Antimicrobial Activity of Glucose Oxidase-immobilized Plasma-activated Polypropylene Films

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Antimicrobial enzyme, glucose oxidase (GOX), was covalently immobilized onto amino- and carboxyl-plasma-activated biorientated polypropylene films (BOPP) via glutaraldehyde and carbodiimide chemistries. N2-plasma + NH3 and N2-plasma + CO2 treatments were utilized to create amino (1.1 nmol/cm2) and carboxyl (0.9 nmol/cm2) groups densities onto the surface of BOPP films. GOX-immobilized onto amino-activated BOPP films using 2.5% glutaraldehyde produced higher enzymatic activities than GOX-immobilized by 0.4% carbodiimide. Further immobilizations were carried out with glutaraldehyde as the coupling agent at temperatures of 4–75°C at pH 5.6 and 7.2. 10 s treatment was sufficient to immobilize GOX at high temperatures in both pH conditions, producing enzymatically active films which remained active over 30 days of storage. GOX covalently immobilized onto BOPP films completely inhibited the growth of Escherichia coli and substantially inhibited the growth of Bacillus subtilis; thus, they may have great potential to be exploited in various antimicrobial packaging film applications. Copyright © 2005 John Wiley & Sons, Ltd.

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INTRODUCTION

Antimicrobial packaging systems can be used to inhibit the growth of harmful microorganisms on the surface of packed food. Antimicrobial activity is possible to accomplish by several methods, including the addition of sachets or pads containing antimicrobial volatiles, incorporation of antimicrobials into the matrix of the packaging material, coating or adsorbing antimicrobials onto the packaging surface, using inherently antimicrobial substances as packaging for raw materials or immobilization of antimicrobial substances onto the packaging surface.1 Immobilization can be carried out via ionic or covalent linkages, thus it requires the presence of suitable functional groups on both the antimicrobial substance and the packaging surface. Examples of antimicrobial substances with functional groups are peptides, enzymes, polyamines and organic acids. Glucose oxidase is one of the antimicrobial enzymes immobilized onto various substrates.1–5

Glucose oxidase is a typical example of oxidoreductase systems that do not themselves possess...
antimicrobial activity. However, the reaction products from reactions catalysed by a given antimicrobial oxidoreductase system exhibit antimicrobial activity. The glucose oxidases produced by moulds such as *Aspergillus niger* and *Penicillium* spp. catalyse the formation of H$_2$O$_2$ and D-glucono-δ-lactone, which then reacts with H$_2$O to form D-gluconic acid. The antimicrobial activity of the system is due to the cytotoxicity of the H$_2$O$_2$ formed, although the lowering of pH by the production of D-gluconic acid may also influence the growth of some microorganisms. Reductive effects have been obtained on the growth of *Salmonella infantis*, *Staphylococcus aureus* and *Clostridium perfringens*. The activity of glucose oxidase against four microorganisms usually present in shrimp has also been studied. Hydrogen peroxide was found to cause growth inhibition of *P. fluorescens*, *A. calcoaceticum* and *H. polymorpha*, while *C. aquaticum* was inhibited by gluconic acid. In addition, glucose oxidase has been used in the food and fermentation industry as well as in biosensors for medical applications and environmental monitoring.

Various immobilization methods have been described, such as ionic and covalent immobilization, cross-linking, graft co-polymerization and entrapment. Covalent binding often exhibits the highest stabilization of enzyme activities because the active conformation of the immobilized enzyme is stabilized. The total activity of covalently immobilized enzyme is lower than the activity of free enzyme. However, the immobilized glucose oxidase retains its activity over a wider temperature and pH range than soluble enzyme. Also the storage stability of immobilized glucose oxidase is notably higher than that of free enzyme. Glucose oxidase has recently been immobilized onto electrochemically prepared poly(aniline-co-fluoroaniline) and chemically modified acrylonitrile co-polymer films, as well as on chitosan gel beads. Immobilization of glucose oxidase can be done, for example, by covalent binding of the amino group of the enzyme and the activated surface via suitable bifunctional reagents. Plasma treatment can be utilized in many ways for modifying the surface properties of food packaging materials to improve both safety and quality of foods. In addition, it can be used to generate suitable anchor groups for active substances. The functional groups that can be utilized in the covalent immobilization of enzymes include amino, carboxyl, hydroxyl and phenolic groups. NH$_3$- and CO$_2$-plasmas have been used to incorporate amine groups and carboxyl groups on polymer surfaces. Bifunctional reagents such as carbodiimides and glutaraldehyde can be used as covalent binding agents between the suitable functional groups. Carbodiimides are generally utilized as carboxyl activating agents for amide bonding with primary amines, whereas glutaraldehyde is used as a coupling agent between amines.

In the present study, the amino and carboxyl groups were generated onto the surface of polypropylene film by NH$_3$- and CO$_2$-plasma activation. Glutaraldehyde and carbodiimide (EDC) were used to covalently immobilize an antimicrobial enzyme, glucose oxidase, onto this kind of novel food packaging film, which was further proved to have both enzymatic and antimicrobial activity.

**MATERIALS AND METHODS**

Glucose oxidase (GOX) (EC 1.1.3.4), G 7141, lyophilized powder, type X-S, 180 units/mg from *Aspergillus niger*, was obtained from Sigma-Aldrich Chemical Co. Inc. (St. Louis, MO, USA). Glutaraldehyde, 25% solution, was obtained from Merck-Schuchardt (Hohenbrunn, Germany) and N-(3-dimethylaminopropyl)-N¢-ethylcarbodiimide hydrochloride (EDC) from Fluka. 2,2′-azinobis[3-ethylbenzothiazoline-6-sulphonate] (ABTS) was obtained from Roche Diagnostics GmbH (Mannheim, Germany), peroxidase type II (from horseradish) from Sigma-Aldrich and D-glucose (analar) from BDH Laboratory Supplies (Poole, UK). Acid orange 7 was obtained from Sigma-Aldrich and toluidine blue O from Merck. Bi-axially orientated polypropylene (BOPP) films (thickness 25µm) were from Innova Films (UK).

**Plasma activation**

Plasma activation was carried out in a dielectric barrier discharge (DBD)-reactor at atmospheric pressure (Figure 1). The configuration consists of
two parallel electrodes (20 × 25 cm²) covered with a dielectric material, in this case glass. The inter-electrode distance was set at 2 mm and the BOPP sheets were placed on the lower electrode. Standard-purity nitrogen was used as inert carrier gas. The flow rate was controlled by mass flow controllers and set at 20 l/min. During activation, NH₃ or CO₂ was added to the nitrogen flow at a rate of 3 l/min. An AC-field with a frequency of 5.6 kHz, generated by a 20 kV/200 mA AC power supply, was applied to the electrodes, giving rise to a transient, spatially uniform glow with a power density of 1 W/m². The BOPP sheets were treated for 0.5 min.

**Enzyme immobilization**

Glucose oxidase was immobilized onto plasma-activated BOPP films in various conditions (temperature 4–75°C; pH 3.6–7.2; time 10 s–1 day). Immobilizations were carried out by either two-phase or one-phase processes (Figure 2).

**Two-phase process.** The film samples were first treated with activating agents (carbodiimide or glutaraldehyde) and washed three times with distilled water. The film samples were then immersed in glucose oxidase solution and finally washed three times with buffer and distilled water.

**One-phase process.** Film samples were immersed in glucose oxidase solution containing either carbodiimide or glutaraldehyde and washed three times with buffer and distilled water.

**Determination of surface densities of amino and carboxyl groups**

The surface densities of the amino and carboxyl groups were evaluated from the uptake of acidic
and basic dyes. For determination of amino groups, film samples (0.5 dm$^2$) were immersed in 20 ml 0.01 g/ml acid orange 7, pH 3, at room temperature for 5 h to yield an ion complex between the amino groups and the acidic dye, and then washed with distilled water, followed by 1 mM HCl to remove non-complexed dye molecules. The dye molecules complexed to the film surface were desorbed into 1 M NaOH and the optical density at 485 nm was measured on the resulting supernatant. Carboxyl groups were complexed with 20 ml 0.01 g/ml toluidine blue O, pH 10, at room temperature for 5 h. Non-complexed dye was removed with distilled water and 1 M NaOH and desorption of dye molecules complexed to the carboxyl groups on the film surface was conducted with 50% acetic acid solution. The dye concentration was determined at 633 nm, using a spectrophotometer, and calculated from the calibration curve. The surface densities of functional groups were calculated assuming that acid orange 7 and toluidine blue O were complexed to equivalent moles of amino and carboxylic groups.

**Enzyme activity assay**

Glucose oxidase activity was measured as follows: the reaction mixture consisted of d-glucose (1.0 m) and ABTS (75 mg) in 150 ml 0.1 M sodium acetate buffer (pH 5.1) in the presence of horseradish peroxidase (5 mg). The reaction was started by adding a 2 cm$^2$ piece of the film or 0.1 ml of the pure enzyme solution. The mixture (total volume 3.7 ml, containing 0.6 ml d-glucose) was incubated for 5 min at room temperature and the colour was monitored spectrophotometrically at 436 nm. The enzymatic activity of glucose oxidase immobilized onto BOPP film ($A_{film}$) was determined according the following equation:

$$A_{film} = \frac{V \cdot \Delta Abs}{\varepsilon \cdot s}$$

where $V$ is the total volume of the assay, $\Delta Abs$ is the difference in the absorbance at 436 nm/min, $\varepsilon$ is the extinction coefficient (29.3 cm$^2$/µmol) and $s$ is the surface area of the film. $A_{film}$ was expressed as units/cm$^2$. One unit of glucose oxidase oxidizes 1.0 µmol/min of β-d-glucose to d-gluconolactone and H$_2$O$_2$.

**Antimicrobial activity**

The antimicrobial activity of the coated films against E. coli (ATCC 11775) and B. subtilis (Merck 1.10649) was measured using an antimicrobial drop test, which was carried out as follows: sheets (not sterilized) were cut into 1.5 ¥ 1.5 cm$^2$ test pieces and each piece was placed into a Petri dish. Pre-grown liquid cultures of the test strains were diluted in sterile peptone saline to approximately 1 × 10$^6$ cfu/ml. A drop of the diluted culture was placed on each test piece. The Petri dishes were placed into a box containing wetted paper to increase the humidity, in order to prevent evaporation of the drops during incubation at 30°C for 24 h. After incubation, 5 ml sterile peptone–saline was added in the Petri dishes and the bacteria were washed from the test pieces by shaking (Infoss AG CH 4103 orbital shaker, 100 r.p.m.) for 5 min at 25°C. The number of surviving bacteria in the test solution was measured by plating on TSB plates and incubating for 24 h at 30°C. The effect of the antimicrobial film was compared to the reference film without added antimicrobials.

The antimicrobial activity of soluble glucose oxidase was determined by incubating E. coli (1.4 ¥ 10$^5$ cfu/ml) and B. subtilis (2.7 ¥ 10$^5$ cfu/ml) in tryptic soy broth with 10 g/l added glucose. Glucose oxidase (Sigma G 7141) was added to obtain activity levels of 1.7–0.0017 U/10 ml. The number of surviving bacteria was determined by plating after 5 h and 24 h of incubation.

**RESULTS AND DISCUSSION**

Glucose oxidase can be immobilized onto plasma-activated BOPP by exploiting either carbodiimide or glutaraldehyde chemistries. Carbodiimide is capable of forming covalent bonds between the carboxylic groups of the activated BOPP and amino groups of the enzyme. The mechanism with glutaraldehyde is based on the formation of imine bonds between the aldehyde groups of glutaraldehyde and the amino groups of the enzyme and the amino-activated substrate.
Functional activity of plasma-treated BOPP surfaces

N$_2$-plasma + CO$_2$ and N$_2$-plasma + NH$_3$ treatments were utilized to create carboxyl group densities of 0.9 nmol/cm$^2$ and amino group densities of 1.1 nmol/cm$^2$, respectively, onto BOPP. A schematic diagram of the immobilization is shown in Figure 3. Glutaraldehyde was used to link amino groups of glucose oxidase to the amino-plasma-activated film, whereas carbodiimide was used to attach amino groups on the enzyme to carboxyl groups on the carboxyl-plasma-activated film. In more detail, EDC carbodiimide activated the carboxyl group of the film to form an active O-acylisourea intermediate, allowing it to be coupled to the amino group of the enzyme. A by-product was released as a soluble urea derivative after substituted by the enzyme, thus no spacer existed between the molecules being coupled. Based on the determination of amino and carboxyl group densities on the surface of plasma-activated BOPP films, the theoretical maximum amounts of immobilized glucose oxidase molecules were $6.62 \times 10^{14}$ and $5.42 \times 10^{14}$/cm$^2$ of activated film, respectively.

Enzymatic activity of glucose oxidase-immobilized films

A two-phase immobilization process was carried out for plasma-activated films as follows: (a) amino-activated circular film samples (7 cm diameter) were treated with 10 ml glutaraldehyde (2.5%) for 30 min, slightly stirring at 25°C, and washed three times with buffer and distilled water; (b) carboxyl-activated circular film samples (7 cm diameter) were treated with 40 mg carbodiimide in 10 ml sodium acetate buffer, pH 4.8, for 1 h, stirring at 4°C, and washed three times with buffer and distilled water, after which the films were immersed in enzyme solution as above.

Correspondingly, a one-phase immobilization process was carried out for plasma-activated films as follows: (c) amino-activated film samples were immersed in 10 ml (0.5 mg/ml) glucose oxidase solution (in acetate buffer, pH 3.6, containing 2.5% glutaraldehyde) for 16 h, stirring at 4°C, and finally washed three times with buffer and distilled water; (d) carboxyl-activated film samples were immersed in 10 ml (0.5 mg/ml) glucose oxidase solution (in acetate buffer, pH 3.6, containing 40 mg carbodiimide) for 16 h, stirring at 4°C, and washed as described above. All immobilized films were stored for 30 days at 4°C or 22°C and the enzymatic activities were periodically measured.

In the case of glutaraldehyde-treated amino-activated films, the one-phase process produced notably higher activities of immobilized enzyme than the two-phase process. With carbodiimide-treated carboxyl-activated films, the two-phase process produced slightly higher enzymatic activity. Overall, the use of glutaraldehyde as a linker between glucose oxidase and amino functional groups of plasma-activated BOPP generated higher enzymatic activities than carboxyl-activated BOPP films enzymatically immobilized via carbodiimide. The enzymatic activities of glucose oxidase immobilized onto plasma-activated BOPP films can be found in Table 1.

For optimizing the activity of immobilized enzyme, the amino-activated films were also immersed in 10 ml (1.0 mg/ml) glucose oxidase solution (in phosphate buffer, pH 7.2, or acetate buffer, pH 5.6, containing 2.5% glutaraldehyde) for 16 h, stirring at 4°C, and washing as described above. Enzymatic activities were 0.0041 and 0.0066 U/cm$^2$, respectively; thus, immobilization in acetate buffer, pH 5.6, produced better activities than with phosphate buffer, pH 7.2.

10 mg/ml glucose oxidase (160 kDa) consisted of approximately $3.8 \times 10^{16}$ molecules. The solution faced the plasma-activated BOPP surface of 40 cm$^2$ having approximately $2.7 \times 10^{16}$ amino groups. Glucose oxidase molecules were coupled with the film surface via glutaraldehyde (2.5%). The
amount of glutaraldehyde molecules in the enzyme solution was approximately $1.5 \times 10^{21}$, thus there was a clear excess of the coupling agent. This was necessary to ensure that all glucose oxidase would be immobilized.

Glucose oxidase, like other enzymes, is sensitive to high temperatures. Thermal inactivation of glucose oxidase is mainly due to the destabilization of chemical interactions and breakage of the hydrogen bonds, which lead to decreased enzymatic activity. However, the thermal stability of immobilized glucose oxidase is an important requirement for its practical applications as food packaging materials. According to the manufacturer, the optimum storage temperature for commercial soluble glucose oxidase used in these studies was less than 0°C. Naturally, this is not a practical processing or storage temperature for packaging films in real life. In most cases the packaging films are exposed to varying time/temperature conditions during their production and storage.

In large-scale roll-to-roll plasma processing, the exposure times are shorter and temperatures higher than in the optimal conditions for typical enzymes described above. Thus, the immobilization experiments in further tests were carried out as follows: NH$_3$ plasma-activated BOPP films were immersed in 15 ml (1 mg/ml) glucose oxidase solution (in phosphate buffer, pH 7.2, or acetate buffer, pH 5.6, containing 2.5% glutaraldehyde) for 10 s, stirring at 25°C, 55°C, 65°C, 75°C, and for 1 day, stirring at 4°C, as a reference, and finally washed three times with buffer and distilled water. As can be seen in Figure 4, the optimum immobilization temperature for the activity of glucose-oxidase is near 65°C. Immobilization in acetate buffer, pH 5.6, produced better activities than with pH 7.2 in phosphate buffer. Immobilized films were stored for 30 days at 4°C or 22°C and the reduction of activities was monitored. As can be seen in Figure 5, the activity of immobilized glucose oxidase remained relatively stable during 30 days of storage at 4°C. Films stored at 22°C lost some of their activity (Figure 6). In every case, immobilized enzyme retained its activity at a clearly detectable level after 1 month of storage.

The enzymatic activities of films produced within this work were slightly lower than those reported in previous studies. Immobiliza-
tion with a suitable spacer molecule could improve the concentration and activity of the immobilized enzyme. Glucose oxidase immobilized onto polyethylene with an alkylene diamino spacer group has been shown to protect the enzyme from structure deformation. In addition, by modifying the microenvironment, including the hydrophobicity and pH, of the support material and optimizing the amount and type of coupling reagents, the activity of immobilized glucose oxidase may be increased.

Antimicrobial activity of glucose oxidase-immobilized films

Soluble glucose oxidase was effective against *E. coli* at activity levels of >0.017 U/ml. However, a complete inhibition, i.e. a cell number reduction of 5 logarithmic units, was obtained in *E. coli* using an enzyme dosage of 0.17 U/ml (Figure 7). Taking into consideration the average size of drops in the antimicrobial drop test and the approximate area they covered, it was roughly estimated that a glucose oxidase activity of >0.003 U/cm² is needed in the films to reach the complete inhibition of *E. coli* in the drop test. As enzymatic activity measurements revealed, most of the films immobilized with glucose oxidase retained their activity level above the limit value of 0.003 U/cm² after 30 days of storage.

Soluble glucose oxidase was effective against *B. subtilis* at activity levels of >0.0017 U/ml. A substantial inhibition (a cell number reduction of 3 logarithmic units) was obtained in *B. subtilis* using an enzyme dosage of 0.17 U/ml (Figure 8). Using the same average drop size as above, a rough estimation for the activity level of glucose oxidase needed in the films to achieve complete inhibition of *B. subtilis* is >0.03 U/cm². As enzymatic activity measurements revealed, none of the films immobilized with glucose oxidase reached an activity level above 0.01 U/cm² during 30 days of storage. On the other hand, most films retained an activity...
level above 0.003 U/cm², which was enough for substantial inhibition of *B. subtilis*.

The results of antimicrobial activity of films immobilized with glucose oxidase were in good accordance with the above-mentioned enzymatic activities. Immobilized films were effective against *B. subtilis* and especially against *E. coli*. When immobilized at pH 7.2, glucose oxidase caused a reduction of 2–5 logarithmic units in *B. subtilis* cell numbers and 5 logarithmic units in *E. coli* cell numbers (Figure 9). When immobilized at pH 5.6, a reduction of 4–6 logarithmic units was obtained in *B. subtilis* and 5 logarithmic units in *E. coli* (Figure 10).

As the immobilized films were rinsed thoroughly with both buffers and distilled water, it is reasonable to believe that all substances (e.g. acetic acid, excess of glutaraldehyde) having possible effects on antimicrobial properties were completely removed. Glutaraldehyde still present on the film surface was fully reacted and thus did not have any antimicrobial or other activity. It was linked between the enzyme and the amino-activated BOPP surface and was completely inert and not capable of leaching from the immobilized enzyme network. Films were not sterilized prior testing in order to avoid decreasing the activity of immobilized glucose oxidase.

The antimicrobial activity of glucose oxidase is considered to be mainly due to the production of hydrogen peroxide. The differences in sensitivities of various bacteria to the system can be explained by differences in catalase activity. Gram-negative bacteria, especially coliforms, are less resistant to hydrogen peroxide than Gram-positive bacteria. The higher sensitivity of *E. coli* as compared to *B. subtilis* is in accordance with these findings.

In conclusion, the glucose oxidase-immobilized N₂-plasma + NH₃-activated BOPP films produced complete inhibition in the growth of *E. coli* as well as a substantial reduction in the growth of *B. subtilis*; thus, these films may have great potential to be exploited in various antimicrobial packaging film applications. Before commercialisation, more detailed research for optimizing the process parameters is required.
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