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Immobilization of Lysozyme on Polyvinylalcohol Films for Active Packaging Applications

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ABSTRACT

A new technique for the immobilization of lysozyme onto the surface of polyvinylalcohol films is presented. The active compound was sprayed along with a suitable bonding agent onto the surface of the cross-linked polymeric matrix. Active compound release tests determined the amount of lysozyme immobilized on the film surface. With the use of *Micrococcus lysodeikticus,* the antimicrobial activity of the films was determined and the results correlated with the amount of immobilized lysozyme. This new technique was effective for immobilizing the enzyme, and the developed films were active against the test microorganism. Results were compared with those obtained with a different immobilizing technique, in which the active compound was bound into the bulk of the polymeric film. As expected, the surface-immobilized lysozyme films have a higher antimicrobial activity than bulk-bound films.

Interest in active food packaging has increased because of the potential function of an active system in prolonging the shelf life of packaged foods *(4).* Several studies have been published on antimicrobial packaging because of its potential to control food spoilage *(2, 13, 18, 19, 23).* One type of antimicrobial packaging material is a polymeric matrix containing an active compound that is released into the packaged foods, in either a controlled or an uncontrolled manner, to prevent the growth of undesired bacteria *(5, 8–11, 16).* When a new packaging system based on the release of the active compound is developed, the amount of diffusion of active substance into the packaged product must be determined because high concentrations of a released compound in food could cause sensorial or toxicological problems, whereas low concentrations would not be effective *(4, 7, 14, 17).* Because of the difficulty in developing an optimum active controlled release system and because of a lack in European legislation concerning the release of active substances from packaging materials, active systems based on the immobilization of an agent into or onto the polymer matrix are highly desirable *(1, 20, 21).*

At present, ionic or covalent immobilization of antimicrobials into or onto polymeric materials is limited. The most important problems to overcome include choosing a suitable spacer molecule able to bind the bioactive agent to the polymer surface and avoiding reduction in antimicrobial activity of the active compound that could result from immobilization. Lysozyme, active against gram-positive bacteria, and chitinase, active against bacteria and fungi, have been covalently immobilized by Hotchkiss *(15)* and Wang and Chio *(24),* respectively. However, their activity after immobilization was too low for practical application. Concerning casting immobilization technique, Soares and Hotchkiss *(22)* and Del Nobile et al. *(12)* developed a polymeric film in which naringinase enzyme, able to reduce the bitter taste of some fruit juices, was immobilized on cellulose acetate and polyvinylalcohol films, respectively, without compromising its activity. Concerning surface immobilization, a recent study describes the immobilization of antimicrobial peptides onto a polystyrene substrate *(3).*

The aim of the present research was to optimize a surface immobilization technique to bond an active compound on the polyvinylalcohol matrix. The results were also compared with those obtained with a different immobilizing technique, in which the active compound was bound into the bulk of the polymeric film.

MATERIALS AND METHODS

The test films were produced with the use of polyvinylalcohol (molecular mass, 70,000 to 100,000 Da; Sigma-Aldrich, Gallarate, Italy) as the polymeric matrix, lysozyme (molecular mass, 14,000 Da; Sigma-Aldrich) as the antimicrobial compound, glyoxal (40%, Riedel de Haen, Gallarate, Italy) as the cross-linking agent, and glutaraldehyde (GA; 50%; Aldrich, Gallarate, Italy) as the bonding agent. The active films were produced by casting, according to the following procedures.

Bulk immobilization film preparation. A 13% (wt/vol) polyvinylalcohol solution in distilled water was autoclaved for 30 min and then cooled to room temperature. After dissolution, the polyvinylalcohol was cross-linked by adding 5 μl of glyoxal and 0.2 ml of hydrochloric acid as reaction catalyst. After homogenizing the polymer solution at a speed of 150 rpm, lysozyme and GA were added to the mixture in different amounts. Because both the amount of bonding agent and active compound and the ratio between the two compounds amount could influence the antimicrobial activity of the investigated film, both process variables

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were considered. For this purpose, for two films (Bb and Cb), the ratio between the bonding agent and the active compound was kept constant, whereas the amount of the two compounds was varied. On the other hand, for two films (Db and Eb) the above ratio was varied. The resulting solution was homogenized at a speed of 150 rpm to uniformly distribute the lysozyme and the binding agent. Glacial acetic acid (2 ml, Sigma-Aldrich) was also added as reaction catalyst. The obtained films were dried at ambient conditions until the solvent was completely evaporated and then further dried under vacuum for 1 day. Films had a thickness of ca. 100 μm. The obtained active bulk films are henceforth named Bb (500 mg of lysozyme and 0.025 ml of GA), Cb (100 mg of lysozyme and 0.005 ml of GA), Db (50 mg of lysozyme and 0.005 ml of GA), and Eb (20 mg of lysozyme and 0.005 ml of GA). As control, films of polyvinylalcohol containing glyoxal, GA, and glacial acetic acid without lysozyme were also prepared, and they will be henceforth referred to as film Ab.

Surface immobilization film preparation. To obtain the films in which the lysozyme was immobilized onto the polymer surface, a 13% (wt/vol) polyvinylalcohol autoclaved solution was cross-linked by adding 5 μl of glyoxal and 0.2 ml of hydrochloric acid as reaction catalyst. The resulting solution, after homogenizing at a moderate speed, was cast onto a Plexiglas plate. Solutions with different concentrations of lysozyme and GA dissolved in 10 ml of distilled water were prepared and sprayed on the wet film to uniformly distribute the antimicrobial compound and the bonding agent. Glacial acetic acid (2 ml) was also added to the films by spraying it onto the wet film as a reaction catalyst. The obtained films were dried at ambient conditions until the solvent was completely evaporated and then further dried under vacuum for 1 day. Films had a thickness of ca. 100 μm. The obtained active films are henceforth named Bs (500 mg of lysozyme and 0.025 ml of GA), Cs (100 mg of lysozyme and 0.005 ml of GA), Ds (50 mg of lysozyme and 0.005 ml of GA), and Es (20 mg of lysozyme and 0.005 ml of GA). As control, films of polyvinylalcohol containing glyoxal, GA, and glacial acetic acid without lysozyme were also prepared, and they will be henceforth referred to as film As.

Film washing. Each film (20 by 11 cm) was immersed into 4.5 liters of distilled water with continuous stirring at room temperature. The amount of lysozyme eventually released from the film in the washing solution was evaluated by monitoring its concentration by means of high-performance liquid chromatography (HPLC) until the equilibrium conditions were obtained. The amount of lysozyme released during washing was tested in triplicate for each film.

Lysozyme release. Films were washed according to the procedure described. The washed samples containing the immobilized lysozyme were brought in contact with 610 ml of water to create a volume/surface ratio of 2:1. The amount of lysozyme eventually released was evaluated by monitoring, by means of HPLC, the concentration of the antimicrobial compound in the surrounding solution. The time interval used to monitor the lysozyme concentration in the outer water solution is the same time interval used for the antimicrobial activity tests. The release tests were run in triplicate in this case as well.

Lysozyme determination. The amount of lysozyme released in water during either washing or release tests was determined by means of HPLC (model 1100, Agilent Technologies GmbH, Waghaeusel-Wiesental, Germany). A C18 reverse-phase column (250 by 4 mm, 5 μm) was used, and a gradient elution with water-acetonitrile gradients (1 ml/min) containing 0.1% trifluoroacetic acid was used. The calibration curve was constructed for peak area against lysozyme concentration of standard solutions from 6 to 300 ppm, with five replicate samples for each lysozyme concentration.

Antimicrobial activity. Lysozyme activity can be determined by measuring the decrease in absorbance of *Micrococcus lysodeikticus* incubated with the film in buffer *(1). M. lysodeikticus* was selected because of its high susceptibility to lysozyme antimicrobial activity. A suspension of lyophilized *M. lysodeikticus* cells (ATCC 4698, Sigma, Milan, Italy) was inoculated in 610 ml of 0.1 M phosphate buffer (pH 6.8) to reach a cell concentration of 10^7 cells per ml. Antimicrobial films, washed according to the above-mentioned procedure, were brought in contact with the obtained suspension. The ratio between the volume of solution and the active surface of the film was 2:1. Cellular lysis was monitored by the decrease in absorbance of *M. lysodeikticus* suspension at 450 nm (spectrophotometer UV 1601, Shimadzu model 1642, Shimadzu Europe Ltd., Duisburg, Germany).

The absorbance of the suspension, continuously stirred and in contact with the antimicrobial films, was monitored until a constant value was reached. Each test was conducted in triplicate. As a control, the decrease of absorbance at 450 nm of a microbial suspension in phosphate buffer without film was also measured.

Quantitative determination of film antimicrobial activity. The Gompertz equation as modified by Zwietering was used to quantitatively determine film antimicrobial activity *(25)*,

$$\bar{A}(t) = K + A \exp\left(-\exp\left\{\left[(d_{\max} 2.7182)\frac{\lambda - t}{A}\right] + 1\right\}\right) \quad (1)$$

where $\bar{A}(t)$ is the normalized absorbance at time t obtained by dividing the absorbance at time t by the initial absorbance; K is the initial value of $\bar{A}(t)$, and as expected, is always about 1; A is the maximum decrease in the normalized absorbance; $d_{\max}$ is the maximum death rate; and λ is the lag time. Equation 1 was fitted to the experimental data, and the value of $d_{\max}$ was taken as a measure of film antimicrobial activity.

Statistical analysis. All analyses were carried out in triplicate. Average values and their standard deviations were calculated. The confidence intervals of the model parameters were evaluated as follows. First, a fit was run with the original data; then, with the standard deviation of the data points, 100 additional fits were run on artificial data sets, which were generated by randomly varying the data around the fitted function. From these additional fits, a distribution of values for each parameter was obtained. The sets of data obtained for each parameter was statistically treated to obtain the 95% confidence interval.

RESULTS AND DISCUSSION

To determine any advantage of using the proposed immobilizing technique, a comparison between the performances of the surface-immobilized lysozyme films and the bulk-immobilized lysozyme films was performed. In particular, the influence of the bonding agent concentration on the amount of active substance bound to the film and the antimicrobial effectiveness of the obtained ''active materials'' were determined for both types of test films.

Lysozyme immobilization technique. The ability of GA to bond lysozyme to the polymeric matrix was determined first. As reported above, each film was washed in

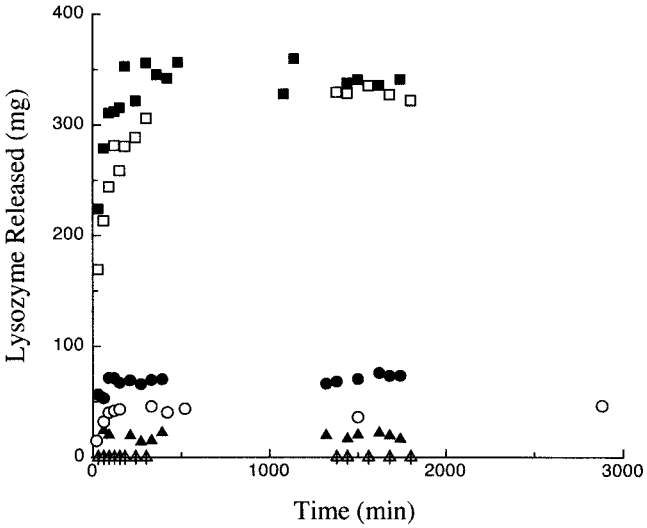

FIGURE 1. *Change over time in the amount of lysozyme released during washing tests from bulk and spray films.* □, *Film Bb;* ○, *film Cb;* △, *film Db;* +, *film Eb;* ■, *film Bs;* ●, *film Cs;* ▲, *film Ds.*

4.5 liters of distilled water to remove the active substance not bound to the polymer. The concentration of lysozyme into the washing solution was monitored until the attainment of equilibrium conditions. The release kinetic of each film is reported in Figure 1 for bulk films and spray films. As can be inferred from the data shown in the figure, films Db and Eb do not release any lysozyme, nor does film Es, whereas films Bb and Cb, prepared by bulk immobilization, and Bs, Cs, and Ds, prepared by spraying immobilization, released a different amount of active substance at a different release rate. As expected, the amount of lysozyme bound to the polymeric matrix increased as the ratio between the amount of loaded lysozyme and amount of bonding agent decreased. From this experimental data, it was possible to determine the washing time needed to completely release the unbound lysozyme for each film (i.e., the time corresponding to the attainment of the asymptotic value). The amount of enzyme released at equilibrium for both bulk and spray films and the corresponding washing time are reported in Table 1. It is worth noting that the molar ratio between the moles of loaded GA and the moles of bound lysozyme maintains similar values for the surface-immobilized lysozyme films, whereas it changes for the bulk-immobilized lysozyme films (Table 2). The Es film, although it was prepared by surface immobilization, doesn't respect this molar ratio because the loaded active compound is lower than the minimal amount that could be immobilized by bonding agent. The bulk and spray films are different because GA can act both as binding agent for lysozyme and as cross-linking agent for the polymeric matrix. In the case of bulk-immobilized lysozyme films, the probability that GA is involved in the above two reactions is much higher than in the case of the surface-immobilized lysozyme films.

To prove that no lysozyme was released after film washing and to provide evidence that the antimicrobial effectiveness of the test films is ascribed exclusively to the immobilized lysozyme, further release tests were run. The active agent in water from films previously washed was monitored for a time equal to that used for the antimicrobial tests. Results confirmed that no lysozyme was released from the washed films during the entire period in which they were brought into contact with the solution.

TABLE 1. *Washing times needed to reach equilibrium and the amounts of released and immobilized lysozyme obtained from washing tests for bulk and spray films*

Film sample	Loaded lysozyme (mg)	Washing time (h)	Lysozyme released into the washing solution (mg)	Lysozyme immobilized (mg)
Bb	500	30	316.58	183.42
Bs	500	9	342.1	157.9
Cb	100	5	47.59	52.41
Cs	100	3	64.34	35.66
Db	50	—	0	50
Ds	50	3	19.06	30.94
Eb	20	—	0	20
Es	20	—	0	20

Antimicrobial effectiveness. As far as the effectiveness of the bound lysozyme is concerned, each spray film was washed for the proper time and then brought in contact with a suspension of lyophilized *M. lysodeikticus* cells (cell concentration of 10^7 organisms per ml). As described in ''Materials and Methods,'' to evaluate the effectiveness of the films, the decrease in absorbance of the suspension because of microorganism death was monitored for each sample. For example, the data obtained in the case of surface-immobilized films are reported in Figure 2. In this graphic, the decreases in absorbance of a microbial suspension in phosphate buffer in the presence of each active film was reported. For the sake of comparison, in the same graph, the results related to a microbial suspension without film and with film not containing the active compound were also reported. Cell concentrations decreased only slightly in the suspensions both without film and in contact with control film, probably because of a natural mortality of the selected

TABLE 2. *The molar ratio between bonding agent and active compound immobilized is reported for each of the test films*

Film sample	Glutaraldehyde (50% solution) (ml)	Glutaraldehyde (mol)	Bound lysozyme (mol)	GA/bound lysozyme (molar ratio)
Bs	0.025	0.000134	1.13×10^{-5}	11.86
Bb	0.025	0.000134	1.31×10^{-5}	10.23
Cs	0.005	2.68×10^{-5}	2.55×10^{-6}	10.51
Cb	0.005	2.68×10^{-5}	3.74×10^{-6}	7.16
Ds	0.005	2.68×10^{-5}	2.21×10^{-6}	12.13
Db	0.005	2.68×10^{-5}	3.57×10^{-6}	7.52
Es	0.005	2.68×10^{-5}	1.42×10^{-6}	18.79
Eb	0.005	2.68×10^{-5}	1.42×10^{-6}	18.79

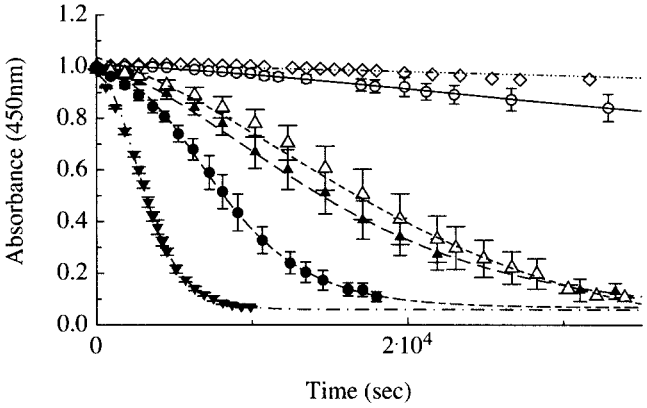

FIGURE 2. *Effectiveness of the different active spray films against* M. lysodeikticus *suspension.* ◇, *Control;* ○, *film As;* ▼, *film Bs;* ●, *film Cs;* ▲, *film Ds;* △, *film Es.*

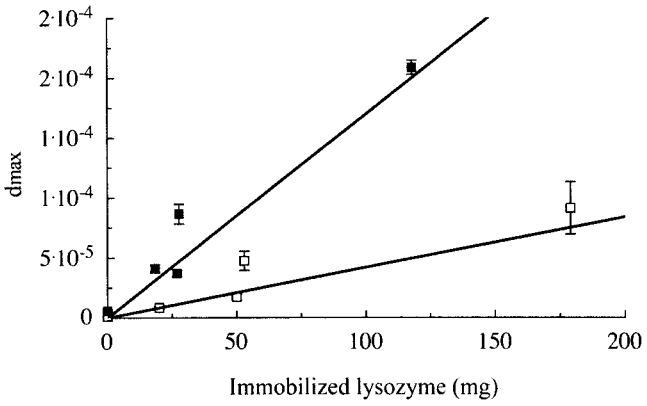

FIGURE 3. *Antimicrobial activity of the test active bulk and spray films plotted as a function of immobilized lysozyme.* ■, *Spray films;* □, *bulk films.*

microorganism. Active films, instead, show a high effectiveness against *M. lysodeikticus* cells. In particular, the rate of microorganism death increases as the amount of bound lysozyme increases.

To quantify the film antimicrobial activity, equation 1 was fitted to the experimental data; the obtained curves are reported in Figure 2. The goodness of fit was evaluated by calculating the relative percent difference ($\bar{E}$%) *(6).* The values of the fitting parameters obtained are reported in Table 3 along with the values of $\bar{E}$%. Among the model's parameters, attention was focused on d_{max} because, as maximal decrease rate of the curve, it was taken as a measure of the antimicrobial activity of the films. In Figure 3, the calculated values of d_{max} are reported as a function of the amount of immobilized active enzyme for both bulk- and surface-immobilized films to show their dependence. As can be inferred from the data shown in the above Figure 3, in both cases, the effectiveness of the films increases, with a linear dependence, as the amount of immobilized lysozyme increases. As expected, the antimicrobial activity of the spray films is higher than that of bulk films. This evidence can be explain by the lysozyme acting as an antimicrobial agent at contact. In the case of bulk-immobilized lysozyme films, the amount of active compound that cannot contact the water solution containing the cells is much higher than in the case of surface-immobilized lysozyme films. It is worth noting that the values of d_{max} related to the bulk films were halved because they represent the efficacy of the films that are active on both their surfaces, whereas the values of d_{max} obtained from the spray films represent the efficacy of films that are active only on one of the two film surfaces. The obtained results confirmed that the surface-immobilized films are desirable packaging systems because the useful amount of immobilized lysozyme is higher than that in a bulk system containing the same amount of active agent.

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TABLE 3. *Parameters and their confidence limits obtained by fitting equation 1 to the experimental data*

Film sample	K	A	d_{max}	λ	$\bar{E}$%
Suspension without film	1.0069	0.1082	3.2057×10^{-6}	1.0281×10^{4}	
	(1.00, 1.02)	(8.85×10^{-2}, 0.133)	(2.24×10^{-6}, 4.35×10^{-6})	(2.29×10^{-4}, 1.49×10^{4})	0.2612
As	1.0300	0.3427	5.8580×10^{-6}	1.3907×10^{-11}	
	(1.02, 1.24)	(0.17, 3.84)	(5.02×10^{-6}, 8.95×10^{-6})	(5.54×10^{-12}, 1.50×10^{-10})	0.3442
Bs	0.9797	0.9206	2.0871×10^{-4}	829.2407	
	(0.93, 1.04)	(0.868, 0.98)	(1.99×10^{-4}, 2.21×10^{-4})	(517.35, 1,113.10)	1.7663
Cs	0.9761	0.9082	8.6439×10^{-5}	2,610.0418	
	(0.88, 1.12)	(0.79, 1.09)	(7.31×10^{-5}, 1.06×10^{-4})	(11.55, 4,345.76)	1.9924
Ds	1.1139	1.1865	3.7026×10^{-5}	1.2851×10^{-11}	
	(1.02, 1.20)	(1.09, 1.29)	(3.19×10^{-5}, 4.26×10^{-5})	(9.58×10^{-12}, 1.65×10^{-11})	3.8324
Es	1.0820	1.0338	4.1010×10^{-5}	1.3864×10^{-11}	
	(1.01, 1.16)	(0.96, 1.12)	(3.46×10^{-5}, 4.83×10^{-5})	(1.14×10^{-11}, 1.62×10^{-11})	2.3688

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