UV-Cured Natural Polymer-Based Membrane for Biosensor Application

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ABSTRACT: Chitosan, a natural product, is inherently biodegradable, biocompatible, and nontoxic. These properties make chitosan ideal for inclusion in matrices designed for use in enzyme immobilization for clinical analysis. This study demonstrates the feasibility of using chitosan in electrochemical biosensor fabrication. The enzyme sulfite oxidase (SOX) was covalently immobilized onto the matrix of chitosan–poly(hydroxyethyl methacrylate) (chitosan–pHEMA), a natural/synthetic polymer hybrid obtainable via UV curing. *p*-Benzoquinone, which served as an electron transfer mediator, was coupled onto the polymer network for activation of the chitosan–pHEMA copolymer, after completion of the photo-induced polymerization reaction. The biological activity of the immobilized SOX and the electroactivity of the coupled *p*-benzoquinone were investigated. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 79: 466–472, 2001

Key words: grafted chitosan;, UV-curable membrane; biosensor; enzyme immobilization

INTRODUCTION

The first enzyme-based biosensor, glucose electrode, was developed in the 1960s. Since then the search for new techniques and matrices for enzyme immobilization applicable to biosensors has continued, driven by the commercialization of effective biosensors. Researchers have keenly pursued overcoming the practical problems of using biosensors.[1–3] Only a minority of the research proposals have been successfully adapted for commercial exploitation,[4,6] attributed either to inadequate stability or to complexity of usage. These may in turn be attributed to inappropriate techniques and/or to the matrices used for immobilization of the enzyme.

Four important types of immobilization approaches have been used frequently for electrochemical biosensor fabrication: the insulation, adsorption, entrapment, and binding techniques.[7,8] Several researchers have reported on the use of the adsorption technique for enzyme immobilization.[9,10] A more recent approach is developing a poly(pyrrole)-based biosensor.[11,12] This method relies on entrapping the enzyme in a conductive poly(pyrrole) matrix. However, there are major

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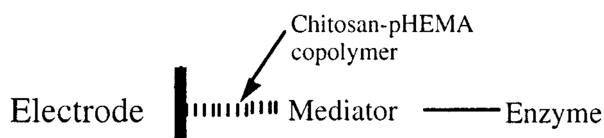


Scheme 1

problems associated with both the adsorption and the entrapment techniques. The latter, the entrapment of enzymes within a water-insoluble gel matrix, involves the formation of a highly crosslinked polymer network in which enzyme molecules are physically held by the three-dimensional polymer matrix. However, it has a major disadvantage: leakage of enzymes from the polymer matrix can occur. With the adsorption method the major disadvantages are weak binding forces and the possibility of enzyme leakage from the carrier during use. Therefore, the stability and reproducibility of sensors fabricated using adsorption and the entrapment methods of immobilization are in doubt.

In this article we report on fabrication of an enzyme-based electrochemical biosensor achieved by UV curing of a polymeric membrane onto an electrode, with an enzyme coupled onto the membrane via a reactive intermediate. As a test case, sulfite oxidase was employed for the analysis of sulfite ions. Sulfite ions are commonly used as oxygen scavengers to prevent oxidation reactions from taking place in foods and beverages. Its various forms have been used extensively as antimicrobials and for prevention against the enzymatic and nonenzymatic browning reactions in foods. Despite its importance, the determination of trace concentrations of sulfite ions is relatively difficult either because of complicated, time-consuming sample preparation or inadequate sensitivity of the various methods used.[13–16]

In this study immobilization was achieved by covalently immobilizing sulfite oxidase onto the UV-cured copolymeric membrane formed by grafting hydroxyethyl methacrylate (HEMA) onto chitosan. The graft copolymer, chitosan–pHEMA, was directly cured onto a platinum disc electrode. During the activation process an electron mediator, *p*-benzoquinone, was introduced onto the copolymer. The enzyme was then coupled onto the mediator, which was covalently bonded to the natural/synthetic polymer matrix, as shown in Scheme 1.

It was expected that problems associated with leakage might be overcome when the enzyme was indirectly immobilized covalently onto the polymer matrix. When activated with *p*-benzoquinone, this natural/synthetic polymer-hybrid material has been found to be a suitable support for immobilizing glucose oxidase for glucose determination.[17]

Chitosan is obtained by deacetylation of chitin, itself extracted from crustacean shells. Chitin, a biopolymer made up of β-(1-4)-2-acetamido-2-deoxy-D-glucose units, is the second most abundant polysaccharide in nature, after cellulose.[18] Its deacetylated form, chitosan, is reported to be nontoxic, biodegradable, and biocompatible.[19] These properties make it a promising matrix in enzyme immobilization systems used in clinical analyses. Poly(hydroxyethyl methacrylate) (poly(HEMA), a hydrogel utilized for biomedical purposes[20] is capable of absorbing water, an important advantage for membranes used in biosensors. However, poly(HEMA) on its own does not constitute a good membrane for biosensor applications, as it has difficulty adhering to the tip of the electrode for a long period of time because of its high retention of water. In addition, hydrogels are often mechanically weak. Grafting poly(HEMA) onto a more resilient polymeric support such as chitosan increases the usefulness of the hydrogel by combining mechanical strength with biocompatibility. Hence, the graft copolymer formed from these two components, each with its own merits, makes it a useful biosensor membrane.

EXPERIMENTAL

Reagents and Materials

Chitosan, with a molar mass of 167.7 g mol^{-1} for the repeat unit and a degree of polymerization of 80,000, was supplied by the Department of Colour Chemistry, University of Leeds (United Kingdom). Hydroxyethyl methacrylate and sulfite oxidase (EC 1.8.3.1.) were procured from Aldrich and *p*-benzoquinone from Merck (Australia). The photoinitiator, 4-(benzoyl-benzyl)-trimethylammonium chloride (BTC) was obtained from Great Lakes Fine Chemicals, Widnes (United Kingdom). Sodium sulfite (AR grade) was from Ajax Chemicals (Australia). All other chemicals were of AR grade unless otherwise indicated. These reagents were used as received. All aqueous reagents were prepared in Milli-Q water.

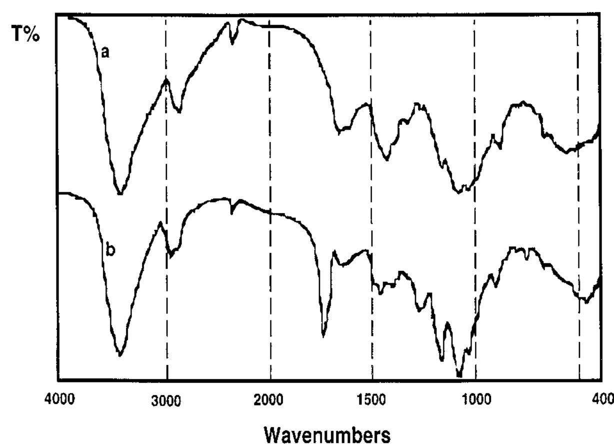


Figure 1 FTIR spectra of (a) chitosan and (b) chitosan–HEMA.

Equipment

A 90-W medium-pressure mercury lamp was used to initiate the polymerization of the copolymer. The FTIR spectra of chitosan and its copolymer were recorded on a 1725x Perkin-Elmer Fourier transform infrared spectrometer using the KBr pellet method. Thermal analyses were carried out using a Du Pont 2000 thermal analyzer in the DSC mode. A Joel JSM-820 scanning electron microscope was used for scanning electron microscopy (SEM) studies. All electrochemical experiments were conducted in a 3-electrode cell comprising a Ag/AgCl (3*M* NaCl) reference electrode, a platinum gauze auxiliary electrode, and a working electrode (1.5 mm platinum disc). Cyclic voltammetry and chronoamperometry were carried out using BAS CV-27 and BAS LC-4C potentiostats, respectively. All data were recorded on a Macintosh computer using the MacLab interface and Chart data-acquisition software (AD Instruments, Australia).

Electrode Preparation

Chitosan (0.1 g) and HEMA in a mole ratio of 1:1 were dissolved, with stirring, in 10 mL of 0.17*M* acetic acid at ambient temperature. This molar ratio was adopted for this study as it had provided the optimum grafting efficiency in our previous research.[17] The water-soluble photoinitiator BTC (0.6% w/w) was then added and thoroughly mixed. 20 μL of the mixture was deposited on a 1.5-mm-diameter platinum disc electrode, and the mixture was subjected to polymerization under a 90 W medium-pressure mercury lamp. The polymeric membrane thus formed was washed with a 2% NaOH solution and then rinsed thoroughly with deionized water. Sulfite oxidase (50 units) was used as received, and 10 μL was immobilized onto the polymeric membrane that had been previously activated with *p*-benzoquinone at 4°C for 12 h, using the method outlined by Brandt et al.[21]

RESULTS AND DISCUSSIO

Characterization of Chitosan–HEMA

Fourier Transform Infrared Spectroscopy Studies

The copolymer chitosan-*co*-HEMA was characterized by Fourier transform infrared spectroscopy (FTIR). Figure 1 depicts the spectra of chitosan and chitosan—HEMA, which were recorded on a 1725x Perkin FTIR spectrometer using the KBr pellet method. The spectrum of chitosan exhibits a band at 1652 cm^{-1} from absorption at the carbonyl group in the polyaminosaccharide. The ap-

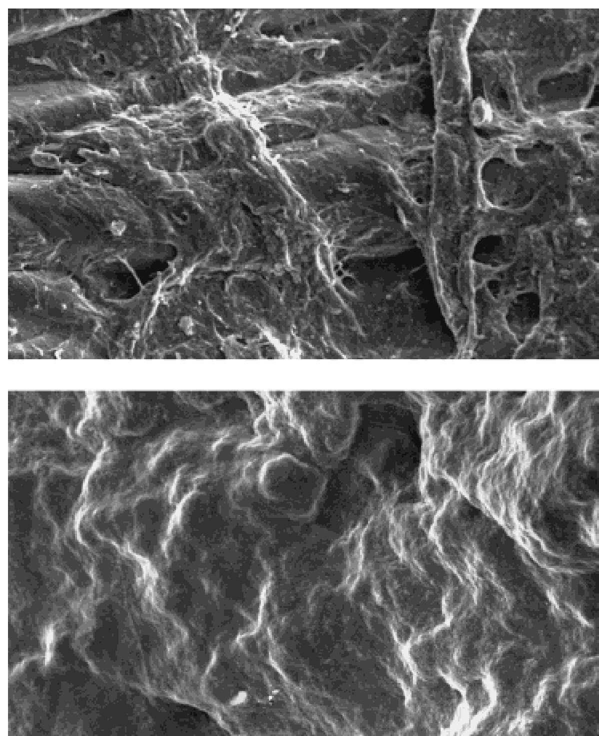
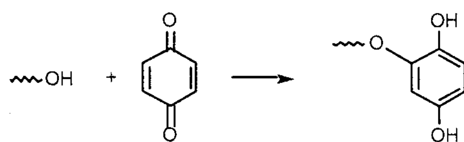
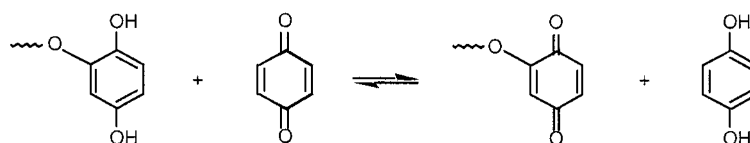


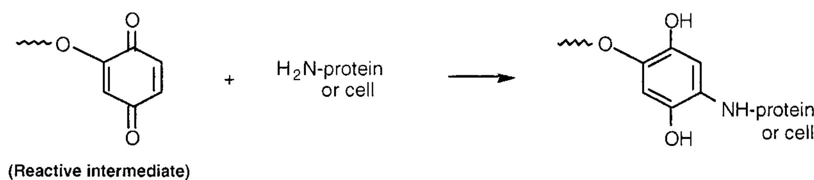
Figure 2 (a) Micrograph of chitosan (× 500 magnification); (b) Micrograph of grafted chitosan (× 500 magnification).

(1) Activation step





(2) Coupling step



Scheme 2

pearance of a new peak in the grafted chitosan spectrum at 1729 cm^{-1} can be attributed to absorption at the carbonyl group of the grafted poly(HEMA) chain.

Scanning Electron Microscopy Studies

Micrographs of chitosan and of grafted chitosan from scanning electron microscopy (SEM) are shown as Figure 2(a,b). The SEM analyses of all samples sputtered with gold were carried out using a Jeol JSM-820 scanning electron microscope fitted with a Mamiya SLR camera. The micrographs show that the grafted chitosan loses some of the structure of the original chitosan. This suggests that that grafting occurred mainly on the chitosan backbone.

Differential Scanning Calorimetry Studies

A knowledge of the thermal stability of the support is important because support systems for immobilized enzymes whose end use is in either the food/drink industry or in biomedical fields would be subject to extensive sterilization prior to enzyme immobilization. Such sterilization would be achieved using high-temperature autoclaving, radiation, and chemical sterilization.

Thermal analyses for this study were done with a Du Pont 2000 thermal analyser in the differential scanning calorimetry (DSC) mode. The apparatus was purged with nitrogen gas, and the heating range was 25–500°C at a rate of 10°C/min. The DSC thermogram for chitosan exhibited an exothermic peak at 305°C. This may correspond to the decomposition of the polysaccharide. An endothermic peak at 409°C appeared in the thermogram for poly(HEMA). This may indicate decomposition of the homopolymer. An exothermic peak appeared between 301°C and 302°C in the chitosan-p(HEMA) thermogram, corresponding to decomposition of the polysaccharide backbone in the copolymer.

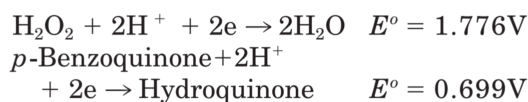
The DSC studies show that the chitosan–pHEMA copolymer is relatively stable thermally,

suggesting this copolymeric membrane may be suitable for the fabrication of gas sensors when higher service temperatures are required.

Dual-Functional *p*-Benzoquinone

Because of the hydroxyl functional group of the HEMA units, the hydrophilic properties of the grafted chitosan are further enhanced. The hydroxyl group can play a significant role in the immobilization of an enzyme. It is activated by *p*-benzoquinone prior to immobilization.

p-Benzoquinone, besides being involved in the activation of the copolymer for the immobilization of sulfite oxidase, also acts as a potential mediator for electron shuttling in the system. The suggested mechanism for the reaction of *p*-benzoquinone with the hydroxyl groups of the copolymer is given in Scheme 2. The coupling route is also shown.



$$H_2O_2 + 2H^+ + 2e \rightarrow 2H_2O \quad E^o = 1.776V$$

$$p\text{-Benzoquinone} + 2H^+ + 2e \rightarrow \text{Hydroquinone} \quad E^o = 0.699V$$

$$H_2O_2 + \text{Hydroquinone} \rightarrow p\text{-Benzoquinone} \quad E^o = 1.077V$$

According to the redox potentials of the sulfite ion-detection system, hydroquinone may be oxidized by hydrogen peroxide generated from an enzymatic reaction. Hence, it may serve as an electron mediator to enhance the current signal.

A cyclic voltammetric technique was employed to evaluate the electrochemical behavior of the immobilized *p*-benzoquinone in relation to its ability to function as an electron mediator. Hydrogen peroxide was selected as the test species since it plays an important role in this electron-shuttling system because of its electrochemical behavior. The cyclic voltammetric responses [Fig. 3(b)] show that the reduction and oxidation reactions of hydrogen peroxide occurred at −0.4 V and +0.6 V, respectively. The anodic peak current of hydroquinone was increased markedly when 3% H_2O_2 was added to the phosphate buffer solution [Fig. 3(b)]. This indicates that hydrogen peroxide can stimulate the oxidation of hydroquinone. This is in contrast to the redox response in the absence of H_2O_2, as shown in Figure 3(a). In other words, enhancement of the current responses may be a result of the existence of *p*-benzoquinone, which catalyses the redox reactions of hydrogen peroxide.

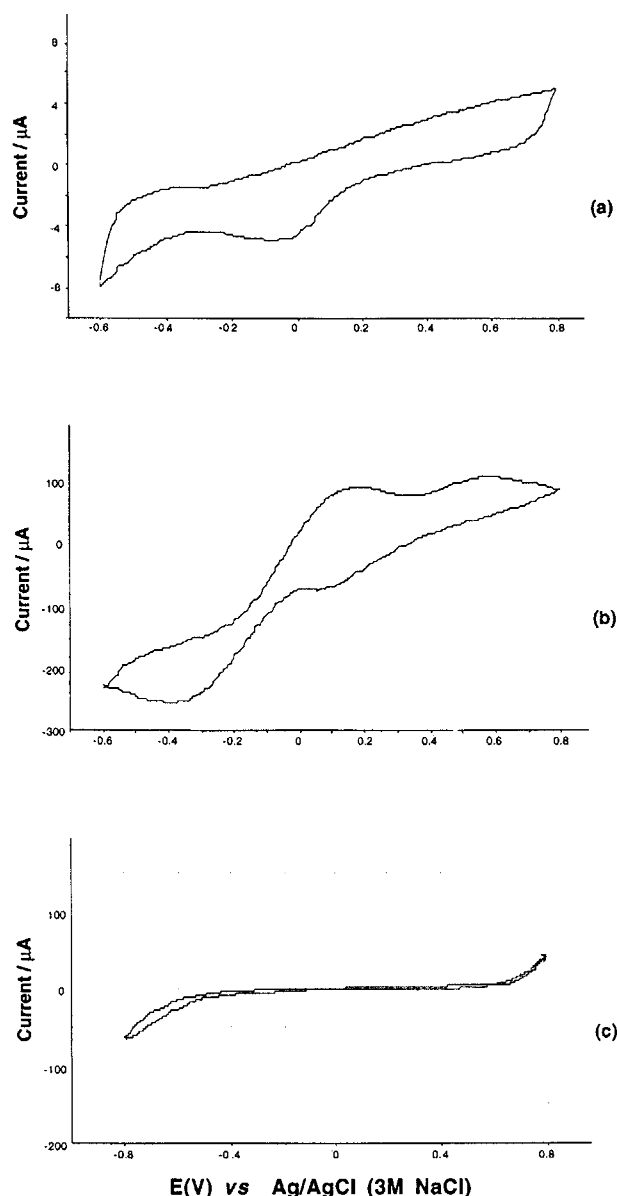


Figure 3 (a) Chitosan/*p*-benzoquinone-modified Pt electrode in 0.1*M* pH 8 phosphate buffer; (b) Chitosan/*p*-benzoquinone-modified Pt electrode in 3% H_2O_2/0.1*M* phosphate buffer; (c) Chitosan modified Pt electrode in 3% H_2O_2/0.1*M* phosphate buffer. Scan rate: 100 mV/s.

In order to confirm this, the cyclic voltammetric responses of a chitosan-modified electrode placed in a hydrogen peroxide solution were obtained [Fig. 3(c)]. These suggest that without *p*-benzoquinone coupling onto the copolymer backbone, the direct reduction and oxidation of hydro-

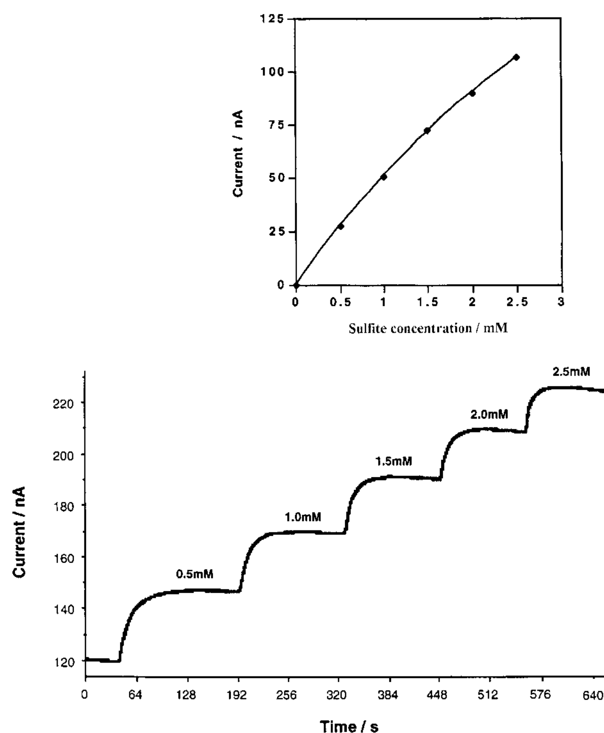


Figure 4 Amperometric response of a chitosan-based sulfite biosensor.

gen peroxide on this polymer-modified electrode surface do not occur. This observation therefore confirmed that the reduction of hydrogen peroxide and the oxidation of hydrogen peroxide in the case of the chitosan/*p*-benzoquinone-modified electrode underwent a mediation/catalysis process or mechanism.

Sulfite Ion Response

In order to demonstrate the feasibility of using a fabricated biosensor in the quantitative determination of sulfite ions, the amperometric response of sulfite ions was studied (Fig. 4). To do this, 5 mM sulfite in 0.1*M* phosphate buffer at pH 8 was used throughout this study. It was found that within the concentration range of 0.5–2.5 mM, the current responses were directly proportional to the concentration of sulfite ions present in the solution. This indicates that the catalytic action of the sulfite oxidase is quantitative. The experiment was repeated, and reproducible amperometric responses were obtained, showing the enzyme was coupled rather than adsorbed onto the copolymeric membrane.

The stability of the biosensor was also investigated. After fabrication of the biosensor, the performance of the electrode was examined immediately. The electrode was then stored in a 0.1*M* phosphate buffer (pH 8) solution at a temperature below 3°C for 24 h. The electrode performance was then reexamined. It was found that the characteristics of the obtained responses were very similar to those of the freshly prepared electrode.

CONCLUSIONS

The enzyme sulfite oxidase can be successfully immobilized onto the UV-cured natural/synthetic copolymeric membrane chitosan–pHEMA through covalent bonding. Chitosan–pHEMA membranes, when activated with *p*-benzoquinone, have been shown to provide a suitable matrix for enzyme immobilization, applicable to biosensor fabrication. *p*-Benzoquinone has the dual functions of activating the copolymer for the immobilization of enzymes and of acting as a mediator for electron shuttling in the system. That SOX can retain its bioactivity when it is covalently bonded to the chitosan–pHEMA matrix shows that fabrication of a chitosan–pHEMA enzyme-based electrochemical biosensor can be achieved using the techniques described.

The effects of environmental factors such as salinity, influence or exposure to metal ions, and the effect of low pH conditions are worthy of further investigation. This is particularly true for food applications in which complex composites of the type described can be used.

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