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Immobilization of bacteriocin nisin into a poly(vinyl alcohol) polymer matrix crosslinked with nontoxic dicarboxylic acid

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ABSTRACT: This work is focused on novel methodology of poly(vinyl alcohol) crosslinking by non-toxic dicarboxylic acid, glutaric acid. The cross-linked system was used as a matrix for immobilization of bacteriocin nisin. Effect of the crosslinking degree on physico-chemical, morphology, mechanical, and thermal properties of poly(vinyl alcohol) films were investigated by using swelling test, Fourier transform infrared spectroscopy, scanning electron microscopy, stress–strain analysis, differential scanning calorimetry, and thermogravimetry. Release profile of the nisin from the cross-linked poly(vinyl alcohol) was studied by high performance liquid chromatography. Antibacterial activity of the prepared systems was tested by agar diffusion test and dilution and spread plate technique. Results showed suitability of glutaric acid as effective crosslinking agent of poly(vinyl alcohol) that acts synergistically with bacteriocin nisin against the tested Gram-positive and Gram-negative bacterial strains. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43674.

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INTRODUCTION

Globalization of the food market and the necessity for fresh and minimally processed food has fueled demand for advantageous packaging materials boasting barrier and protective properties.[1,2] Consequently, greater attention is being paid to antibacterial packaging. This has been widely investigated as a response to consumer-driven demand or trends in industrial production towards fresh, tasty, minimally preserved convenience foods with an extended shelf-life and guaranteed quality.[1–3]

Antimicrobial properties for plastic packaging are brought about through modification with relevant natural-based substances.[4–6] Of particular interest currently is the use of bacteriocins and other biologically derived antimicrobials in packaging materials. This pertains to a broad spectrum of bacteriocins, such as nisin, inhibit Gram-positive food-borne pathogens and spoilage microbes; in addition to Gram-negative bacteria if further processing is undertaken.[7] Out of the recognized bacteriocins, nisin is the only one to have been conferred the status of generally recognized as safe (GRAS) and to receive approval from the US Food and Drug Administration.[6,8] Several forms of nisin are commercially produced and commonly added into food products as preservatives. However, the use of nisin is limited by its structural instability, stemming from loss of activity due to interactions with food and cell matrices. Nevertheless, it is possible to overcome this instability by incorporating it in polymers.[7,9]

Commercially available synthetic polymer poly(vinyl alcohol) (PVA) is frequently used in packaging applications for food, cosmetic and pharmaceutical products, mostly due to its biodegradability, biocompatibility and non-toxicity.[3,10–13] Cross-linked PVA boasts good chemical, thermal and mechanical stability. As a consequence of its hydrophilic nature, PVA has to be modified to minimize swelling in water or atmospheric moisture when fabricated for aqueous applications. Methods for improving the properties of PVA include freezing, heat treatment, irradiation, chemical crosslinking or combinations of these techniques.[14] The insolubility of PVA by chemical crosslinking with dialdehydes, an example being glutaraldehyde, has been well documented.[14–17] Nevertheless, this synthetic crosslinking reagent has been reported as highly cytotoxic, which may impair the biocompatibility of cross-linked materials.[16]

Chemical crosslinking with a non-toxic substance is a suitable alternative procedure for commonly used toxic aldehydes.[18,19] All multi-functional compounds capable of reacting with the hydroxyl group can be used as a cross-linker of PVA.[12] One such option relates to dicarboxylic acids, including

Additional Supporting Information may be found in the online version of this article.

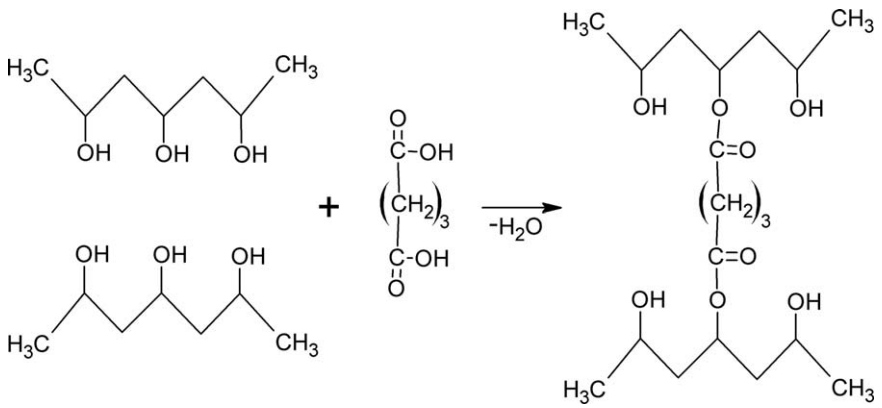

Figure 1. The theoretical reaction scheme of PVA with GA.

low-molecular glutaric acid (GA).[20] The theoretical reaction scheme for cross-linked PVA by GA is illustrated in Figure 1.

Investigation has already taken place on the crosslinking interaction of PVA and GA or poly(propylene imine) with cotton cellulose and GA at high temperature.[20,21] However, no literature has reported on the behavior of thermally unstable bacteriocin nisin in PVA composite film, cross-linked with GA in one step without thermal treatment.

So as to propose herein a self-active antibacterial film for either food packaging or medical purposes, fully hydrolyzed PVA was compounded with GA by the solvent casting technique. Apparent solubility studies were performed as well as research on structural characterization, and mechanical and thermal properties. Antibacterial activity was tested against *S.taphylococcus aureus* and *E.scherichia coli.*

EXPERIMENTAL

Materials

Poly(vinyl alcohol) (PVA, Mowiol 6-98, $M_w \sim 47{,}000$ g mol^{-1}, degree of hydrolysis 98%, CAS:9002-89-5), glutaric acid (GA, 99%, CAS:110-94-1), and nisin from *Lactococcus lactis* (NIS, 2.5% balanced sodium chloride and denatured milk solids, CAS:1414-45-5) were purchased from Sigma–Aldrich, USA. Methyl alcohol (CAS:67-56-1) and hydrochloric acid (HCl, 35%, CAS:7647-01-0) were of analytical grade and sourced from Penta Chemicals, Czech Republic. All the chemicals were used as received without further purification. The bacterial species, *S. aureus* (CCM4516) and *E. coli* (CCM4517), were provided by the Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic. The media required for the microbiological studies (Mueller Hinton Agar, Nutrient broth with 1% Peptone; Plate Count Agar; Soybean Casein Digest broth with Lecithin and Polysorbate 80) were obtained from HiMedia Laboratories, India.

Sample Preparation

PVA films cross-linked with various concentrations of GA with NIS (PVA/GA/NIS) and without NIS (PVA/GA) were prepared using the solvent casting technique as described below.

First, PVA was dissolved in 0.02M HCl (10% water solution) at 90 °C for 30 min under continuous stirring. Once the PVA had completely dissolved, the solution was allowed to cool to room temperature.

Second, GA was added to achieve the desired concentration (X_{GA}) of 0–100% for the theoretical crosslinking agent (Table I). Regarding the subsequent PVA solution, its theoretical concentration of the crosslinking agent was estimated through eq. (1), in terms of the molar ratio of the crosslinking agent to PVA.[22]

$$X_{GA} = \frac{W_{GA} \cdot M_{PVA} \cdot 2}{W_{PVA} \cdot M_{GA}} \cdot 100 \quad (1)$$

The theoretical concentration of the crosslinking agent (X_{GA}) is a function of the total weight of the crosslinking agent utilized (W_{GA}), the molecular weight of a single PVA chain (M_{PVA}), the total weight of PVA used (W_{PVA}), and the molecular weight of the crosslinking agent (M_{GA}).

The PVA solution containing the crosslinking agent and methanol (4% v/v) was stirred continuously for an additional 16 h to achieve formation of bonds between the PVA and GA chains.

Finally, the NIS powder was dissolved in 0.02M HCl and filtered using a syringe microfilter (PTFE, 0.45 μm). An adequate amount of NIS solution was added into the PVA/GA solution and stirred continuously for 15 min to prepare homogeneous polymer samples. Then the samples were poured into an acrylic mold and dried at 35 °C for 24 h in a temperature controlled incubator. All obtained films were stored in silica gel containing desiccator and placed inside sealed polyethylene bags to avoid moisture accumulating. The thickness of the resultant product was 100–130 μm. The eventual concentration of NIS in the polymer sample equaled 150 μg g^{-1}. The samples were designed

Table I. Crosslinking Agent Mass in the Feed

Sample	GA (g/10 g PVA)	Sample	GA (g/10 g PVA)
PVA/0% GA	0.0000	PVA/40% GA	5.6217
PVA/5% GA	0.7027	PVA/60% GA	8.4326
PVA/10% GA	1.4054	PVA/80% GA	11.2434
PVA/20% GA	2.8109	PVA/100% GA	14.0543
PVA/25% GA	3.5136		

as PVA/*x*GA/NIS and PVA/*x*GA, where *x* defined the degree in per cent of PVA crosslinking.

Characterization

Scanning Electron Microscopy. Scanning electron microscopy (SEM) examination was performed on PVA/0GA/NIS and PVA/60GA/NIS composite fractured surfaces in order to evaluate the degree of homogeneity and to gain insight into the internal structure of the composites. The films were observed under a scanning electron microscope (Vega II/LMU, Tescan, Czech Republic). The films were cooled to beneath −60 °C in a freezer box, then were broken and placed onto the SEM sample stub.

Degree of Swelling and Solubility. The PVA, PVA/*x*GA and PVA/*x*GA/NIS films were cut into square pieces of 1.5 × 1.5 cm^2 and dried until constant weight (W_1) was reached. Each piece was immersed in demineralized water at the temperature of 25, 50, and 70 °C. The specimens were removed after predetermined time intervals. Subsequently, surface moisture was carefully removed using paper napkin and the weight of the sample film was measured (W_2). The degree of swelling (DS) was calculated as follows:

$$DS = \frac{W_2 - W_1}{W_1} \cdot 100 \quad (2)$$

Following the last DS measurement, the swelled films were dried again until reaching constant weight (W_3) at 60 °C. Solubility (S) was calculated via the following equation:

$$S = \frac{W_1 - W_3}{W_1} \cdot 100 \quad (3)$$

Attenuated Total Reflectance/Fourier Transform Infrared Spectroscopy (ATR/FTIR). FTIR spectroscopy analysis was carried out to evaluate differences between the structure of pure PVA, PVA/*x*GA and PVA/*x*GA/NIS films. Spectra measurements were performed on a Nicolet 320 FTIR (Nicolet Instrument Corporation, Charleston, WV), using a Zn-Se crystal and OMNIC software (Thermo Fisher Scientific, Waltham, MA), over a range of 4000–650 cm^{-1}. A uniform resolution of 2 cm^{-1} was maintained in all cases.

Surface Properties. The surface water contact angle (WCA) of dry PVA and PVA/*x*GA composite films were determined on a contact angle system—See System 7.0 (Advex Instruments, Czech Republic)—to evaluate the hydrophilicity of the films. To minimize experimental error, the contact angle was measured at three random locations for each sample and the average value subsequently reported.

Static Tensile Measurements. Tensile experiments were carried out with the aid of a tensile testing machine, an M350-5CT device (TESTOMETRIC, UK), in accordance with the ISO 527-1,3:2012 standard.[23,24] The initial length of samples was 100 mm, while the width equaled 10 mm and thickness measured about 100–130 μm. The moving speed of the crosshead was 100 mm min^{-1}. Measurements were performed at the temperature of 25 °C. The values were calculated as averages of six specimens for each plastic film. All the specimens were conditioned in a temperature-humidity controlled chamber at 25 °C and 50% RH for 10 days.

Table II. Gradient of Chromatographic Method

Mobile phase A (%)	Mobile phase B (%)	Time (min)
20	80	0-5
80	20	5-20
20	80	20-25
20	80	25-40

Differential Scanning Calorimetry. The PVA, PVA/*x*GA and PVA/*x*GA/NIS films were analyzed on a differential scanning calorimeter (DSC), a Mettler Toledo DSC1 STAR testing machine with STAR^e Default DB V.9.20 software, in order to assess differences in thermal properties. The samples were dried at 60 °C for 14 h prior to testing. The dried samples (8–10 mg), sealed in aluminum pans, were heated from −20 °C to 230 °C at a heating rate of 10 °C min^{-1}, which was followed by a holding step at 230 °C for 1 min and the process of cooling to −20 °C. After maintaining this temperature for 1 min, a second heating cycle was conducted under the same conditions. The melting temperature (T_m) was determined as the peak value for melting endotherm from the first heating cycle. The value for glass transition temperature (T_g) was determined from the second heating cycle at the mid-point stepwise increase of the specific heat associated with glass transition.

Thermogravimetry. Thermogravimetric measurements (on a TGA Q500, TA Instruments, USA) were performed at a heating rate of 10 °C min^{-1} from 25 to 750 °C under a nitrogen atmosphere (100 mL min^{-1}). The results were evaluated using the V20.13 Build 39 program.

Antibacterial Properties. The antibacterial activity of PVA/*x*GA and PVA/*x*GA/NIS films against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacterial strains were evaluated by an agar disk diffusion test, as well as the dilution and spread plate technique. Six and three independent measurements were made for disk diffusion test and dilution and spread plate technique, respectively.

Release Profile. High performance liquid chromatography with detection by a UV-VIS detector, which is a suitable method for determining nonvolatile compounds,[25] was selected for quantitative determination of NIS. PVA and PVA/*x*GA/NIS films (ca. 0.5 g) were kept in 10 mL of demineralized water at 25 °C at predefined time intervals. A 0.5 mL of aqueous media was collected for analysis by HPLC (HPLC Shimadzu Prominence LC20A Series equipped with thermostatted column compartment and UV-VIS detector). The measurement parameters were as follows: Column XSelect, CS C18 (5 μm, 4.6 × 250 mm^2, Waters) with a precolumn (Reprosil 100 C18, 50 × 4 mm^2, 5 μm, Watrex), column temperature: 40 °C; mobile phase acetonitrile with 0.05% (v/v) trifluoroacetic acid (TFAA) (A), water with 0.05% (v/v) of TFAA (B). The gradient method is shown in Table II. The release experiments were led three times for each type of film.

RESULTS AND DISCUSSION

The prepared films were smooth and transparent up to the crosslinking degree 40%. The transparency of the specimens

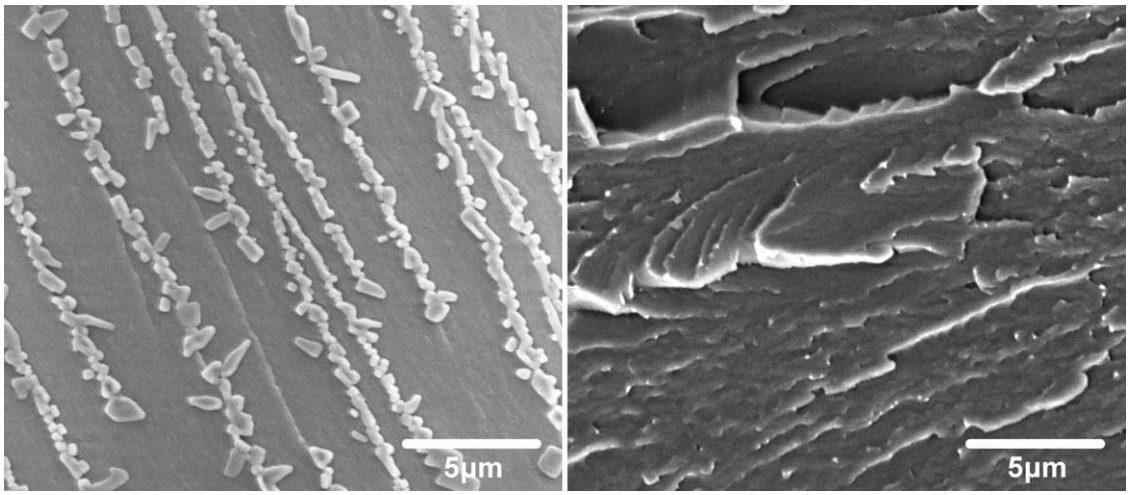

Figure 2. SEM images of PVA/0GA/NIS (left) and PVA/60GA/NIS (right).

with the crosslinking degree above 60% was significantly reduced. The photographs of the films are provided as supporting material (Supporting Information Figure S1). A scanning electron microscope was used to study the morphology of the PVA/0GA/NIS and PVA/60GA/NIS composite fractured surfaces, so as to evaluate the degree of homogeneity and gain insight into the internal structure of the composites. Sodium chloride crystals derived from the nisin modifier are visible on the fractured surface of the film in Figure 2 (left). This indicates that sodium chloride crystals can be produced in the process of forming film.[26] However, the cross-linked polymer film (Figure 2, right) evinced a smooth fractured surface. Basfar *et al.* and Gohil *et al.* reported similar findings with PVA cross-linked by glutaraldehyde and maleic acid, respectively.[12,27] As shown in Figure 2 (right), increased crosslinking of PVA with GA may produce a more compact polymer structure.[20] These results corresponded with findings on mechanical properties shown below.

The degree of crosslinking in a polymer structure has a significant influence on the different properties of a polymer film, like swelling and ration mechanical strength.

The effect exhibited by GA modified PVA through water interaction on the resultant films was studied gravimetrically according to eq. (2). Table III shows the dependence of the degree of swelling (DS) for pure PVA film and samples with different levels of crosslinking with GA over time at different temperatures. Despite the potential resumption of nisin stabilized by NaCl, which may react with the PVA matrix, the low amount of nisin

Table III. Swelling Behavior of PVA-Based Films

Temperature (°C)	Time (hour)	PVA	PVA/10GA	PVA/20GA	PVA/40GA	PVA/60GA	PVA/100GA
25	15	132.1 ± 5.5	57.2 ± 2.8	21.8 ± 0.6	16.2 ± 0.1	4.2 ± 0.2	[a]
	20	131.8 ± 4.6	58.7 ± 3.8	22.4 ± 0.2	16.6 ± 0.1	4.3 ± 0.5	[a]
	40	129.8 ± 4.7	59.5 ± 4.0	23.2 ± 0.2	17.3 ± 0.1	4.5 ± 0.1	[a]
	45	128.9 ± 4.6	60.0 ± 4.5	23.6 ± 0.0	17.1 ± 0.0	4.5 ± 0.3	[a]
	160	125.4 ± 4.3	62.6 ± 4.1	26.3 ± 0.2	18.6 ± 0.3	5.4 ± 0.3	[a]
50	15	202.9 ± 0.0	72.4 ± 0.5	30.5 ± 0.6	19.9 ± 0.9	6.3 ± 0.4	[a]
	20	194.5 ± 0.1	75.2 ± 0.1	33.1 ± 0.1	21.5 ± 0.8	4.8 ± 2.7	[a]
	40	195.3 ± 0.0	79.4 ± 0.5	38.6 ± 0.6	22.3 ± 2.9	8.2 ± 0.6	[a]
	45	[b]	80.2 ± 0.2	39.5 ± 0.2	24.0 ± 1.5	8.9 ± 0.4	[a]
	160	[b]	96.6 ± 4.2	68.6 ± 0.2	39.5 ± 1.3	25.7 ± 0.8	[a]
70	15	[b]	116.4 ± 1.5	61.9 ± 1.7	34.9 ± 1.2	16.9 ± 0.0	[a]
	20	[b]	126.0 ± 2.4	69.3 ± 2.4	39.2 ± 0.8	21.6 ± 0.3	[a]
	40	[b]	135.3 ± 0.6	85.3 ± 1.6	48.8 ± 0.4	37.6 ± 0.2	[a]
	45	[b]	134.9 ± 0.2	87.5 ± 0.8	49.6 ± 0.1	38.9 ± 0.0	[a]
	160	[b]	242.5 ± 14.1	212.1 ± 0.2	126.5 ± 1.7	113.0 ± 2.2	0.6 ± 0.1

[a] No swelling.
[b] Dissolved sample.

Table IV. Solubility Behavior of PVA-Based Films

	0GA	10GA	20GA	40GA	60GA	100GA
PVA/xGA	82.9 ± 1.2	70.6 ± 0.0	64.8 ± 0.2	64.3 ± 0.1	57.6 ± 0.1	28.6 ± 0.1
PVA/xGA/NIS	52.4 ± 0.3	55.0 ± 0.4	31.5 ± 1.2	32.9 ± 3.7	25.7 ± 4.1	26.2 ± 1.0

modifier displayed no notable effect on the degree of swelling of the final polymer films.[28] In the case of the pure PVA film, merely a significant influence was exerted on temperature. The samples measured at 50 °C could only be analyzed within 40 h of the experiment, while at 70 °C complete dissolution of the film occurred within the first 24 h of the experiment. These results are consistent with the theoretical predictions stated by thermodynamic laws, and with the experimental results of other authors.[29,30]

Increasing the crosslinking degree leads to significant decrease in the DS of the examined samples. The maximum DS after 160 h of the experiment increases in parallel with rising temperature. These results reveal the formation of covalent bonds between the PVA chain, which limits interaction between macromolecules and hydrophilic solvent molecules—water.

A solubility test was conducted following the swelling experiments (after 160 h) at 25 °C. Solubility was calculated according to eq. (3). Table IV shows the dependence of solubility on the degree of crosslinking with GA. The course of the crosslinking reaction was confirmed again by solubility decreasing in parallel with a rising degree of crosslinking for PVA/*x*GA and PVA/*x*GA/NIS. Reduced solubility for PVA films with NIS may also slightly result from interactions between NaCl (protein stabilizer) and the PVA matrix relating to formation of insoluble complexes.[31]

FTIR-ATR analysis was used to identify functional groups and interaction between PVA, GA and NIS. FTIR-ATR spectra for PVA/*x*GA are shown in Figure 3. FTIR-ATR spectra for the pure components reveal characteristic absorption bands pertaining to PVA and GA, which correspond to spectra as presented in the literature. Characteristic peaks for PVA can be found at wavenumbers 3323 cm^{-1} (O—H stretching band), 2936 cm^{-1} (C—H stretch vibration band) and 1429 cm^{-1} (CH_2 deformation band), together with an acetal bridge peak for C—O—C at 1150–1085 cm^{-1}.[11,32]

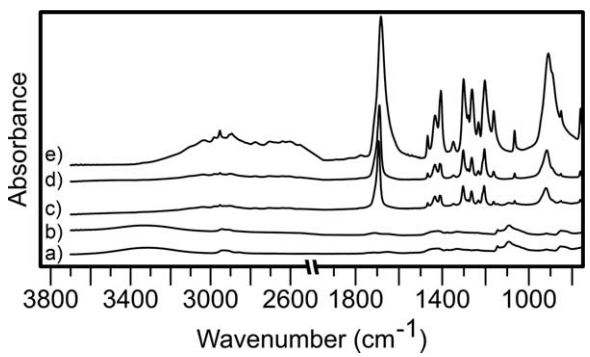

Figure 3. FTIR-ATR spectra of (a) PVA, (b) PVA/20GA, (c) PVA/40GA, (d) PVA/100GA and (e) pure GA.

A noticeable belt at around 2955 cm^{-1} is visible in the spectrum belonging to GA, which represents a —CH_2 stretch chain superimposed upon an O—H stretch band. The peak at 1718 cm^{-1} relates to the C=O stretch group, while that at 1203 cm^{-1} pertains to C—OH in-plane bending bonds originating from carboxyl groups. This corresponds with the results of Mitra *et al.*[33] NaCl shows no absorption spectrum via this method.

Structural characterization of the sample PVA/*x*GA films via FTIR-ATR confirms findings made by the formation of ester bonds. This is represented essentially by a downward trend in the bands at 3323 and 2955 cm^{-1} (O—H), 1150 and 1085 cm^{-1} (C—OH), which clearly indicate the elimination of free OH groups in the polymer structure. The course of the crosslinking reaction is also confirmed by the presence of a characteristic absorption band at 1718–1729 cm^{-1} (C=O). The rising degree of crosslinking consequently triggered increases in the peak at 920 cm^{-1}, in addition to symmetrical movement of the chain (C—O—C) and a rise in the peak at 1718 cm^{-1} (C=O). Similar results were presented by Ceia *et al.* in their study of PVA crosslinked by glutaraldehyde.[32]

The spectra of the measured samples containing NIS reveal no specific interactions between the polymer matrix and the modifier. The presence of NIS does not exhibit any discernible influence on the FTIR-ATR spectra for the samples due to the low concentration of modifier in the PVA matrix.

The effect of the crosslinking reaction between PVA and GA on the surface properties of the prepared films was also tested by measuring the surface contact angle with water (WCA). The results are shown in Table V. It can be seen that an increasing degree of crosslinking occurs in conjunction with a steep decrease in WCA, thereby furthering the hydrophilicity of the surfaces studied. This might result from the roughness of the surface of the random forms contributing to the rise in hydrophobicity of the surface. However, the slight contact angle of

Table V. The Influence of The Degree of Crosslinking of PVA Films on WCA

Sample	WCA (°)
PVA	68.2 ± 0.7
PVA/40GA	34.2 ± 3.5
PVA/60GA	25.9 ± 1.8
PVA/100GA	22.2 ± 2.4

Table VI. Mechanical Properties of PVA Films with Various Crosslinking Degree

Sample	Young modulus (MPa)	Tensile strength (MPa)	Elongation at break (%)
PVA	670 ± 50	39 ± 4	189 ± 17
PVA/5GA	216 ± 20	35 ± 4	174 ± 16
PVA/15GA	82 ± 8	28 ± 4	189 ± 22
PVA/20GA	46 ± 4	23 ± 3	293 ± 30
PVA/25GA	48 ± 4	21 ± 2	211 ± 22
PVA/40GA	17 ± 2	8 ± 1	92 ± 12
PVA/80GA	8 ± 1	2 ± 0	36 ± 4

the surface on the cast films is a consequence of the smoothness of the latter.[34]

The influence of crosslinking reaction values on the mechanical properties of PVA films is listed in Table VI as the dependence of Young's modulus (E), tensile strength and elongation at break on the degree of crosslinking with GA. The results clearly show significant reduction in E as well as tensile strength, thereby demonstrating the progress of the crosslinking reaction and action of the GA plasticizer—i.e., the formation of transverse bonds; although such decrease permits flexibility in chain mobility.[35,36] Elongation at break, which is determined at the point where the film breaks under tensile stress, provides data on the flexibility and stretch capacity of the film. As Table VI shows, elongation at break diminishes alongside rise in the amount of GA in the cross-linked film. The similar results have also been reported for PVA-glutaraldehyde blend films by other researchers.[37,38] The increasing elongation at break could be due to formation of a more rigid and compact network with an increasing content of crosslinking agent, consequently reducing the number of hydrophilic hydroxyl groups in the polymer chains. This is also confirmed by the reduction in solubility concurrent with escalation in GA content.[37]

Crosslinking with GA by up to 25% appears to improve mechanical properties and practical applicability. Above this threshold there is no significant increase in the efficacy of the crosslinking reaction. On the contrary, excess GA significantly impairs the mechanical properties of the film.

The effect of the crosslinking reaction between PVA and GA can also be seen in measurements recorded by the differential scanning calorimeter (DSC); see Table VII. While pure PVA exhibited for the first heating cycle a melting temperature (T_m) maximally of 185 °C and a glass transition temperature (T_g) for the second heating cycle of 113 °C, cross-linked systems showed significantly lower values for both. As a general rule, any structural feature that reduces segmental mobility of polymer chains or free volume gives rise to increase in T_g and T_m.[37] However, in this case a significant drop of T_g was observed. Moisture and plasticizers content plays an important role in connection with this. Water is a known plasticizer, hence also diminishes T_g.[11] The same behavior can be expected from eventually unreacted GA. Such decline in mobility of the polymer chains and closely related lesser value for T_g is visible in samples containing NIS, arising through the interaction introduced between the PVA and salt ions. In contrast, melting point values actually go up due to the presence of NaCl that originates in commercially available NIS.[39]

Thermogravimetry was carried out for pure GA, pure PVA film, and PVA/xGA films at temperatures from 25 °C to 750 °C. The effect of the crosslinking reaction on the thermal stability of PVA-based films is evident from the TGA curves as illustrated in Figure 4; the corresponding thermal degradation temperatures are given in Table VIII. Figure 4 clearly displays the shift in initiating thermodegradation processes to higher temperatures, which is in contrast to pure PVA. All the analyzed samples showed a degradation multistage, while PVA actually had two such stages,[40] and cross-linked systems demonstrated 3 degradation stages. Generally, decomposition of the samples fell into four temperature ranges. The first interval (up to 260 °C) comprised decomposition of thermally unstable GA and proteins (if present), evaporation of water, possible dehydration and the potential to form polyenes with the capacity for depolymerization, which were decomposed in the second interval at temperatures of up to 380 °C.[12,33,41] Degradation of higher carbon compounds occurred at temperatures above 380 °C.[41] The observed temperature ranges correspond to the thermal properties of chitosan/PVA films cross-linked by glutaraldehyde and dicarboxylic itaconic acid.[42]

The antibacterial activity of the samples was studied via the agar diffusion method, and the dilution and spread plate technique against both Gram-positive *S. aureus* and Gram-negative *E. coli* bacterial strains.

Evaluation of the antibacterial activity of various film samples using the first of the aforementioned methods is shown in Figure 5, as an average of 20 measurements with the width of the inhibition zones. Figure 5 displays that sample PVA/0GA had no effect against *E. coli* and *S. aureus*. In contrast, sample PVA/100GA/NIS, which contains 150 μg of nisin per g of film and GA corresponding to 100% crosslinking, acted against both

Table VII. Glass Transition Temperature (T_g) and Melting Temperature (T_m) of PVA-based Films and GA

Sample	T_g (°C)	T_m (°C)
PVA/0GA	113.0	185.0
PVA/20GA	92.9	168.0
PVA/40GA	82.9	162.0
PVA/80GA	49.7	157.1
PVA/0GA/NIS	121.6	184.1
PVA/5GA/NIS	115.4	182.3
PVA/10GA/NIS	107.8	189.1
PVA/20GA/NIS	97.2	208.0
PVA/40GA/NIS	95.7	212.5
PVA/60GA/NIS	65.5	205.2
PVA/100GA/NIS	49.5	212.3
GA	53.3	98.4

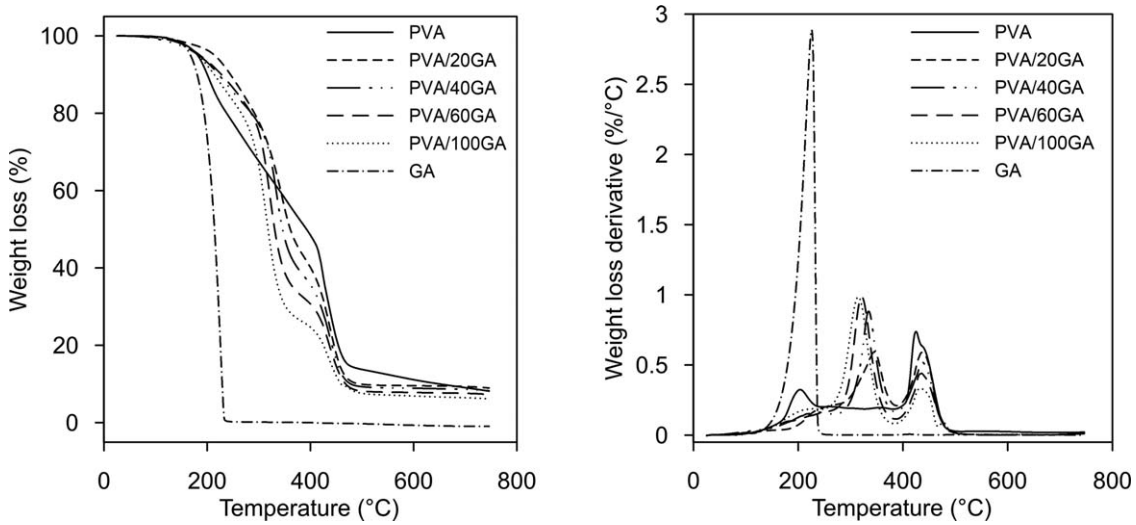

Figure 4. TGA (left) and DTG (right) curves of PVA, PVA/20GA, PVA/40GA, PVA/60GA, PVA/100GA and pure GA.

bacterial strains (Supporting Information Figure S2). An important finding was that the GA in the polymer system did not bring about antibacterial activity against Gram-negative *E. coli* in samples with up to the degree of 40% crosslinking of GA. It is clear that the response to the Gram-negative strain was caused by the synergistic effect of NIS and GA at lower concentrations. The nature of the given bacteria also plays an important role. Gram-positive bacteria (*S. aureus*) do not possess such a complex structure of cell wall as Gram-negative ones do (*E. coli*). This could explain the better resistance of Gram-negative bacteria to antibacterial agents.[10] Another factor is that nisin is widely known to exert antibacterial activity primarily against Gram-positive but not Gram-negative bacteria.[43] Nevertheless, Helander *et al.* demonstrated interaction by nisin with organic acids, indicating that nisin can act in a protective manner on Gram-negative bacteria.[44] Non-cross-linked PVA films showed no antibacterial activity.[10] Both antibacterial tests presented diminishing bacterial growth alongside increasing GA concentration in all cases, as mentioned previously by other authors.[45]

It is known that NIS is susceptible to degradation over time if not sufficiently stabilized. The efficacy of the films against *E. coli* and *S. aureus* after 7 and 21 days is shown in Figure 6. The results for films with a lower degree of GA crosslinking show that there is a slight reduction in antibacterial activity against both of the bacterial strains over time. In contrast, a higher degree of crosslinking (40% or more) provides a stable system with long-lasting antibacterial effects. Correia *et al.* reported that the PLGA-nisin matrix is able to continuously deliver nisin for more than 2 weeks, a finding which corresponds to our results.[9] The synergy of the antibacterial activity of nisin (especially against Gram positive *S. aureus*) and GA at higher crosslinking degree is evident also here.

The second procedure for testing antibacterial activity was the modified spread plate technique and dilution method, where samples were exposed to bacterial suspension in test tubes for 24 h. After an additional 24 h of cultivation on agar, the resultant colony counts were deduced for each dilution. The test was repeated again after 3 weeks to detect any change in the

Table VIII. Thermal degradation temperatures of the samples expressed of peaks of DTG curves (Figure 4)

Sample	Tp_1	Tp_2	Tp_3
PVA/0GA	–	357.0	425.0
PVA20GA	265.4	345.4	436.2
PVA/40GA	235.4	334.5	437.4
PVA/60GA	230.8	321.2	435.6
PVA/100GA	225.3	314.7	432.3
GA	225.6	–	–

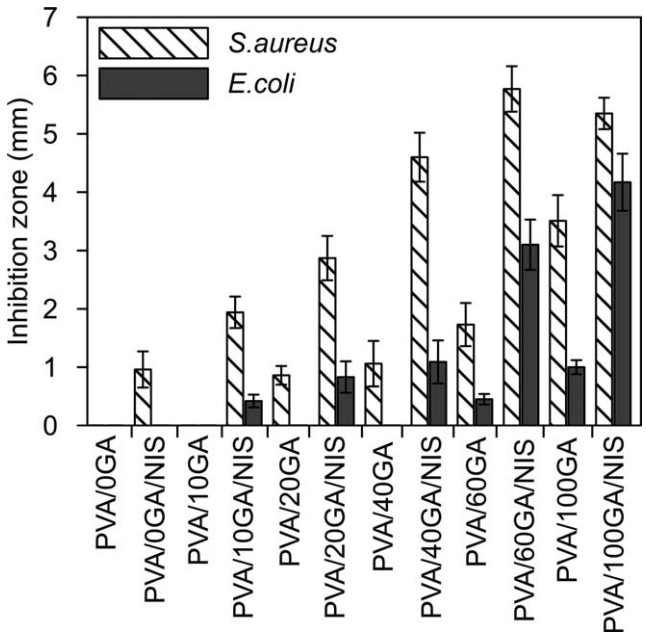

Figure 5. Inhibition zone of samples against Gram-positive *S. aureus* (striped bars) and Gram-negative *E. coli* (grey bars).

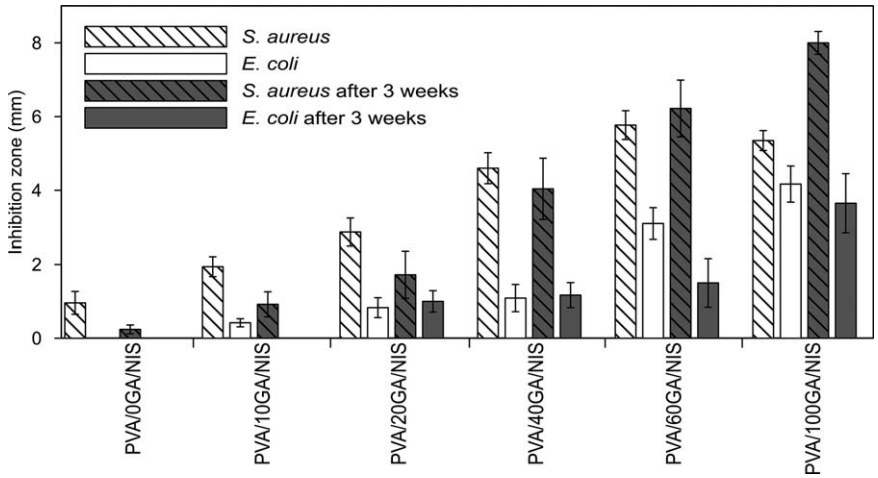

Figure 6. The effect of aging of antibacterial films depending on the increasing crosslinking degree against *S. aureus* (striped bars) and *E. coli* (unstriped bars); provided for the first week after production (white bars) and after 3 weeks (grey bars).

Table IX. Aging effect on antibacterial activity of NIS containing PVA films with various crosslinking degree

	(CFU/ml)			
	E. coli		*S. aureus*	
Sample	After 1 week	After 3 weeks	After 1 week	After 3 weeks
PVA/0GA/NIS	$2.9{\cdot}10^{6}$	$5.0{\cdot}10^{6}$	$1.4{\cdot}10^{4}$	$2.1{\cdot}10^{4}$
PVA/5GA/NIS	0	$1.3{\cdot}10^{4}$	$1.1{\cdot}10^{3}$	$3.0{\cdot}10^{3}$
PVA/10GA/NIS	0	$4.2{\cdot}10^{3}$	$4.5{\cdot}10^{1}$	$1.8{\cdot}10^{3}$
PVA/20GA/NIS	0	$1.3{\cdot}10^{3}$	$7.0{\cdot}10^{1}$	$1.1{\cdot}10^{2}$
PVA/40GA/NIS	0	$5.0{\cdot}10^{0}$	0	$8.2{\cdot}10^{1}$
PVA/60GA/NIS	0	0	0	0
PVA/100GA/NIS	0	0	0	0

efficacy of the films. Table IX presents results from said spread and dilution method, clearly highlighting the effect of the NIS and GA combination in the film samples. The results obtained tally with those of the agar diffusion method, as detailed above.

The release profile of NIS from the PVA/*x*GA/NIS samples was monitored for 10 days at 25 °C. The amount of nisin released at different times was determined by HPLC. As can be seen from Figure 7, increase in the degree of crosslinking leads to slower release of NIS. An initial burst release phenomenon appeared in each cumulative release curve within the first 4 h, mainly due to dissolution of nisin on or near to the surfaces of the films. After 4 h, the cumulative release curves (in per cent) exhibited a gradual rise in accumulative release, as the entrapped nisin molecules would actually require greater time for release from the inner core of the polymer matrix.[13] It was established that the most intensive release occurred during the first 24 h.[46] Figure 7 also illustrates that the polymer matrix, which had been cross-linked to 100%, permitted release of NIS after a lag phase of about 1.5 h. Hence, the results indicate that it is possible, by means of a cross-linked polymer, to create a system facilitating gradual release of NIS. These findings correlate with Alishahi, where NIS was immobilized to chitosan

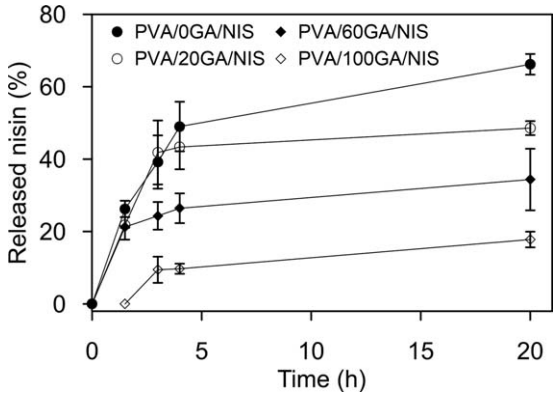

Figure 7. The release profile of NIS from PVA/0GA, PVA/20GA, PVA/60GA, and PVA/100GA.

nanoparticles through electrostatic interactions between the chains.[46]

CONCLUSIONS

The modified poly(vinyl alcohol) composites with antibacterial nisin were prepared by immobilizing the same into a crosslinked polymer matrix, which was carried out by utilizing dicarboxylic acid glutaric at different levels of crosslinking degree. Structural characterization by FTIR reveals an esterification reaction between PVA and GA. Moreover, crosslinking esterification reactions between both components in the systems were demonstrated through studying the degree of swelling, solubility and water contact angle. Testing also involved investigating the efficacy of PVA crosslinking on that of immobilization and antibacterial activity using bacteriocin nisin.

Results indicate that GA is indeed a suitable crosslinking agent for PVA, which acts synergistically with bacteriocin nisin against the tested Gram-positive and Gram-negative bacterial strains. As regards the mechanical properties of the systems studied, it is preferable to maintain lesser degrees of PVA crosslinking with GA, thereby not bringing about significant adverse changes to the samples in terms of Young's modulus, tensile stress and strength. Nevertheless, a higher degree of crosslinking is interesting from the perspective of antibacterial activity and controlled release of nisin from the PVA matrix. In relation to this, nisin in combination with GA (up to 20%) facilitates effective antibacterial systems for modifying PVA.

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