

Immobilization of glucose oxidase on chitosan-based porous composite membranes and their potential use in biosensors

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ABSTRACT

The glucose oxidase (GO_x) enzyme was immobilized on chitosan-based porous composite membranes using a covalent bond between GO_x and the chitosan membrane. The chitosan-based porous membranes were prepared by the combination of the evaporation- and non-solvent-induced phase separation methods. To increase the membrane conductivity, carbon nanotubes (CNTs) were added to the chitosan solution. The resulting membranes were characterized in terms of water permeability, surface morphology and surface chemistry. Enzyme immobilization was performed on the chitosan membranes with and without activation using glutaraldehyde (GA). Three different configurations of working electrodes were evaluated to investigate the potential use of the modified membranes in biosensors. The results show that enzyme immobilization capacity was greater for membranes that had been activated than for membranes that had not been activated. In addition, activation increased the stability of the enzyme immobilization. The immobilization of GO_x on chitosan-based membranes was influenced by both pH and the concentration of the enzyme solution. The presence of CNTs significantly increased the electrical conductivity of the chitosan membranes. The evaluation of three different configurations of working electrodes suggested that the third configuration, which was composed of an electrode-mediator-(chitosan and carbon nanotube) structure and enzyme, is the best candidate for biosensor applications.

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1. Introduction

Enzyme-based electrochemical biosensors are currently used in areas such as health care, food safety and environmental monitoring [1,2]. Although the performance of an enzyme-based biosensor depends on the function of the transducer, the heart of a biosensor is the enzyme, which converts the substrate into product(s), providing electronic signals to the transducer. The transducer then converts the electronic signals into an easily measurable signal, which can be displayed in the form of the desired units. Consequently, enzyme immobilization is of great importance and is necessary for successful biosensor fabrication. Another important factor is the electron transfer from the enzyme center to the surface of the electrode. This transfer should be as fast as possible to provide a rapid response and accurate measurements.

The immobilization of enzymes on solid supports is used to increase the thermostability and operational stability of the enzymes and for enzyme recovery [3]. Several methods for enzyme immobilization have been proposed, and these methods can

be classified into physical and chemical methods [4–11]. Physical methods, which include adsorption, encapsulation and entrapment, are simple, but the interactions between the support and the enzymes are relatively weak, leading to enzyme leakage. Entrapment in a conductive matrix has also been used [5,6]. Other techniques, including entrapment in an organic polymer [7,8], entrapment on carbon-polymer electrodes [9] and the sol-gel method, have also been developed [10,11]. Chemical methods are relatively complex, but due to the formation of covalent bonds between the enzyme and the substrate, the stability of enzyme immobilization should be higher than that obtained when using physical methods.

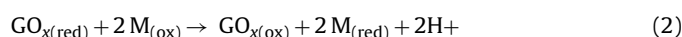
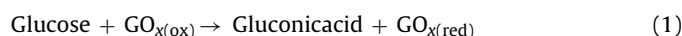
Chitosan (CS), a (1 → 4)-2-amino-2-deoxy-β-D-glucan a derivative of chitin, is a linear hydrophilic polysaccharide that has received much attention in biological fields [2,12]. It is an attractive biocompatible, biodegradable and non-toxic nature biopolymer that has an excellent film-forming ability. In addition, CS can be used as a modification agent because it has abundant $-\text{NH}_2$ and $-\text{OH}$ functional groups, which can react with bioactive molecules. Because of these characteristics, chitosan has been used as a substrate for the covalent immobilization of enzymes [13,14]. However, due to chitosan's relatively low conductivity, the transfer of electronic signals to the transducer can be a problem and can decrease the performance of chitosan-based biosensors.

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In this study, glucose oxidase (GO_x), which is used in glucose biosensors, was used as a model of enzyme. It is important to note that glucose biosensors represent the largest market for biosensors. It has been reported that glucose biosensors dominate commercial biosensor products and account for approximately 85% of the entire world market for biosensors [1]. Glucose biosensors are a diagnostic tool used to measure the blood sugar levels of diabetic patients and are an important component of closed-loop glycemic control systems that regulate a person's blood glucose level. There are currently three generations of glucose biosensors that can be used as models for development [1,2]. The first-generation biosensors are based on the monitoring of the oxygen consumed by the enzyme-catalyzed reaction or the detection of the hydrogen peroxide that is produced. The fundamental weakness of this type of biosensor is that the performance of the sensor depends on the concentration of oxygen in the blood and requires a high over-potential. To solve this problem, the second-generation biosensors were developed in which the role of oxygen in the reaction is fulfilled by an electron transfer mediator.

The reaction of second-generation biosensors can be described as follows [1,2]:



where $\text{M}_{(\text{ox})}$ and $\text{M}_{(\text{red})}$ are the oxidized and reduced forms of the mediator, respectively. The reduced form is reoxidized at the electrode, giving a current signal (proportional to the glucose concentration) while regenerating the oxidized form of the mediator (3). The problem that often arises with second-generation biosensors is the release of mediators from the space between GO_x and the electrode. The third-generation biosensors appear to eliminate the use of an electron transfer mediator and use electrodes formed by conductive organic salts. These electrodes can oxidize the reduced GO_x directly on the electrode surface. However, the mechanism of electron transfer is complex and is still debated. It also allows electroactive interference and poisoning [1,2,15].

Various nanomaterials, including gold nanoparticles and carbon nanotubes (CNTs), have been used as electrical connectors between the electrode and the redox center of GO_x in biosensor applications [15,16]. Since the discovery of CNTs in 1991, these particles have received much attention due to their unique electrical and mechanical properties [17].

The objective of this study was to develop a method for high-stability enzyme immobilization and an electrode configuration for potential applications in biosensors. Initially, a model of enzyme (GO_x) was immobilized via covalent bonding onto chitosan-based porous composite materials. To increase the conductivity of the CS, CNTs were added. The developed method was then used for biosensor fabrication. An electron transfer mediator (ETM) was also incorporated to facilitate electron transfer. The leakage of ETMs was minimized by both possible chitosan-ETM bonding and the resistance of the chitosan membrane pores.

2. Experimental

2.1. Materials

Chitosan (190000–310000 Da, 75–85% degree of deacetylation (DD)), glucose oxidase (GO_x) from *Aspergillus niger* (EC 1.1.3.4; type XS; 245,900 units g^{-1}) and glutaraldehyde (50 wt% in H_2O) were purchased from Sigma–Aldrich. KH_2PO_4 and Na_2HPO_4 (chemicals for phosphate buffer), ferrocene ($\text{C}_{10}\text{H}_{10}\text{Fe}$), acetic acid, sulfuric acid and NaOH were purchased from Merck. The CNTs were from Bayer Material Science, Germany. The platinum working electrode, Ag/AgCl reference electrode and platinum auxiliary electrode were purchased from ALS Japan.

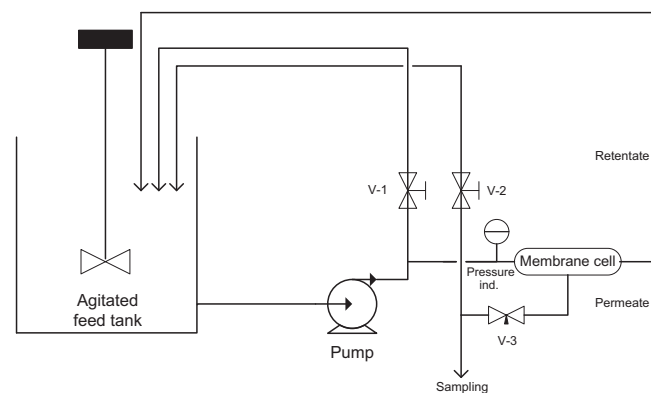


Fig. 1. Schematic diagram of the cross-flow filtration apparatus used in this study.

2.2. Methods

2.2.1. Preparation of porous chitosan membranes

Porous chitosan membranes were prepared using the phase separation method. A chitosan solution with a certain concentration (in 1% v/v acetic acid) was cast onto a glass substrate to form a film with a thickness of 300 μm . Precipitation (phase separation) was performed by evaporation (evaporation-induced phase separation, EIPS) or immersion in a non-solvent (non-solvent-induced phase separation, NIPS) or a combination of these two methods. In the EIPS method, the solvent in the cast membrane was evaporated at room temperature to promote precipitation. In the NIPS method, the cast membrane was coagulated by immersion in 2% (w/w) sodium hydroxide (NaOH) as the non-solvent. In the EIPS and NIPS combination method, the solvent in the cast membrane was first evaporated for 24 h and then coagulated in 2% (w/w) NaOH for ~48 h. The resulting membranes were rinsed and dried.

The chitosan–CNT composite membranes were prepared by the following procedure: Chitosan with a certain concentration was dissolved in an acetic acid solution (1% v/v) with stirring. The homogeneous chitosan solution was left without stirring until no bubbles were observed. CNTs (0.1 g) were then added to the homogeneous chitosan solution, and a homogenizer (IKA T10 basic ULTRA-TURRAX with 14,000 rpm) was used to obtain a homogenous dispersion. The membranes were then prepared using the same method as for the chitosan membranes.

2.2.2. Membrane characterization

The water permeability, surface chemistry and surface morphology of the membrane were determined.

2.2.2.1. Water permeability measurements. The water permeability of the chitosan membrane was measured using a homemade laboratory scale for a filtration test [18]. The apparatus consisted of a feed tank, a pump, a pressure indicator and a flat sheet membrane cell. The experimental apparatus is schematically shown in Fig. 1. The membrane area used in this experiment was 52 cm^2 . The permeate and retentate were returned back to the feed tank to maintain the feed concentration. All permeability measurements were performed at room temperature ($30 \pm 2^\circ\text{C}$). The membrane was first compacted by filtering water through the membrane for 0.5 h at a pressure of 1 bar. The membrane permeability (L_p) was calculated using the following equation:

$$L_p = \frac{Q}{A\Delta P} \quad (1)$$

where Q is the permeate flow rate, A is membrane area and P is transmembrane pressure.

The water permeability measurements were then used for the determination of the membrane pore size using the Hagen–Poiseuille equation:

$$L_p = \frac{J}{\Delta P} = \frac{\varepsilon r^2}{8\eta\Delta x} \quad (2)$$

where L_p is the membrane permeability, J is the permeate flux, ΔP is the transmembrane pressure difference, Δx is the thickness of the membrane, τ is the tortuosity, η is the viscosity, r is the pore radius and ε is the membrane porosity.

2.2.2.2. Membrane morphology. The surface morphology of the tops of the membranes was visualized using a Supra 35 VP field emission scanning electron microscope (FESEM). The outer surface of the sample was sputter coated with gold/palladium for 1 min before analysis.

2.2.2.3. Surface chemistry. The membrane surface chemistry was analyzed using a Varian Excalibur series 3100 Fourier transform infrared (FTIR) spectrometer. A total of 64 scans were taken at a resolution of 4 cm^{-1} and a temperature of $21 \pm 1^\circ\text{C}$.

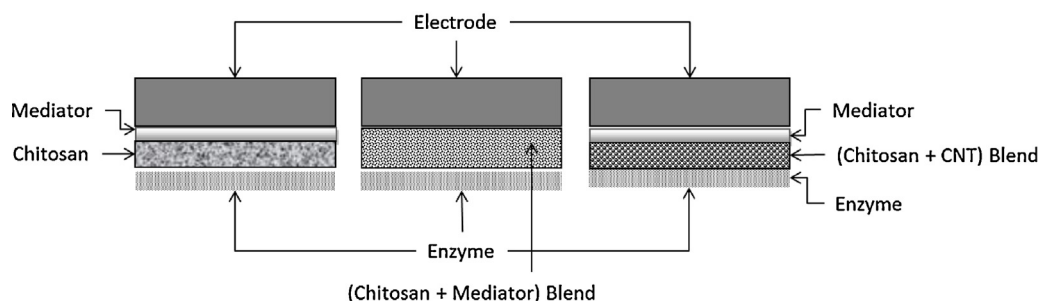


Fig. 2. Working electrode configuration schemes. E (top): the electrode, M: media electron transfer, Cs: chitosan, CNT: carbon nanotubes, Ez: enzymes.

Varian's Resolution Pro 4.0 was used to record the membrane spectra relative to the corresponding background spectra.

2.2.3. Enzyme immobilization

Enzyme immobilization was performed by incubating the CS membrane with GO_x solution (in phosphate buffer) for 24 h. For enzyme immobilization on activated membranes, the CS membranes were initially activated by exposing the membrane surface to a glutaraldehyde solution (GD) (1% w/v) for 1 h. The effects of pH and the concentration of enzyme solution on the binding capacity of the enzyme were investigated. The binding capacity of GO_x was determined by measuring its initial concentration in the buffer solution and its concentration after incubation with the CS membrane for a certain time. The enzyme concentration was measured using a spectrophotometric method at 453 nm. The binding capacity was then deduced from the mass balance and expressed in terms of the amount of enzyme bound to the membrane divided by the membrane area.

The enzymatic activity was measured using the method proposed by Yang et al. [19]. Briefly, the selected amount of GO_x was added to 30 ml of phosphate buffer (KH_2PO_4 – Na_2HPO_4 solution, pH 5) containing 1 g of glucose, and the solution was bubbled with air for 15 min at $30 \pm 2^\circ\text{C}$. Then, 30 ml of 0.1 M NaOH was added to the solution to quench the reaction, followed by titration using 0.1 M HCl to determine the amount of excess NaOH. A unit (U) of GO_x is defined as the amount of enzyme required to oxidize 1 μmol of β -D(+)-glucose to D-gluconic acid and H_2O_2 per minute at pH 5 at 30°C (see Ref [19] for more details).

2.2.4. Study of enzyme stability

The stability of the GO_x immobilized on porous chitosan membranes was investigated by soaking the membrane in phosphate buffer solution at pH 5 for 5 days. The concentration of GO_x in the buffer solution was measured and used as an indicator of the immobilization stability.

2.2.5. Preparation of the working electrode

The working electrode was prepared using a method to obtain both covalent immobilization of GO_x on the porous chitosan membrane and fast electron transfer to the surface of the electrode. Three configurations were evaluated. In the first configuration, the electrode was wetted using a ferrocene solution as the ETM agent. Thereafter, the electrode was coated with a chitosan membrane and activated using GD. The enzyme immobilization step was then performed. The second configuration was similar with the first configuration, but in the second configuration, chitosan was blended with ferrocene and the electrode was coated with a chitosan–ferrocene blend membrane. In the third configuration, CNTs were incorporated into the chitosan–ferrocene blend membrane. A schematic diagram of the configuration of the working electrode developed in this study is presented in Fig. 2.

2.2.6. Electrochemical behavior

Electrochemical behavior was investigated in a 3-electrode cell comprising a Ag/AgCl (saturated KCl solution) reference electrode, a platinum auxiliary electrode and the developed working electrodes (cf. Section 2.2.5). Voltammetry was used to monitor the reaction through the chitosan membrane at the working electrode. The data were recorded on a personal computer using EZware software with the LabVIEW Runtime Engine connected to an EZstat potentiostat (Nuvant System, United State). A schematic diagram of the experimental apparatus is presented in the Supplemental material (A).

3. Results and discussion

3.1. Preparation and characterization of porous chitosan membranes

Three different methods (EIPS, NIPS and combination of the two) for the preparation of porous chitosan membranes were evaluated. Two different solvents, hydrochloric acid and acetate acid, were

used to dissolve the chitosan. It is important to note that both solvents have been used in previous studies (e.g., [12]). Dissolution should be faster for systems with greater interaction between the solute and the solvent. The performances of HCl and acetic acid for chitosan dissolution were compared. HCl should dissolve chitosan faster than acetic acid due to its greater interaction with chitosan. HCl would be ionized completely in water and should have a stronger interaction with chitosan, which is cationic, than acetic acid. Nevertheless, the experimental results showed that the dissolution of chitosan using HCl was slower than the dissolution using acetic acid. The reason for this phenomenon may be due to the inorganic character of Cl^- , which not only is mobile but also lacks the hydrocarbon backbone of the organic anions [20]. Thus, acetic acid was used for further experiments.

The membrane preparation experiments showed that EIPS and NIPS alone could not be used to prepare chitosan membranes. Phase separation, necessary for successful membrane preparation, did not occur completely in either method. Membranes were then prepared using a combination of EIPS and NIPS. The cast membrane was first evaporated at room temperature overnight and then coagulated in 2% sodium hydroxide for at least 48 h. Using this method, complete phase separation could be achieved, and chitosan membranes could be produced (see Supplemental material B). In all further experiments, the chitosan membranes were prepared using combination of the EIPS and NIPS methods.

The effects of the chitosan concentration on the membrane characteristics were investigated. In general, the experimental results showed that the higher the chitosan concentration, the smaller the resulting pore size. In addition, stronger (not easily broken) membranes and membranes that were easier to remove from the substrate were obtained. However, chitosan concentrations higher than 2% resulted in membranes with low permeability, indicative of a smaller pore size. Fig. 3 shows that the permeability of

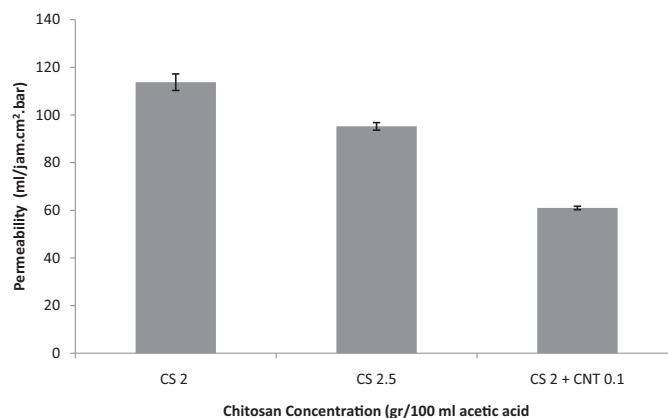


Fig. 3. Effect of chitosan concentration on the porous chitosan membrane permeability.

a porous chitosan membrane prepared with a concentration of 2% was greater than that of a chitosan membrane prepared with a concentration of 2.5% (113 ml/h cm² bar and 95 ml/h cm² bar, respectively). It is important to note that the membrane pore size should be smaller than the size of GO_x. However, a pore size that is too small will limit the mass transfer of the products of the enzymatic reaction to the electrode. Based on permeability data and using the Hagen–Poiseuille equation (Eq. (2)), the membranes' pore sizes were then determined and were found to be 0.0082 μm (= 8.2 nm) and 0.0075 μm (= 7.5 nm) for the membranes prepared using 2% and 2.5% chitosan, respectively. Both membranes had a pore size smaller than the size of GO_x. It has been reported that GO_x has a size of $12 \times 10 \times 0.5$ nm [21]. Because the membrane prepared from 2% CS had a higher permeability than that prepared with 2.5% CS, in all further experiments a chitosan concentration of 2% was used.

To increase the membrane conductivity, CNTs were incorporated into the chitosan solution before the membrane was prepared. The presence of CNTs in the chitosan polymer made the resulting membrane darker than the membrane produced without CNTs. The conductivity measurements showed that the pure chitosan membranes, pure CNTs and CNT-chitosan composite membranes exhibited average conductivities of 0.4×10^{-3} , 8.2×10^3 , and 0.95×10^1 S/cm, respectively. In addition, the membrane permeability decreased with the addition of CNTs. This reduction in permeability is due to the hydrophobic properties of the CNTs [22].

The surface morphology of the chitosan membrane was visualized using SEM. Fig. 4 shows the surface morphology of the chitosan membranes with and without CNTs. The chitosan membrane prepared without CNTs had a smoother surface and a larger pore size than the chitosan membrane with CNTs. The presence of CNTs in the chitosan membrane prepared with CNTs was clearly observed. However, the CNTs were not evenly distributed, and clustering of the CNT nanoparticles was observed.

The membrane surface chemistry was characterized using FTIR. The absorption bands of functional groups within the wave number range of 500 to 4500 cm⁻¹ were identified (Fig. 5). Typical chitosan material has an absorption band at ~ 1552 cm⁻¹ from the amine bond (NH₂) and an absorption band corresponding to the C=O carbonyl group (from the carboxylic group) at ~ 1643 cm⁻¹. The results obtained from this characterization agree well with those previously reported by Hefian et al. [23] and Wu et al. [17]. In general, the presence of CNT nanoparticles did not change the IR spectrum of the chitosan membrane.

3.2. Enzyme immobilization and binding capacity

In this study, the effects of chitosan activation, the pH and the enzyme concentration on the binding capacity were investigated.

3.2.1. Effect of activation

Two different chitosan membranes, i.e., with and without activation, were used for GO_x immobilization. The binding capacity was used as an indicator of the effectiveness of enzyme immobilization. This experiment was also performed using the chitosan-CNT blend membrane. Fig. 6 shows the experimental results.

It was obvious that the GO_x binding capacity of the chitosan membrane was increased approximately two-fold by activation. A similar phenomenon was observed for the chitosan-CNT blend membrane. The activation of CS using GD can result in the formation of imine groups on the membrane's surface. This explanation is supported by an increase in the band at ~ 1661 cm⁻¹ (Fig. 7). The presence of imine functional groups would increase the likelihood of covalent binding between the enzyme and the chitosan membrane.

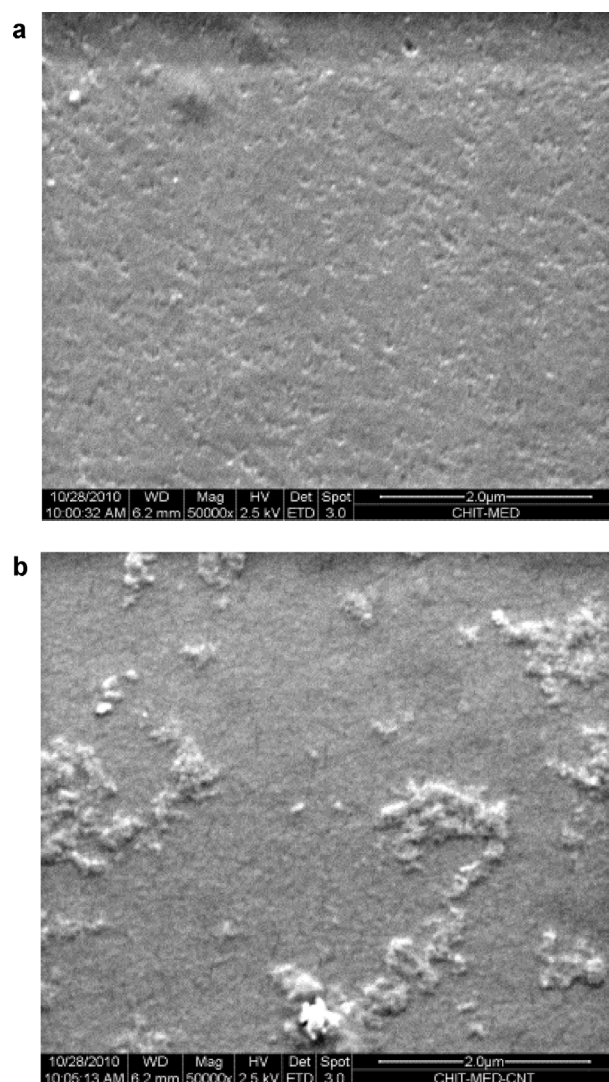


Fig. 4. Visualization of surface morphology using SEM. (a) Chitosan only (b) Chitosan – CNT.

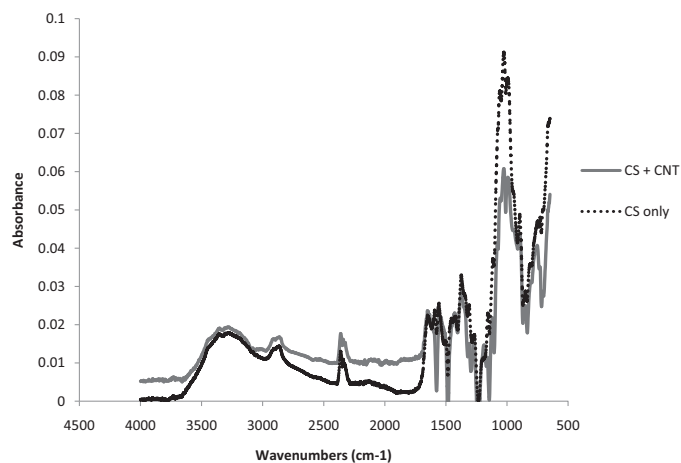


Fig. 5. Chemical composition characterization of the porous chitosan membrane by using FTIR. (a) Medium MW 2 g/ml (b) Medium MW 2 g/ml + 1 g of CNT.

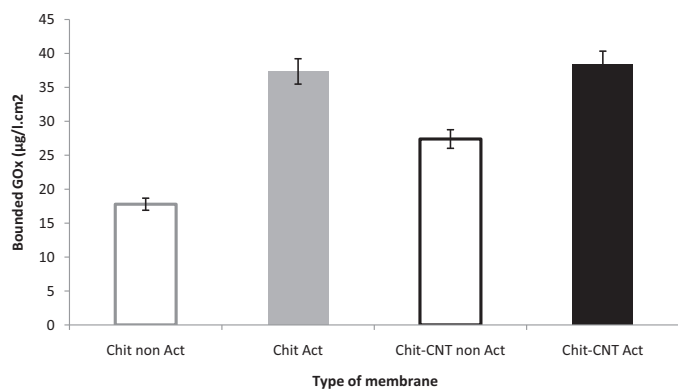


Fig. 6. Binding capacity during enzyme immobilization using different chitosan based membranes.

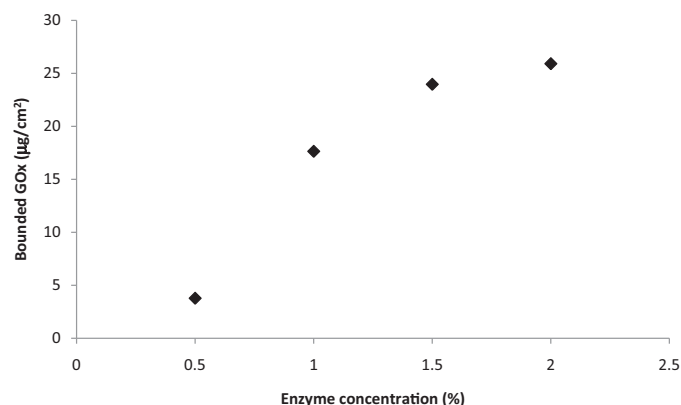


Fig. 9. Effect of enzyme concentration on the enzyme-binding capacity.

condition) decreased the binding capacity. These results are similar to the results obtained in previous studies [24,25]. At a pH greater than ~4.3 (the isoelectric point of GO_x), GO_x should have a negative charge and be electrostatically attracted to chitosan. However, because the isoelectric point of chitosan is 6.3, the solubility of hydrated chitosan decreases above this pH [26]. Consequently, the electrostatic attraction between GO_x and chitosan will decrease at pHs above 6.3. Another possible explanation is that there is electrostatic repulsion between GO_x molecules that have already bound to the CS membrane and unbound free GO_x molecules with the same charge.

3.2.3. Effect of the enzyme concentration

The effect of the enzyme concentration on the binding capacity was investigated using an activated chitosan membrane. As shown in Fig. 9, the increase in the enzyme concentration within the range of 0–1.5% significantly increased the binding capacity. Above the concentration of 1.5%, a slight increase was observed. It seems that the adsorption resulted predominantly in monolayers (at concentrations less than 1.5%) and that there was a small contribution of enzyme-enzyme interactions at high concentrations (>1.5%). A similar phenomenon was observed in a study of dextran adsorption on a polyether sulfone membrane [27].

3.3. Study of enzyme immobilization stability

The study of the enzyme immobilization stability was performed by soaking in buffer solution a chitosan-based membrane on which GO_x had been immobilized (Fig. 10). As shown in Fig. 10, the activated chitosan membrane had a higher stability, as indicated by the lower enzyme concentration in the buffer solution

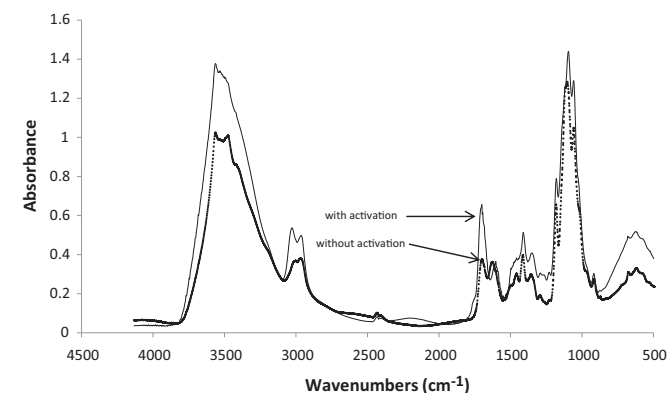


Fig. 7. Chemical composition characterization of the porous chitosan membrane by using FTIR (with and without activation).

Fig. 6 also shows that the presence of CNTs in the chitosan membranes increased the enzyme binding capacity. This effect was more pronounced for the unactivated chitosan membrane. Two possible explanations are the increase in the specific surface area of the membrane due to the CNT nanoparticles and the hydrophobic character of the CNTs.

3.2.2. Effect of pH

To investigate the effect of pH on the binding capacity of GO_x , activated chitosan membranes were soaked in an enzyme solution at different pHs. As shown in Fig. 8, the highest binding capacity was achieved at pH 5. An increase in the pH (to alkaline

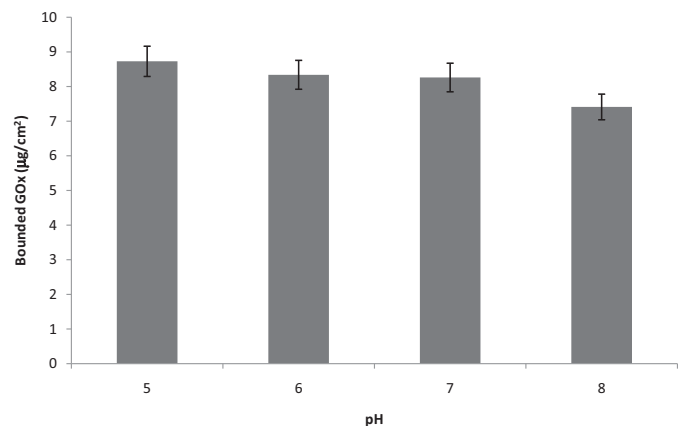


Fig. 8. Effect of pH on the enzyme-binding capacity.

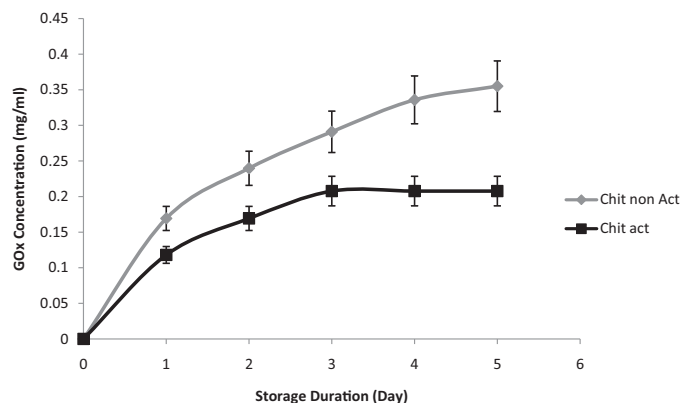


Fig. 10. Effect of storage duration on the the concentration of GO_x in buffer.

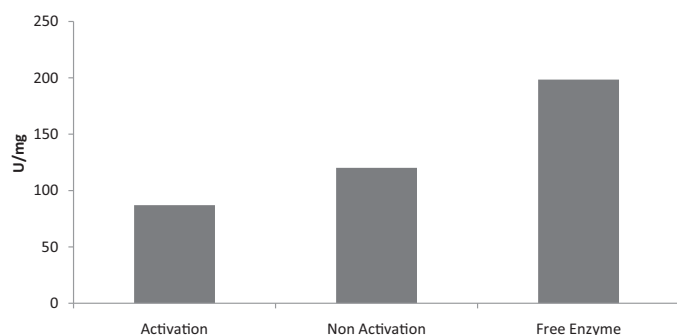


Fig. 11. Enzyme activity of immobilized enzyme and free enzyme.

after the same soaking time. The lower concentration of enzyme in the buffer solution indicates that fewer individual enzyme molecules were released from the CS membrane. Nevertheless, the release of enzyme into the buffer solution was still observed for the activated membrane. This phenomenon could be explained as follows: the attachment of enzyme molecules to the chitosan membrane could occur via physical and chemical (covalent bond) interactions. Enzymes bound by physical interactions are more likely to be released into the buffer solution than those bound by chemical interactions. The proportion of chemical interactions was greater for the activated chitosan membranes than the unactivated membranes. Consequently, the amount of enzyme released into the buffer solution was greater for the unactivated membrane. This result aligns with the results obtained by Hanafeld et al. [28] and Ulbricht et al. [29]. In addition, because imine bonds can be hydrolyzed to yield the corresponding amine- and carbonyl-containing compounds (aldehyde), covalently bonded enzymes could also be released.

In addition to the enzyme stability, the enzyme activity was measured, and the results are presented in Fig. 11. The highest activity was observed for the free enzyme, and the activity of the immobilized enzyme was much higher for the chitosan membrane that was not activated using GA than for the activated chitosan membrane. The performance of an enzyme should be evaluated not only based on its activation (Fig. 11) but also on the amount of enzyme present (Fig. 10).

3.4. Electrochemical behavior

The electrochemical behavior is related to the redox potential, the electrochemical reaction of an analyte solution and the reversibility of a reaction at a given scan rate [30]. In this work, the electrochemical behavior of activated chitosan membranes (prepared from a 2% chitosan solution) after enzyme immobilization was investigated. Chitosan membranes were used in different electrode configurations.

Fig. 12 shows the comparison of the electrochemical behavior of the chitosan membranes with and without glucose in the test solution. In the presence of glucose, there was a peak at the potential of ~ 1.8 V (0.9 mA), which indicates that the rate of glucose oxidation into gluconic acid was greater. This result suggests that GO_x catalyzed the oxidation of glucose.

The experimental results for the electrochemical behavior of three different configurations (cf. Fig. 2) are presented in Fig. 13. The first configuration had an oxidation peak at a potential of 1.8 V (1.3 mA), whereas the second and third configurations had oxidation peaks at potentials of 1.7 V (1.3 mA) and 1.95 V (2.3 mA), respectively. This result suggests that the third configuration, which used a chitosan-CNT blend membrane, has the best performance for potential applications in biosensors. The presence of

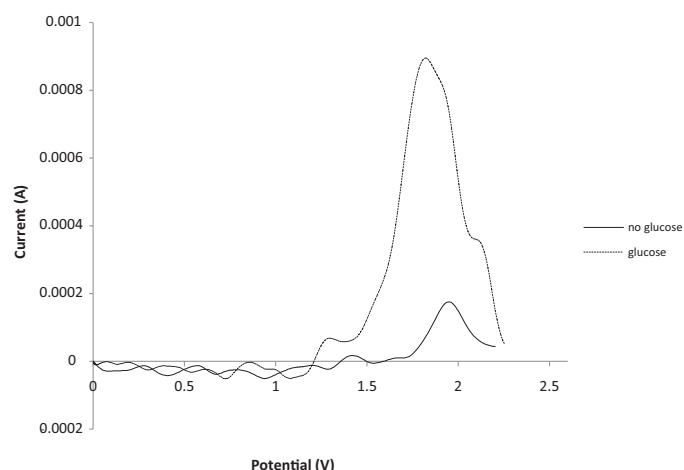


Fig. 12. Electrochemical behavior of chitosan membrane with and without glucose in the test solution. (First configuration electrode, scan rate of 0.01 V/s, potential range from 0 to 2.5 V).

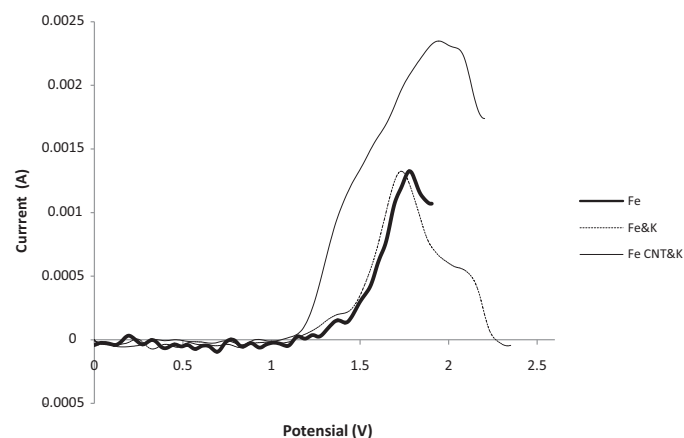


Fig. 13. Comparison of linear voltammogram biosensor configuration 1, 2, and 3.

CNTs improved the electrochemical behavior of the chitosan membrane, which is required for high-performance biosensors.

4. Conclusions

Chitosan-based composite membranes were prepared using a combination of evaporation-induced phase separation and non-solvent-induced phase separation. The effects of the solvent and the chitosan concentration on membrane preparation were investigated. The membrane characteristics, the performance of the enzyme immobilization and the electrochemical behavior were also investigated. The results of the membrane characterization assays suggest that the best chitosan membrane for GO_x immobilization is the chitosan membrane prepared from 2% chitosan using acetic acid as the solvent. Incorporating CNTs into the chitosan membrane to increase the membrane conductivity was successfully performed. The membranes (without and with activation using glutaraldehyde) were used for the immobilization of glucose oxidase (GO_x). The results suggest that membrane activation has significant impacts on the enzyme immobilization capacity and stability. Membrane activation increased the immobilization capacity by approximately two-fold. GO_x immobilization on chitosan-based membranes was also influenced by both pH and the enzyme concentration. The electrochemical behavior suggests that the working electrode, which was composed of electrode-mediator-(chitosan and CNTs) and enzyme, is the best for biosensor applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.enzmictec.2013.02.005>.

References

- [1] Wang J. Glucose biosensors: 40 years of advances and challenges. *Electroanalysis* 2001;13:983.
- [2] Dzyadevych SV, Arkhypova VN, Soldatkin AP, El'skaya AV, Martelet C, Jaffrezic-Renaul N. Amperometric enzyme biosensors: past, present and future. *Irbm* 2008;29:171–80.
- [3] Cetinus SA, Oztop N. Immobilization of catalase on chitosan film. *Enzyme and Microbial Technology* 2000;26:497.
- [4] Korell U, Lenno RB. A sulphite biosensor, coupling of sulphite oxidase (EC 1.8.3.1) to a TTFTCNQ electrode. *Electroanalytical Chemistry* 1993;351:137.
- [5] Adeloju SB, Barisci JN, Wallace GG. Electroimmobilisation of sulphite oxidase into a polypyrrole film and its utilisation for flow amperometric detection of sulphite. *Analytica Chimica Acta* 1996;332:145.
- [6] Adeloju SB, Shaw SJ, Wallace GG. Polypyrrole-based amperometric flow injection biosensor for urea. *Electroanalysis* 1996;6:865.
- [7] Zhang Y, Hu Y, Wilson GS, Sirat DM, Poitout V, Reach G. Elimination of the acetaminophen interference in an implantable glucose sensor. *Analytical Chemistry* 1994;66:1183.
- [8] Bartlett PN, Cooper JM. A review of the immobilization of enzymes in electropolymerized films. *Journal of Electroanalytical Chemistry* 1993;362:1.
- [9] Cespedes F, Alegret S. New materials for electrochemical sensing II. Rigid carbon-polymer biocomposites. *Trends in Analytical Chemistry* 2000;19:276.
- [10] Miao Y, Tan SN. Amperometric hydrogen peroxide biosensor with silica sol-gel/chitosan film as immobilization matrix. *Analytica Chimica Acta* 2001;437:87.
- [11] Sampath S, Lev O. 3D organized self-assembled monolayer electrodes: a novel biosensor configuration. *Advanced Materials* 1994;9:410.
- [12] Krajewska B. Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. *Enzyme and Microbial Technology* 2004;35:126.
- [13] NG LT, Guthrie JT, Yuan YJ, Zhao H. UV-cured natural polymer-based membrane for biosensor application. *Journal of Applied Polymer Science* 2000;79:466.
- [14] Chen G, Wang Y, Yang P. Amperometric biosensor coupled to capillary electrophoresis for glucose determination. *Microchimica Acta* 2005;150:239.
- [15] Wang J. Carbon-nanotube based electrochemical biosensors: a review. *Electroanalysis* 2005;17:7.
- [16] Wang Y, Wei W, Liu X, Zeng X. Carbon nanotube/chitosan/gold nanoparticles-based glucose biosensor prepared by a layer-by-layer technique. *Materials Science and Engineering C* 2009;29:50.
- [17] Wu Z, Feng W, Feng Y, Liu Q, Xu X, Sekino T, et al. Preparation and characterization of chitosan-grafted multiwalled carbon nanotubes and their electrochemical properties. *Carbon* 2007;45:1212.
- [18] Susanto H, Widiasta IN. Ultrafiltration fouling of amylose solution: behavior, characterization and mechanism. *Journal of Food Engineering* 2008;95:423.
- [19] Yang YM, Wang JW, Tan RX. Immobilization of glucose oxidase on chitosan-SiO₂ gel. *Enzyme and Microbial Technology* 2004;34:126.
- [20] Ahonkhai EI, Arhewoh IM, Okhamafe AO. Effect of solvent type and drying method on protein retention in chitosan-alginate microcapsules. *Tropical Journal of Pharmaceutical Research* 2006;5:585.
- [21] Otsuka I, Yaoita M, Nagashima S, Higano M. Molecular dimensions of dried glucose oxidase on a Au(111) surface studied by dynamic mode scanning force microscopy. *Electrochimica Acta* 2005;50:4861.
- [22] Tzeng Y, Huang TS, Chen YC, Liu C, Liu YK. Hydration properties of carbon nanotubes and their effects on electrical and biosensor applications. *New Diamond and Frontier Carbon Technology* 2004;14:193.
- [23] El-hefian EA, Nasef MM, Yahaya AH. The preparation and characterization of chitosan/poly(vinyl alcohol) blended films. *E-journal of chemistry* 2010;7:1212.
- [24] Wang X, Zhu KX, Zhou HM. Immobilization of glucose oxidase in alginate-chitosan microcapsules. *International Journal of Molecular Sciences* 2011;12:3048.
- [25] Bankar SB, Bule MV, Singhal RS, Ananthanarayan L. Glucose oxidase—An overview. *Biotechnology Advances* 2009;27:489.
- [26] Li XF, Feng XQ, Yang S, Wang TP, Su ZX. Effect of molecular weight and concentration of chitosan on antifungal activity against *aspergillus niger*. *Iranian Polymer Journal* 2008;17:834–52.
- [27] Susanto H, Ulbrich M. Influence of ultrafiltration membrane characteristics on adsorptive fouling with dextrans. *Journal of Membrane Science* 2005;266:132.
- [28] Hanafeld U, Gardossib L, Magnerc E. Understanding enzyme immobilization. *Chemical Society Reviews* 2009;38:453.
- [29] Ulbricht M, Papra A. Polyacrylonitrile enzyme ultrafiltration membranes prepared by adsorption, cross-linking, and covalent binding. *Enzyme and Microbial Technology* 1997;20:61.
- [30] Grieshaber D, MacKenzie R, Voros J, Reimhult E. Electrochemical biosensors – sensor principles and architectures. *Sensors* 2008;8:1409.