; Katrukha, G. S.; Netrusov, A. I. A comparison of the properties of bacteriocins formed by *Lactococcus lactis* sbsp. *lactis* strains of diverse origin. *Appl. Biochem. Microbiol.* **2007**, *43*, 604–610.
(21) Sanjurio, K.; Flores, S.; Gerschenson, L.; Jagus, R. Study of the performance of nisin supported in edible films. *Food Res. Int.* **2006**, *39*, 749–754.
(22) Liu, L. S.; Finkenstadt, V. L.; Liu, C.-K.; Jin, T.; Fishman, M. L.; Hicks, K. B. Preparation of poly(lactic acid) and pectin composite films intended for applications in antimicrobial packaging. *J. Appl. Polym. Sci.* **2007**, *106*, 801–810.
(23) Liu, L. S.; Jin, T. Z.; Finkenstadt, V. T.; Liu, C.-K. Liu; Cooke, P. H. Cooke; Coffin, D. R.; Hicks, K. B.; Samer, C. Antimicrobial packaging materials from poly(lactic acid) incorporated with pectin-Nisaplin® microparticles. *J. Balk. Tribol. Assoc.*, **2009**, *15*(2), 237−252.
(24) Jin, T.; Zhang, H. Biodegradable polylactic acid polymer with nisin for use in antimicrobial food packaging. *J. Food Sci.* **2008**, *73*, M127–134.
(25) Salmaso, S.; Elvassore, N.; Bertucco, A.; Lante, A.; Caliceti, P. Nisin-loaded poly-L-lactide nano-particles produced by CO_2 anti-solvent precipitation for sustained antimicrobial activity. *Int. J. Pharm.* **2004**, *287*, 163–173.
(26) Chen, F.; Liu, L. S.; Cooke, P. H.; Hicks, K. B.; Zhang, J. Performance enhancement of poly(lactic acid) and sugar beet pulp composites by improving interfacial adhesion and penetration. *Ind. Eng. Chem. Res.* **2008**, *47*, 8667–8675.
(27) Callister, W. D. *Materials Science and Engineering*, 5th ed.; Wiley: New York, 2000; pp 800−801.

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