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Covalent Immobilization of Lysozyme on Ethylene Vinyl Alcohol Films for Nonmigrating Antimicrobial Packaging Applications

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ABSTRACT: The objective of this study was to develop a new antimicrobial film, in which lysozyme was covalently attached onto two different ethylene vinyl alcohol copolymers (EVOH 29 and EVOH 44). The EVOH surface was modified with UV irradiation treatment to generate carboxylic acid groups, and lysozyme was covalently attached to the functionalized polymer surface. Surface characterization of control and modified films was performed using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and dye assay. The value of protein loading after attachment on the surface was 8.49 μg protein/cm^2 and 5.74 μg protein/cm^2 for EVOH 29 and EVOH 44, respectively, after 10 min UV irradiation and bioconjugation. The efficacy of the EVOH−lysozyme films was assessed using *Micrococcus lysodeikticus*. The antimicrobial activity of the films was tested against *Listeria monocytogenes* and was similar to an equivalent amount of free enzyme. The reduction was 1.08 log for EVOH 29−lysozyme, 0.95 log for EVOH 44−lysozyme, and 1.34 log for free lysozyme. This work confirmed the successful use of lysozyme immobilization on the EVOH surface for antimicrobial packaging.

KEYWORDS: *lysozyme, EVOH, immobilization, UV irradiation, L. monocytogenes*

INTRODUCTION

Microbial growth in packaged foods not only represents a significant food safety concern but also contributes to the increasing amount of food wasted due to microbial spoilage organisms. Antimicrobials may therefore be used in food products to inhibit or retard microbial growth in foods in order to extend product shelf life.[1] When they are added directly to food formulations, antimicrobial agents cause an immediate reduction in the bacterial populations; however, this immediate activity is often followed by a decline in activity when the concentrations of active antimicrobials decrease to values below the minimal inhibitory concentration due to degradation, interaction with food, or dispersion in the food matrix. Further, antimicrobial agents may exert only limited activity against injured cells or against the growth of surviving cells.[2] Many products are subject to microbial contamination at the food surface,[3] and therefore, the bulk addition of antimicrobials to such foods may be excessive to achieve the desired control. An alternative is to develop an antimicrobial active packaging system by incorporation of antimicrobial agents either throughout the bulk of the packaging material to enable controlled release of the active agent or by immobilization onto the packaging surface to provide a direct contact of the antimicrobial with the food surface. A broad range of natural compounds have been researched as antimicrobial agents for use in food packaging, such as organic acids, essential oils, peptides, enzymes, etc.[4]

The incorporation of antimicrobials throughout the bulk of packaging matrices has been used to control microbial contamination by allowing for diffusion of the antimicrobial through the packaging in a time-released manner.[5] Many approaches to antimicrobial active packaging follow this approach, yet it suffers certain drawbacks: (a) it is often difficult to release these antimicrobials in a controlled way to maintain the antimicrobial activity; (b) the presence of the agents in the polymer matrix may lead to changes in the functional properties of the films; and (c) biologic antimicrobials such as peptides and enzymes are difficult to incorporate into many packaging films due to incompatibility with the film manufacturing process (thermomechanical stress or solvent miscibility). There would therefore be both functional and regulatory advantages in an antimicrobial packaging material in which the active agent is covalently bound onto the material's surface such that it was unlikely to migrate into the food.[6−8] Modification of film surfaces by chemical or physical means followed by covalent conjugation have been used for attachment of active agents that are active when in contact with foods, yet do not migrate and are not consumed.[9−13]

Lysozyme is an antimicrobial enzyme and is one of the most studied natural antimicrobial agents with packaging applications.[1] It is classified as GRAS (generally recognized as safe) by the Food and Drug Administration (FDA) and as a food additive by the European Union (E 1105) with bacteriostatic, bacteriolytic, and bactericidal activity.[14] It is characterized by a single polypeptide chain. The antimicrobial activity depends on the ability to hydrolyze the beta 1−4 glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine. These bonds are present in peptidoglycans, which comprise 90% of the Gram-positive bacteria cell wall, making them very susceptible to lysozyme antimicrobial activity.[15] Lysozyme has been immobilized on different support using entrapment, adsorption, and surface conjugation methods.[10,16] However, the use of

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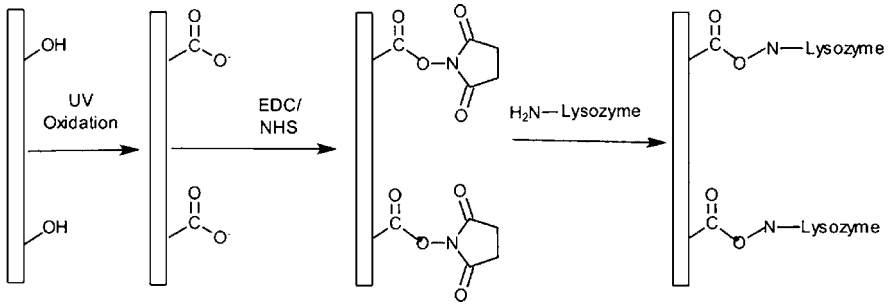

Figure 1. Scheme of the functionalization process: surface modification of EVOH and subsequent carbodiimide-mediated conjugation of lysozyme.

covalently immobilized lysozyme as an antimicrobial agent for food packaging applications is limited.

Ethylene vinyl alcohol (EVOH) copolymers are approved for food contact and have been used as matrices for the development of active packaging systems. EVOH has also been shown to be heat sealable and can be used to coat active agents onto another polymer support film (e.g., polyethylene, polypropylene).[17−21] These copolymers are also good candidates for surface modification due to the presence of hydroxyl groups. Given the applicability of EVOH films for food packaging applications, the antimicrobial nature of lysozyme, and the limitations of current immobilization methods, the objective of this study was to develop a new antimicrobial packaging film based on the covalent attachment of lysozyme to the surface of EVOH films.

■ MATERIALS AND METHODS

Materials. Films of ethylene vinyl alcohol (EVOH) copolymers (75 μm thick) with a 29% ethylene molar content (EVOH 29) and with a 44% ethylene molar content (EVOH 44) were kindly provided by The Nippon Synthetic Chemical Company (Osaka, Japan). Lysozyme, from chicken egg white, as lyophilized powder, ≥98%, with about 50,000 U of protein per mg, and *Micrococcus lysodeikticus*, ATCC 4698, were purchased from Sigma-Aldrich (St. Louis, MO). *Listeria monocytogenes* (*LM* 21) FSL-J1-048 was obtained from M. Wiedmann, Cornell University.

Film Preparation. EVOH films were cut to 1 × 2 cm^2 pieces and were sequentially cleaned by sonication in isopropyl alcohol, acetone, and deionized water (10 min per repetition). The cleaned EVOH were dried overnight over anhydrous calcium sulfate at room temperature (25 °C).

Surface Modification. *UV Irradiation.* EVOH films were irradiated, after a 5 min lamp warm-up period, in an open glass Petri dish for 1, 5, 10, and 15 min in a Jelight Co. model 42 UVO Cleaner (Irvine, CA), which emits 28 mW/cm^2 light at 254 nm at a distance of 2 cm. After the treatment, the films were turned over and the other sides were exposed to UV light under the same conditions. This method was used to oxidize and create carboxylic acid functional groups on both film surfaces.

Bioconjugation. Immediately after UV treatment, a conjugation solution was prepared with 5 × 10^{-2} M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (ProteoChem, Inc. Denver, Co) and 5 × 10^{-3} M *N*-hydroxysuccinimide (NHS) (Fisher Scientific, Pittburgh, PA) in 0.1 M pH 5.2 2-(*N*-morpholino)-ethanesulfonic (MES) (Fisher Scientific, Pittburgh, PA) buffer (Figure 1). Concentrations represent molar excesses of 100× and 10× for EDC and NHS, respectively, compared to the determined mole quantity of surface carboxylic acid groups. EVOH films (4 cm^2/mL) were shaken in the MES conjugation buffer for 30 min at room temperature (ca. 25 °C). After that films (4 cm^2/mL) were placed in 0.1 M pH 7.0 MES buffer to which lysozyme was added to get a final enzyme concentration of 1 mg/mL. Films were agitated in lysozyme solution for 2 h at room temperature, rinsed in copious amounts of deionized water, and dried with purified air.

Surface Analysis. *FTIR Analysis.* The surface modification was studied by using ATR-FTIR spectroscopy before and after the bioconjugation on an IRPrestige-21 FTIR spectrometer (Shimadzu Scientific Instruments, Inc., Kyoto, Japan) with sample compartments and a diamond ATR crystal. Each absorbance spectrum represents 32 scans at a 4 cm^{-1} resolution using Happ−Genzel apodization, taken against a background spectrum of an empty ATR crystal. The resultant spectra were analyzed with KnowItAll Informatics System 8.1 (BioRad, Hercules, CA) and processed with SigmaPlot 12.0 (Systat Software, Inc., Chicago, IL).

Quantification of Surface Carboxylic Acids. Toluidine blue O (TBO) (Fisher Scientific, Pittsburgh, PA) dye assay was used to quantify the number of available carboxylic acids according to the method described by Kang et al.[22] and Uchida et al.[23] with some modifications. TBO solution (0.5 mM) was made in deionized water and the pH adjusted to 10.0 with NaOH. Control and UV-treatment films were immersed in the TBO solution and shaken for 2 h at room temperature (25 °C). Then, the films were rinsed 3 times with deionized water adjusted to pH 10.0 with NaOH to remove noncomplexed dye. To desorb the complexed dye on film surfaces, films were submerged in 50 wt % acetic acid solution for 15 min. Absorbances of the acetic acid solutions were measured at 633 nm using a Synergy 2 microplate reader (BioTek Instruments, Winooski, VT) and compared with a standard curve made of TBO dye in 50 wt % acetic acid solution.

Analysis of Protein Loading on Films. Lysozyme immobilized on the surface of EVOH films was quantified using a modified version of the bicinchoninic acid assay (BCA, limit of detection (LOD) of 0.5 μg/mL). In brief, each film was submerged in 3 mL of BCA (Thermo Scientific, Rockford, IL) working reagent and shaken for 30 min at 60 °C. Absorbances of developed color were measured at 562 nm[24,25] using a Synergy 2 microplate reader (BioTek Instruments, Winooski, VT), and a standard curve of bovine serum albumin was used to calculate protein mass per film sample area.

Antimicrobial Activity. *Lysozyme Effectiveness Assay.* *Micrococcus lysodeikticus* (Sigma-Aldrich, St. Louis, MO) was used to assess the activity of free and immobilized lysozyme.[7,10,26] The antimicrobial efficacy of free and immobilized lysozyme was determined by the hydrolysis of *M. lysodeikticus* according to the method described by Shugar.[27] The LOD from lysozyme with *M. lysodeikticus* is 5 μg/mL. Briefly, a decrease in absorbance at 450 nm of a solution of *M. lysodeikticus* in buffer corresponds to antimicrobial activity. The reaction mixture was prepared by suspending 9 mg of dried *M. lysodeikticus* cell in 25 mL of 0.1 M potassium phosphate buffer, pH 7.0, which was diluted to a final volume of 30 mL with the same buffer. For the free enzyme control, 100 μg of free lysozyme was kept in contact with 3 mL of suspended lyophilized *M. lysodeikticus* and shaken until a constant absorbance value was reached. This amount of free lysozyme was selected since it is in the range of bonded lysozyme on the treated film surface (96 μg on a total surface area of 4 cm^2 of film). Separately, three pieces of EVOH−lysozyme immobilized films (EVOH 29 and EVOH 44, 4 cm^2 per film considering both sides of a 1 × 2 cm^2 film) were placed in a glass tube containing 3 mL of

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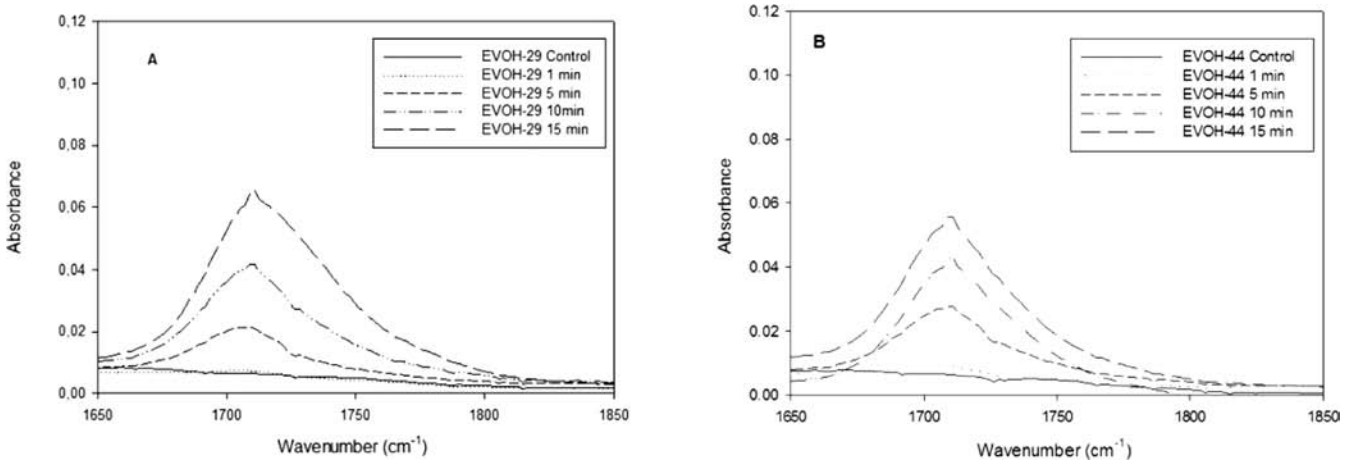

Figure 2. ATR-FTIR spectra of UV-treated EVOH 29 (A) and EVOH 44 (B) films in the 1650 to 1850 cm^{-1} range. Spectra shown here are representative of four replicates collected from three independent films per treatment (control, 1, 5, 10, and 15 min).

suspended lyophilized *M. lysodeikticus* and shaken until a constant absorbance value was reached (ca. two hours). The antimicrobial efficacy of each substrate was determined by monitoring the decrease of absorbance at 450 nm (BioTek Instruments, Winooski, VT), and extrapolating the linear rate to determine the unit activity per gram of protein. One unit produced a change in absorbance at 450 nm of 0.001 per minute at pH 7.0 at 25 °C using *M. lysodeikticus* as substrate, in a 3 mL suspension mixture. EVOH films treated for 10 min by UV irradiation were chosen as the optimal sample and used for this antimicrobial assay. Native EVOH substrates were also tested as controls. Each test was performed in triplicate.

Bacterial Cultures. *Listeria monocytogenes* (*LM* 21) FSL-J1-048 was obtained from M. Wiedmann, Cornell University. *L. monocytogenes* was selected as a model microorganism to demonstrate antimicrobial activity of the immobilized lysozyme films because it is a Gram-positive bacterium and with relevance to food safety. The strain was stored in Tryptone Soy Broth (TSB) with 20% glycerol at −80 °C until needed. For experimental use, the stock was maintained by monthly subculture at 4 °C on slants of Tryptone Soy Agar (TSA). To obtain early stationary phase cells, a loopful of strain was transferred to 10 mL of TSB and incubated at 37 °C for 18 h, prior to each experiment.

Determination of MIC against L. monocytogenes. The lowest concentration that inhibited the growth of the pathogen microorganism was reported as the minimum inhibitory concentration (MIC).[20] 100 μL of cell cultures of *L. monocytogenes* in stationary phase, with an optical density of 0.9 at 600 nm, was diluted in 10 mL of TSB and incubated at 37 °C for 4 h until exponential phase was reached, corresponding to an optical density of 0.2 at 600 nm (10^5 CFU/mL) measured with a UV−vis spectrophotometer using TSB as blank. 100 μL of exponential phase cells was inoculated in tubes with 10 mL of TSB and 100 μL of lysozyme solutions at concentration ranging between 100 and 800 μg/mL. A control tube without lysozyme was used as a blank. Turbidity was measured after 24 h of incubation. A volume of 100 μL of those tubes in which there was no growth after 24 h of incubation was inoculated on solid medium TSA to differentiate the MIC. Tests were performed in triplicate.[28]

Antimicrobial Activity of EVOH Films with Lysozyme. Antimicrobial activity of EVOH films with lysozyme was tested in liquid media. 100 μL of exponential phase microorganism (cultured as previously described) was inoculated into tubes with 10 mL of TSB. Ten films (cut into pieces of 1 × 2 cm^2) were placed in a glass tube containing 10 mL of TSB. The same amount of free lysozyme (340 μg/mL) was added to additional glass tubes with 10 mL of TSB. Tubes were incubated with rotation at 37 °C for 24 h. Depending on the turbidity of the tubes, serial dilutions with peptone water were made and plated on TSA. Colonies were counted after incubation at 37 °C for 18 h. Results were expressed as log CFU/mL. All experiments were performed in triplicate. Tubes without films served as controls. Additional controls of EVOH films, EVOH films after UV treatment, and EVOH films exposed to lysozyme without UV treatment were performed and determined to exhibit no antimicrobial activity.

Migration Testing. A test was conducted to ensure that lysozyme was not released from the film into liquid media. EVOH 29−lysozyme and EVOH 44−lysozyme were placed in a set of glass tubes containing 3 mL of distilled water at room temperature and shaken for 2 h. The amount of protein in the rinsewater and the antimicrobial activity of the rinsewater were determined at different time intervals by sampling and analysis through the BCA and *M. lysodeikticus* assays as described above. The experiments were performed in triplicate.

Statistical Analysis. One-way analyses of variance were carried out using the SPSS189 computer program (SPSS Inc., Chicago, IL, USA). Differences in pairs of mean values were evaluated by the Tukey test for a confidence interval of 95%. Data are represented as mean ± standard deviation.

RESULTS AND DISCUSSION

As reported above, the aim of this work is to develop antimicrobial packaging materials in which lysozyme is covalently immobilized onto the surface of a polymer packaging film. A two-step procedure was carried out to attach the lysozyme (Figure 1). In the first step, EVOH was exposed to UV irradiation to generate carboxylic acids. In the second step, lysozyme was covalently attached via EDC/NHS. The surface characterization was studied with ATR-FTIR and dye assay before and after the bioconjugation. The antimicrobial efficiency of the EVOH/lysozyme films was assessed against Gram-positive bacteria *M. lysodeikticus* and *L. monocytogenes*.

Surface Analysis. *Surface Modification of EVOH Films.* UV oxidation was used to modify the surface of EVOH films and produce primarily carboxylic acid onto the surface.[29,30] Following UV oxidation, ATR-FTIR was used to evaluate the changes in the polymer surface. As Figure 2 shows, a new absorbance band at 1700−1725 cm^{-1} appeared after oxidation which is attributable to the introduction of carboxylic acid groups. An increase in the number of carboxylic acids (increment in absorbance at 1700−1725 cm^{-1}) was observed with increasing the duration of the UV treatment. A qualitative comparison between films shows that the number of carboxylic acids available at the EVOH 29 surface was higher than that at the EVOH 44 surface (Figures 2A and 2B). EVOH copolymers are composed of two segment chains: one with hydrophilic behavior, because of the hydroxyl substituent, and the other

one with hydrophobic character, coming from ethylene. EVOH 29 and EVOH 44 contain 29% mol and 44% mol of ethylene, respectively. As hydroxyl groups are more susceptible to oxidation, a higher molar percentage of alcohol segments in EVOH 29 (which has a higher molar percentage of hydroxyl substituent monomer than EVOH 44) resulted in a higher amount of carboxylic acid on the polymer surface after UV irradiation.

Quantification of Surface Carboxylic Acids. The TBO assay was used to quantify the amount of carboxyl groups on the surface of the EVOH 29 and EVOH 44 films (Figure 3). A high value was observed in the EVOH 29 control samples, which would not be expected to contain surface carboxylic acid groups. It is known that EVOH copolymers are obtained by hydrolysis of ethylene vinyl acetate copolymer and that as the molar percentage of acetate groups increases the degree of hydrolysis decreases. Therefore, this initial carboxylic acid value obtained by the TBO assay may be caused by the presence of acetate substituents. The values for both films significantly increased with the length of the treatment in both films. However, the carboxylic acid values observed for EVOH 29 films after 10 and 15 min of UV treatment were not significantly different ($p > 0.05$), resulting in 70.35 and 75.54 nmol/cm^2 respectively. A similar trend was seen for the EVOH 44 samples, with values of 37.43 and 47.50 nmol/cm^2 after 10 and 15 min of treatment respectively. The results indicate that there were significantly more carboxyl groups on the EVOH 29 than with EVOH 44, which verifies the qualitative data observed with FTIR. It is likely that the EVOH 29 copolymer had more carboxylic acids after UV irradiation than the EVOH 44 copolymer due to the greater molar percentage of vinyl acetate monomers present in the copolymer. An increase in carboxylic acid density is valuable in that it provides a greater number of bioconjugation points for lysozyme immobilization.

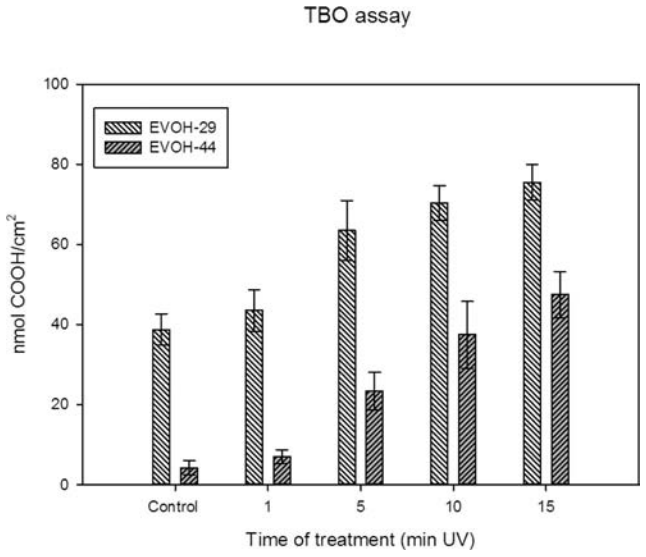

Figure 3. Available carboxylic acid surface density values obtained from TBO assay. Values are means and standard deviation of four independent films conducted on different days ($n = 8$).

Lysozyme Attachment to Modified Films. Lysozyme was covalently attached to oxidized EVOH films using carbodiimide chemistry. The isoelectric point of the enzyme (pI = 11.35) promotes association with the negatively charged modified film surface under conjugation conditions (pH 7.0) while the EDC/NHS results in covalent attachment. After bioconjugation, ATR-FTIR spectroscopy was performed to evaluate the surface chemistry changes. Figure 4 shows the ATR-FTIR spectra of UV-treated films after the lysozyme immobilization step. Films after 1 min UV treatment were not tested as this short treatment time resulted in no significant increase in the number of carboxylic acids (Figure 3). As can be seen, a new absorption band appeared at 1550−1650 cm^{-1} for both EVOH films which was not present before the lysozyme addition (see Figure 2A and Figure 2B). These bands correspond to the primary and secondary amine groups of the enzyme, indicating that the lysozyme was successfully attached to the EVOH 29 and EVOH 44 film surfaces. The increase in absorbance at 1550−1650 cm^{-1} of lysozyme immobilized onto films exposed to increasing lengths of UV exposure also suggests that the amount of lysozyme immobilized increases with the density of carboxyl groups formed in film surface.

Analysis of Protein Loading on Films. The BCA assay was performed to quantify the amount of protein on the surface of the EVOH 29 and EVOH 44 films. The data presented in Figure 5 for both EVOH 29 and EVOH 44 showed significant increases in attached protein loading with the length of UV treatment. However, no significant differences in the amount of lysozyme immobilized could be observed between EVOH 29 samples UV-treated for 10 and 15 min (8.49 and 9.69 μg of protein/cm^2 respectively), in good agreement with the results of carboxylic surface density. We hypothesize that, in general, the amount of protein covalently linked to the surface increases with the number of COOH groups. However, it is likely that there is a maximum protein immobilization density that can be achieved, beyond which the anchorage of protein molecules to the polymer surface can restrict the access of other molecules to free COOH substituents by steric hindrance. This effect may explain the observation that while 15 min UV irradiation on EVOH 29 samples resulted in an increase in COOH groups, it did not further increase the amount of immobilized protein. These results confirmed the successful lysozyme immobilization on the surface of EVOH films. Moreover, the release test confirmed that no protein was found in the liquid media (data not shown), indicating that the enzyme was strongly attached to the polymer and therefore enzyme molecules are unlikely to migrate into the solution.

Antimicrobial Activity. *Efficacy of Free and Immobilized Lysozyme against M. lysodeikticus.* Free lysozyme and EVOH 29 and EVOH 44 films to which lysozyme was covalently attached were put in contact with a suspension of *M. lysodeikticus* to quantify enzyme activity. For free enzyme activity assays, 100 μg of lysozyme was added to 3 mL of suspension. Three films were used in each test tube for immobilized lysozyme activity assays. At a total surface area of 12 cm^2 per tube, this corresponded to 102 μg in the 3 mL suspension for the EVOH 29 sample (at 8.49 μg/cm^2 protein) and 69 mg in the 3 mL suspension for the EVOH 44 sample (at 5.75 μg/cm^2 protein). The decrease in absorbance of the *M. lysodeikticus* suspension was monitored as a function of time to quantify the antimicrobial efficacy and enzymatic activity of the films. As can be seen in Figure 6, a clear decrease in the absorbance values was detected for both EVOH films as well as for the free enzyme. The free enzyme showed a high effectiveness against *M. lysodeitkticus*, with an initial activity of 1.6×10^3 units/mg of protein. Growth inhibition progressed with exposure time. The activity of lysozyme was significantly reduced following immobilization, resulting in mean activities

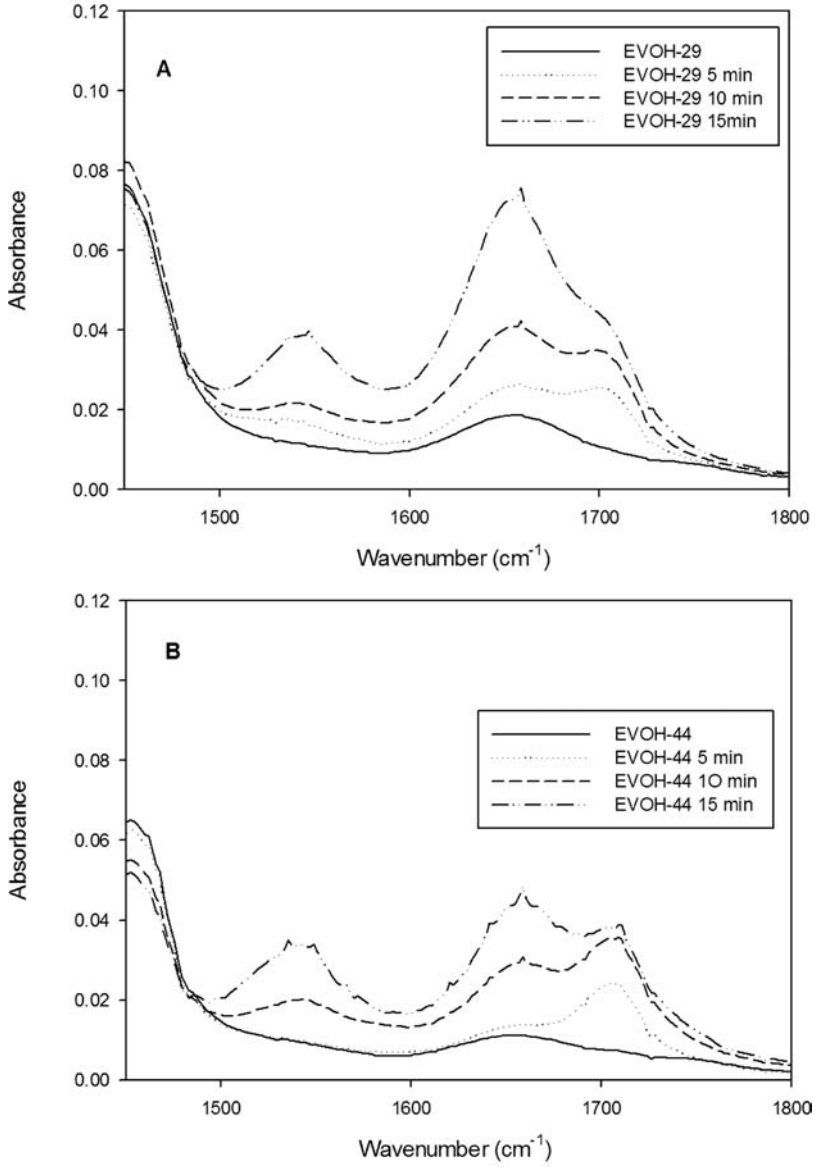

Figure 4. ATR-FTIR spectral of UV-treated films of EVOH 29 (A) and EVOH 44 (B) after the lysozyme immobilization step in the 1400 to 1800 cm^{-1} range. Spectra shown are representative of four replications collected from three independent films per UV treatment (control, 5, 10, and 15 min).

of 120 units/mg and 125 units/mg for lysozyme immobilized on EVOH 29 and EVOH 44, respectively. The loss of activity can be a consequence of the denaturation of the enzyme or of limitations to substrate accessibility due to orientation, steric hindrance, or diffusion limitations.[31] Nevertheless, growth inhibition progressed with exposure time to reach similar values as the free lysozyme sample. No bacterial growth inhibition was observed when testing the migration liquid media after film removal (data not shown), which indicates that the observed efficacy is the result of direct contact with the surface attached lysozyme. This is an important distinction demonstrating the nonmigratory nature of this work's antimicrobial packaging film. Had lysozyme released from the film to the liquid media, inhibition would have continued after film removal. Conte et al. reported that lysozyme was not released from some active surfaces, but the films were still able to act against the bacteria when the surfaces of the lysozyme films come in contact with the suspension.[7]

MIC of Lysozyme against L. monocytogenes. The bactericidal effect of lysozyme against *L. monocytogenes* was studied in liquid media. The lowest concentration that inhibited the pathogen microorganism was reported as the minimum inhibitory concentration (MIC). There are many factors that could affect the MIC value, including temperature, inoculum size, and type of microorganism.[32] Lysozyme at concentrations of 17 μg/mL in TSB inhibited the growth of *L. monocytogenes*. The antimicrobial properties are associated with the hydrolysis of peptidoglycan in the Gram-positive bacteria cell wall.[33,34] Lysozyme is more active against Gram-positive bacteria than against Gram-negative, because their cell wall is mainly composed of peptidoglycan (90%). In the case of Gram-negative organisms, they have a greater defense system and only 5−10% of the cell wall is constituted by peptidoglycan.[35] To improve the activity of lysozyme toward Gram-negative bacteria, it can be associated with membrane destabilizing

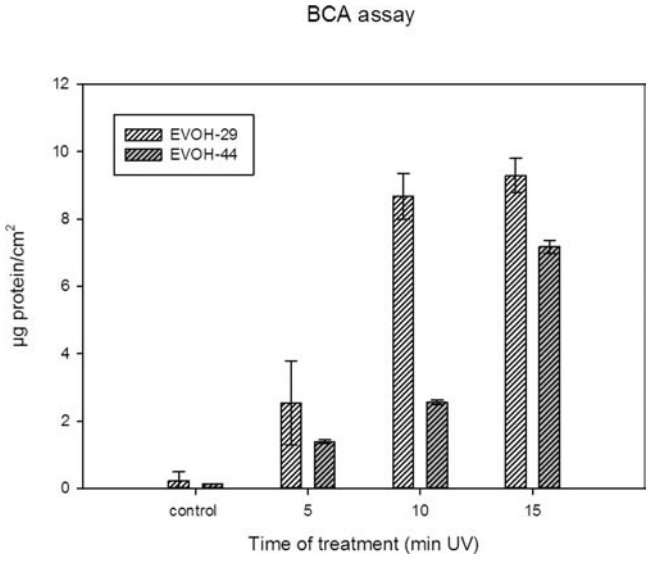

Figure 5. Protein concentration on film surface according to the BCA assay. Values are means of four independent films conducted on different days (n = 8, ±SD).

agents.[36] This higher resistance of Gram-negative bacteria has also been observed with other antimicrobial compounds.[37,38]

Antimicrobial Activity of Free and Immobilized Lysozyme against L. monocytogenes. The antimicrobial activity of the films was tested against *L. monocytogenes* (Tables 1 and 2). As shown in Table 1 no significant differences were observed between EVOH films, EVOH films after UV treatment, and EVOH films exposed to lysozyme without UV treatment. Table 2 shows that the free enzyme (34 µg/mL of lysozyme) produced a reduction of 1.34 log in 10 mL of TSB after 24 h at 37 °C compared with the inoculated TSB control. Incubation of 10 films of EVOH 29–lysozyme containing a total of 340 µg of immobilized enzyme in 10 mL of TSB resulted in a 1.08 log reduction under the same conditions. Similarly, incubation of 10 films of EVOH 44–lysozyme films with a total of 230 µg of immobilized enzyme in 10 mL of TSB produced a 0.95 log reduction after 24 h at 37 °C. No significant differences were observed between the free enzyme, EVOH 29–lysozyme, and EVOH 44–lysozyme samples, which may be due to the amount of immobilized enzyme in direct contact with the microorganisms at the film/medium interface being above a minimum threshold for both EVOH films. Compared to the relative efficacy of the immobilized lysozyme films against *M. lysodeikticus*, the antimicrobial activity of the films against *L. monocytogenes* was more analogous to the free enzyme. This discrepancy may be due to the size or shape of the organisms tested, the growth phase, or diffusion restrictions as a function of medium viscosity.

These results indicate that EVOH–lysozyme films (in which the enzyme lysozyme is covalently immobilized on the surface

Table 1. Antimicrobial Effectiveness of Control Films against *L. monocytogenes* at 37 °C Expressed as Logarithm of Colony Forming Units (log(CFU/mL))

	log(CFU/mL)
control without film	8.92 ± 0.08
EVOH 29	8.75 ± 0.03
EVOH 44	8.84 ± 0.06
EVOH 29–UV treatment	8.97 ± 0.07
EVOH 44–UV treatment	8.92 ± 0.06
EVOH 29–lysozyme without UV	9.00 ± 0.03
EVOH 44–lysozyme without UV	9.00 ± 0.02

Table 2. Antimicrobial Effectiveness against *L. monocytogenes* at 37 °C Expressed as Logarithm of Colony Forming Units (log(CFU/mL)) and Log Reduction Value (LRV)

	log(CFU/mL)	LRV
control	9.21 ± 0.04	
lysozyme	7.86 ± 0.09	1.34
EVOH 29–lysozyme	8.13 ± 0.10	1.08
EVOH 44–lysozyme	8.25 ± 0.06	0.95

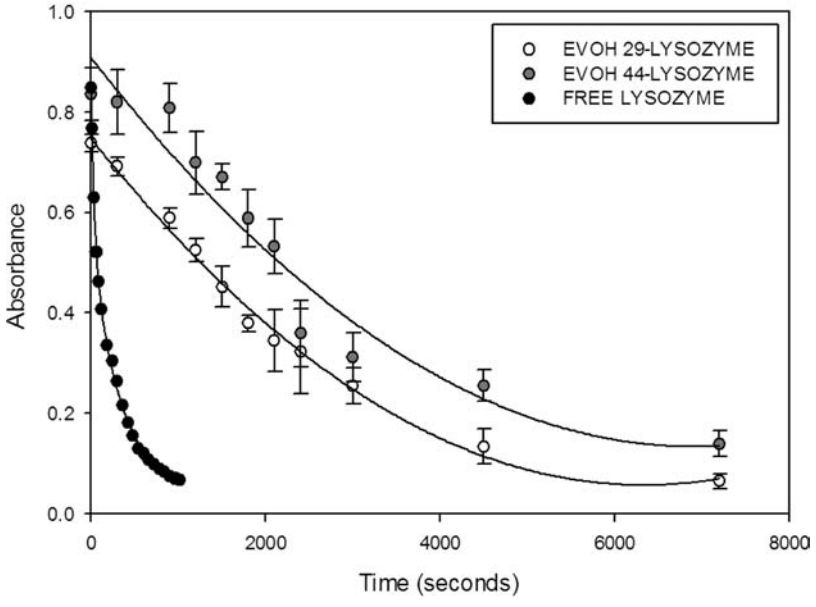

Figure 6. Effectiveness of EVOH 29–lysozyme and EVOH 44–lysozyme films, 10 min UV treatment lysozyme immobilization, against *M. lysodeikticus* suspension.

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of EVOH films) can be applied to reduce the growth of Gram-positive bacteria (e.g., *M. lysodeikticus* and *L. monocytogenes*) without migration of the lysozyme from the film. While there was an observed decrease in enzymatic activity after immobilization of the enzyme on the polymeric film, it is interesting to note that the immobilized enzyme films retained similar antimicrobial activity to an equivalent amount of free enzyme over a 24 h incubation period (1.34 log reduction for free lysozyme, 1.08 log reduction for an equivalent mass of covalently immobilized lysozyme). The application of these films as the internal surface of a package for direct contact with a fluid product has potential to improve stability of products without the addition (or release) of preservatives. Ongoing research is evaluating the effectiveness of these films against real food products at both room temperature and refrigerated temperatures to better elucidate for which food products the developed antimicrobial films will be effective.

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Notes

The authors declare no competing financial interest.

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