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Development and analytical application of a glucose biosensor based on glucose oxidase/*O*-(2-hydroxyl)propyl-3-trimethylammonium chitosan chloride nanoparticle-immobilized onion inner epidermis

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ABSTRACT

A glucose biosensor comprising a glucose oxidase/*O*-(2-hydroxyl)propyl-3-trimethylammonium chitosan chloride nanoparticle (O-HTCC NP)-immobilized onion inner membrane and a dissolved oxygen (O_2) sensor has been successfully developed. The detection scheme is based on the depletion of dissolved O_2 content upon exposure to glucose. The decrease in O_2 level was monitored and related to the glucose concentration. The biosensor shows linear response to glucose from 0.0 to 0.60 mM with a detection limit of 50 μM ($S/N = 3$). The effect of O-HTCC NP and enzyme loading, pH, temperature, and phosphate buffer concentration on the sensitivity of the biosensor was studied in detail. The biosensor exhibits fast response time (70 s), good repeatability (3.2%, $n = 10$) and storage stability (90% of initial sensitivity after 3-week storage). Common interferents including acetic acid, lactic acid, propionic acid, butyric acid, folic acid, methanol, glycine, DL-α-alanine and DL-cysteine do not cause significant interferences on the biosensor. The proposed biosensor method was successfully applied to determine the glucose content in real samples such as orange juice, red wine and tea drink and the results were comparable to that obtained from a spectrophotometric method. The glucose recovery test demonstrates that the proposed glucose biosensor offers an excellent, accurate and precise method for the determination of glucose in real samples.

1. Introduction

In recent years there is a growing interest in the development of glucose biosensor based on immobilized enzyme since rapid, accurate and in situ determination of glucose in food industry, medical and bioanalysis fields is of paramount importance (Mizutani and Yabuki, 1997; Wang, 1999; Choi, 2005). Considerable attention has been focused on immobilizing glucose oxidase (GO_x) on different solid matrices which are subsequently positioned on a transducer surface to produce workable biosensors. This enzyme immobilization process is crucial as it can determine the analytical performance of a glucose biosensor (Zhang et al., 2005). As such, finding the compatible enzyme matrices and effective cross-linking agents for improving the functionality, sensitivity and repeatability of biosensors has always been an important subject for researchers. An excellent immobilization support and opportune technological design are essential to preserve the biological activities of enzymes (Wink et al., 1997).

Enzyme immobilization is commonly performed by physical adsorption (Niculescu et al., 2004), covalent attachment (Li et al., 1998), entrapment (Kwan et al., 2004; Tag et al., 2000), or cross-linking (Yang et al., 2006; Wen et al., 2007; Zhang et al., 2007). On the other hand, various inorganic materials (Hasse and Scholz, 2004; Manesh et al., 2008; Wang et al., 2009), organic polymers (Rauf et al., 2006; Li and Lin, 2007) and natural biomaterials (Yang et al., 2006; Wen et al., 2007; Kumar and D'Souza, 2009) have been exploited for immobilization of enzymes and their use in biosensor fabrication. Among these methods and matrices, the effective ways to improve stability, repeatability, reproducibility and activity of immobilized enzymes are to incorporate the enzymes into biomaterials by cross-linking as they are more biocompatible with each other since biomaterials in living organisms are composed

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of biomolecules such as carbohydrates, lipids and proteins. So far, bamboo inner shell membrane, eggshell membrane and onion inner epidermal membrane have been proved to be useful biomaterials for enzyme immobilization (Yang et al., 2006; Zhang et al., 2006; Kumar and D'Souza, 2009). For effective enzyme immobilization, it is essential to find a suitable cross-linking agent to prevent enzyme leaching from the solid support. Glutaraldehyde and chitosan have been successfully used as a gluey agent, while the sensitivity of the biosensor with chitosan is two times more sensitive than glutaraldehyde (Wen et al., 2007). The use of chitosan, however, is generally limited to acidic conditions since it has poor solubility above pH 6.5 where chitosan loses its cationic nature (Jae et al., 2003). In order to circumvent this problem, chitosan can be functionalized with quaternary ammonium salt to form cationic chitosan. For instances, choline dichloride comprising a trimethylammonium moiety reacted with chitosan to yield highly cationic chitosan (Curti et al., 2003.). Chitosan was converted to *O*-(2-hydroxyl)propyl-3-trimethylammonium chitosan chloride (O-HTCC) nanoparticle (NP) and this O-HTCC NP was proved to be a useful protein carrier material (Sun and Wan, 2007).

In this study, we explore the use of O-HTCC NP for immobilization of an enzyme for biosensing. The major advantage of our proposed method is to employ a new alternative cross-linking agent and biomaterial as an enzyme immobilization platform. The inner epidermal membrane of the onion bulk scales was chosen as the bio-platform to immobilize enzyme since it possesses excellent gas and water permeability for substrates and products to diffuse in and out of the membrane; very similar to those of the above-mentioned biomaterials. Onion membranes mainly consist of elongated tubular cells, blunt or tapering ends along with numerous guard cells (Scott et al., 1958; Bruce and Hepworth, 2004). The structural features of the inner epidermis cell wall can possibly provide a biocompatible environment for enzyme immobilization. This natural membrane is mechanically stronger than other natural membranes for biosensors because of its microfibrillar cellulosic structure. Thus, it should be an ideal biocompatible platform for enzyme immobilization (Kumar and D'Souza, 2009).

Glucose oxidase (GO_x) was selected for investigation since it can recognize glucose target molecules quickly and accurately in complicated systems. In addition, GO_x has been widely studied and applied in the field of biosensor (Newman and Turner, 2005; Manesh et al., 2008; Wang et al., 2009). In this work, the quantitative determination of glucose in real samples is based on the enzymatic oxidation of glucose by dissolved oxygen (O_2) as depicted in Eq. (1). GO_x is a flavoprotein which catalyzes the oxidation of β-D-glucose by molecular O_2 to δ-gluconolactone which subsequently spontaneously hydrolyzes to gluconic acid (Zoldak et al., 2004):

$$\text{D-glucose} + O_2 + H_2O \xrightarrow{GO_x} \text{D-gluconic acid} + H_2O_2 \quad (1)$$

The aim of this work is to explore the use of GO_x/O-HTCC NP-immobilized onion inner membrane for quantitative determination of glucose. The enzyme-immobilized membrane was fabricated by gelating GO_x with O-HTCC NP on an inner epidermal onion membrane and then positioned it on the surface of an O_2 sensor. The biosensor response is defined as the decrease in dissolved O_2 concentration which is linearly related to the glucose concentration in the standard solutions. The effects of pH, phosphate buffer concentration and temperature on the response of the biosensor were studied in detail. To our knowledge, this is the first report on using onion membrane to immobilize both GO_x and O-HTCC NP to achieve higher enzyme activity, better detection sensitivity and stability. Our proposed biosensor method has been successfully applied to the determination of glucose in some beverage and wine samples.

2. Experimental

2.1. Materials and reagents

Glucose oxidase (EC 1.1.3.4 from *Aspergillus niger*) with a specific activity of 245 units per milligram of solid was obtained from Sigma (St. Louis, MO, USA). Benzaldehyde, chitosan, glycidyltrimethylammonium chloride, glutaraldehyde solution (25%, w/w) and sodium tripolyphosphate were obtained from Aldrich (Milwaukee, WI, USA). All other reagents were of analytical-reagent grade and used without further purification. The buffer solution for preparing the glucose solution was 200 mM monosodium dihydrogen phosphate–disodium hydrogen phosphate solution at pH 7.4. A glucose stock standard solution (0.50 M) was prepared in pH 7.4 phosphate buffer (200 mM) and was allowed to mutarotate for 24 h at room temperature before use. All solutions were prepared with doubly distilled water. Fresh onions (*Allium cepa* L.) were purchased from a local market.

2.2. Preparation of O-HTCC nanoparticle

The synthesis of O-HTCC NP was based on a previous method (Sun and Wan, 2007) with slight modifications. 3.0 g of chitosan was dissolved in 100 mL of 10% acetic acid solution. After the addition of benzaldehyde (15.8 g), the solution was stirred for an hour and then left for 20 h in vacuum (55–60 °C). The resulting solution was adjusted to pH 7.0 with a 1 M NaOH solution and filtered. The *N*-benzylidene chitosan precipitate was washed with methanol several times. 9.0 g of *N*-benzylidene chitosan and 9.0 g of glycidyltrimethylammonium chloride were added to 50 mL of isopropyl alcohol. The resulting mixture was stirred at 70 °C for 16 h to precipitate the *o*-quaternary ammonium-*N*-benzylidene chitosan. The *o*-quaternary ammonium-*N*-benzylidene chitosan was collected and washed with methanol and acetone successively. 3.0 g of *o*-quaternary ammonium-*N*-benzylidene chitosan was added to 50 mL of 0.25 M ethanolic HCl solution and then stirred for 24 h at room temperature. After most of the ethanol was removed by vacuum, 15 mL of H_2O was added to the mixture and followed by adding excess acetone to precipitate the O-HTCC. The solvent was removed under vacuum at 80 °C to obtain O-HTCC. The crude O-HTCC product was further purified: 1.5 g of O-HTCC was dissolved in 40 mL of water and then the solution was poured into acetone to obtain the O-HTCC precipitate. The precipitate was collected and washed with acetone several times. The precipitate was then dissolved in distilled water again and dialyzed against water for 3 days, concentrated, precipitated in acetone, and dried under vacuum at 80 °C for 48 h to get the purified O-HTCC product.

10 mg of the purified O-HTCC was dissolved in 10 mL of acetic acid. Then 0.80 mL of sodium tripolyphosphate aqueous solution (4.0 mg mL^{-1}) was added to the stirred O-HTCC solution at room temperature. Opalescent suspension immediately appeared and the solution was filtered to obtain the O-HTCC NP. The O-HTCC NP was dried under vacuum and kept in a dry box until further use. The size of O-HTCC NPs was determined to be ca. 35.3 nm in sodium tripolyphosphate aqueous solution (Sun and Wan, 2007).

2.3. Immobilization of GO_x/O-HTCC NP on onion membrane

The procedure to immobilize GO_x and O-HTCC NP on an onion inner epidermis membrane was similar to a previous report (Kumar and D'Souza, 2009). An onion was cut into half, bulb scales were separated and inner epidermis from outer fleshy scale was stripped. Onion membranes were placed in a clean watch glass, cut into a circular pieces of 1.5 cm diameter and stored at 4 °C. An aliquot of 100 μL of 0.8% (w/v) GO_x (prepared in 200 mM phosphate buffer at pH 7.4) was added on the onion membrane. After 30 min, 20 μL

of 0.3% (w/v) O-HTCC NP solution as a gelation agent was dropped onto the surface of the membrane to mix with GO_x uniformly by a glass rod. The GO_x/O-HTCC NP-immobilized membrane was dried at room temperature for 1 h. The GO_x/O-HTCC NP-immobilized membrane was immersed in and washed with a phosphate buffer (pH 7.4) for 5 min to remove any loosely bound enzyme and then kept at 4 °C until further use.

2.4. Scanning electron microscopy of onion membrane

The scanning electron micrographs (SEM) of the onion inner epidermis with and without the immobilized GO_x/O-HTCC NP were captured by a Quanta 200 scanning electron microscope (FEI Company, Hillsboro, OR, USA).

2.5. Assembly of glucose biosensor and determination

The GO_x/O-HTCC NP-immobilized onion inner epidermis was positioned on the surface of a Pasco CI-6542 O_2 sensor (Pasco Scientific, Roseville, CA, USA) and kept in a steady position by an *O*-ring. The O_2 sensor was immersed into a stirred 5.0 mL phosphate buffer solution (200 mM, pH 7.4). A series of volumes of standard (0.50 M) or sample glucose solution were injected into the phosphate buffer with a syringe. The dissolved O_2 signal was captured and processed by a datalogger system consisting of a Science Workshop 500 interface, serial cables, a power supply, and control software (Pasco Scientific, Roseville, CA, USA). The data were logged in a computer for real-time display and processing. The working principle of O_2 sensor has been previously described by Wen et al. (2007).

3. Results and discussion

3.1. Microstructure of onion inner membranes without and with GO_x/O-HTCC NP

The microstructures of onion inner membranes without and with immobilized GO_x/O-HTCC NP were studied. An onion inner membrane is mainly composed of regular fibrillar lacunaes with clear stripes as depicted in Fig. 1a. When the onion membrane was immobilized with GO_x/O-HTCC NP, the onion fibers still retain their integrity which is more or less the same as the fresh one. It is clear that lumps of GO_x/O-HTCC NP are deposited on the onion fibers (Fig. 1b). The SEM supports that GO_x/O-HTCC were successfully immobilized onto the onion membrane. It is well known that enzyme can be immobilized on solid substrates via the electrostatic interaction between the positively charged polyelectrolytes and the negatively charged enzyme molecules (Wei et al., 2003; Choi, 2005; Miscoria et al., 2006). Fig. S1 (Supplementary Material) depicts the chemical structure of O-HTCC which is a quaternized chitosan derivative possessing a positive charge per monomer unit. The immobilization of GO_x on O-HTCC NP can be interpreted by the strong electrostatic attraction between the cationic O-HTCC NP and the anionic GO_x. The major advantages of our enzyme immobilization method are three folds: first, O-HTCC NP have larger specific surface area which means more enzyme molecules loading; second, O-HTCC NP could still maintain its cationic nature at pH > 6.5 as compared to chitosan (Jae et al., 2003), inferring that GO_x/O-HTCC NP would be stable at a wider pH range; finally, the enzyme molecules immobilized on the O-HTCC NP/onion membrane could retain their bioactivity because of the good biocompatibility of onion membrane with enzyme.

3.2. Response behavior of glucose biosensor

The principal detection mechanism of the glucose biosensor relies on the enzymatic oxidation of glucose by O_2 as shown in Eq. (1). An O_2 electrode acting as an O_2 transducer was employed to measure the rate of O_2 consumption. The analytical signal captured by glucose biosensor is the decrease in the dissolved O_2 content in pH 7.4 phosphate solution with glucose. A typical response curve of glucose biosensor is displayed in Fig. 2. The O_2 content decreased with the addition of glucose and the decrease in the O_2 level was found to be proportional to the glucose concentration. A linear calibration curve plotting the decrease in the O_2 level against the concentration of glucose is shown in the inset of Fig. 2.

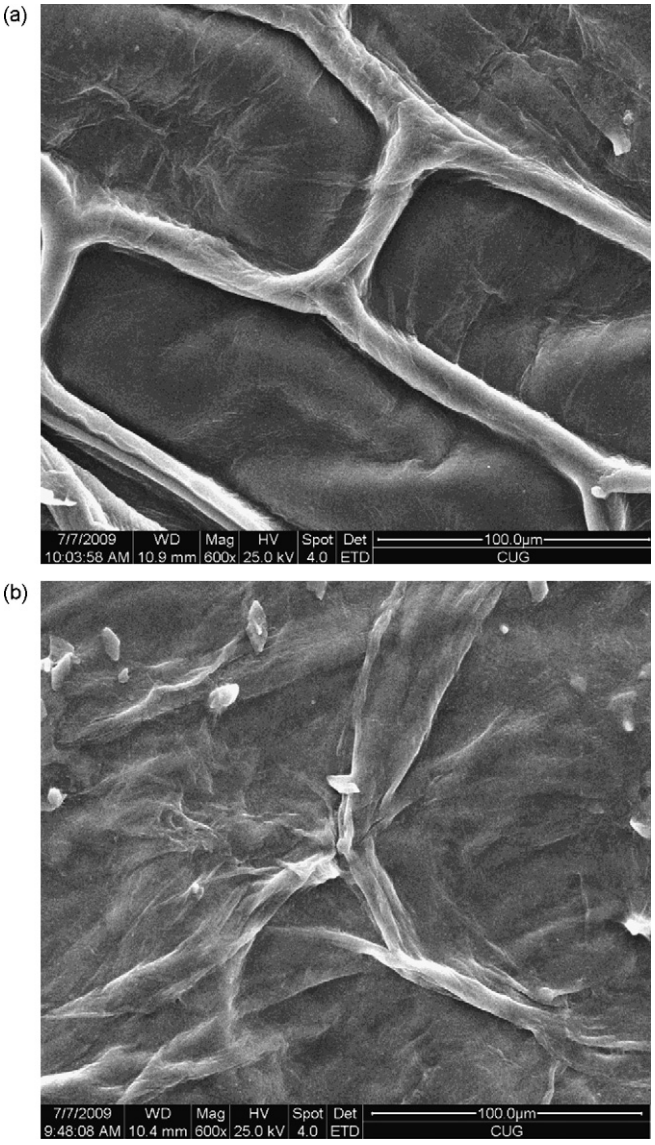

Fig. 1. Scanning electron micrographs of the onion inner membranes without (a) and with (b) immobilized GO_x/O-HTCC NP.

3.3. Effect of the amount of O-HTCC NP

In order to determine the optimal amount of O-HTCC NP used as a gelating agent for immobilizing GO_x, 0.10, 0.30 and 0.50% (w/v) were studied. The biosensor showed highest sensitivity to glucose when the amount of O-HTCC NP was 0.30% (w/v). When the amount of O-HTCC NP was below 0.3% (w/v), the sensitivity was lower. If the amount of O-HTCC NP was too low, some enzyme molecules may not be able to bind with the O-HTCC NP resulting in leaching of un-immobilized enzyme molecules. By contrast, when the amount of O-HTCC NP was too large, thicker and larger clumps or

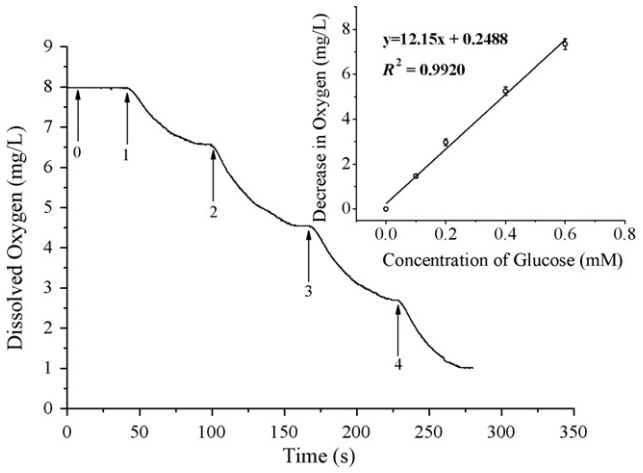

Fig. 2. Typical response curve of the glucose biosensor at pH 7.4 phosphate buffer (200 mM) after each successive addition of various volumes of 0.50 M glucose standard: (0) 0.0; (1 and 2) 1.0; and (3 and 4) 2.0 μL. The inset displays the linear calibration curve for glucose from 0.00 to 0.60 mM. Error bars are 95% confidence intervals calculated from three replicate measurements.

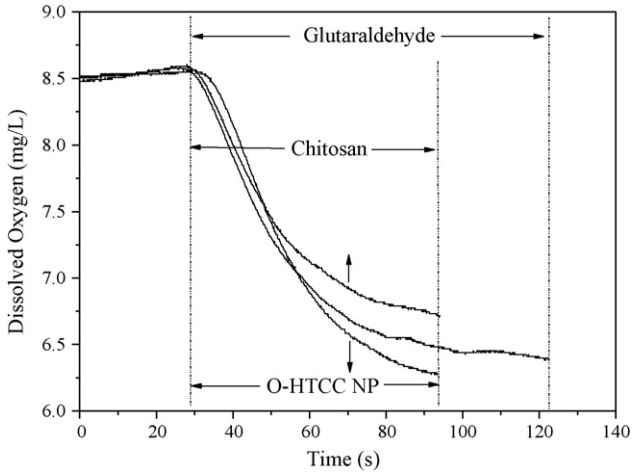

Fig. 3. Comparison of performance of GO_x immobilized onion membrane using O-HTCC NP, chitosan and glutaraldehyde by measuring the decrease in O_2 content of a 0.20 mM glucose standard in phosphate buffer (pH 7.4).

clusters of GO_x/O-HTCC NP were deposited on the onion fibers of the membrane which slow down the gas and water permeability of membrane and then eventually prolong the response time (>100 s).

3.4. Effect of enzyme loading

Since the response of the glucose biosensor depends strongly on the amount of the immobilized enzyme, any change in enzyme concentration would influence its sensitivity (Wen et al., 2007). Various amounts of GO_x (0.20, 0.40, 0.60, 0.80, 1.0, 1.2, and 1.5%, w/v) were immobilized on the onion inner membrane to determine its optimal response sensitivity. The sensitivity, that is the decrease in dissolved O_2, was measured by exposing the biosensor to 0.20 mM glucose in phosphate buffer at pH 7.4. In general, it was found that the sensitivity of the glucose biosensor increased with the increase of enzyme loading on the onion inner membrane. However, not much increase in the sensitivity of the biosensor was observed when the concentration of GO_x was higher than 0.80% (w/v). It is obvious that larger enzyme loading will improve sensitivity as more GO_x molecules are immobilized on the onion membrane. However, when O-HTCC NP is overloaded with enzyme, the excess GO_x will not bind with O-HTCC NP; as a result, they just simply leach out of the membrane. As such, the optimal concentration for enzyme immobilization was chosen as 0.80% (w/v) in this work.

3.5. Comparison of performance of GO_x immobilized onion membrane using O-HTCC NP, chitosan and glutaraldehyde

Glutaraldehyde and chitosan are common and useful cross-linking agents for immobilizing enzymes on solid substrates (Wu et al., 2004; Yang et al., 2006; Wen et al., 2007; Zhang et al., 2007). In order to compare the use of O-HTCC NP, chitosan and glutaraldehyde, biosensors fabricated from GO_x-immobilized onion membranes using 0.3% (w/v) O-HTCC NP (20 μL), 0.3% (w/v) chitosan (20 μL) and 2.0% (w/v) glutaraldehyde (10 μL) were exposed to 0.20 mM glucose (pH 7.4), respectively as depicted in Fig. 3. It was observed that the glucose biosensor fabricated with O-HTCC NP showed higher sensitivity than that of chitosan and glutaraldehyde. It is plausible that glutaraldehyde could deactivate the immobilized enzyme (Sirkar et al., 2000) whereas the enzyme immobilization capability of chitosan is reduced because of its loss of cationic nature at >pH 6.5 (Jae et al., 2003). In addition, the glucose biosensor fabricated with O-HTCC NP possessed faster response time (70–90 s) than that of glutaraldehyde (response time > 100 s). The membrane with glutaraldehyde retained 60% of its original activity after 7-day of storage whereas the membrane with O-HTCC NP still maintained 90% of its initial activity after 3-week of storage at 4 °C. The shell-life of our GO_x/O-HTCC NP membrane is comparable to a biosensor reported in a recent article (Kumar and D'Souza, 2009). These results demonstrate that O-HTCC NP is a better enzyme immobilization matrix than that of glutaraldehyde and chitosan.

3.6. Effect of pH

The response of the glucose biosensor depends on the activity of immobilized GO_x which is associated with the pH of the ambient environment. The effect of pH on the performance of biosensor was studied at various pH (4.0–10.0) phosphate buffer solutions when the biosensor was subject to 0.20 mM glucose standards as shown in Fig. S2. The biosensor exhibited relatively stable and higher sensitivity at pH 6.0–8.0 and maximum sensitivity at pH 7.4. The response decreased at lower (4.0–5.0) or higher (9.0–10.0) pH, attributing to the decrease in enzyme activity of the immobilized GO_x/O-HTCC NP. In fact, similar observations have reported the drop of the activity of free and immobilized GO_x on the poly(*N*-vinylimidazole) hydrogels at higher pH values (>8.0) (Pekel et al., 2003). As a result, the optimal pH was chosen at 7.4 in this study. In addition, it was observed that the response of the biosensor slightly dropped when the pH was 5.0 or 9.0, inferring that our biosensor can function satisfactorily over the pH range from 5.0 to 9.0 and has a relatively broad pH working range.

3.7. Effect of temperature

The analytical performance of enzyme-immobilized onion inner membrane is temperature-dependent. Lower or higher temperature would influence not only the enzyme activity but also shorten the lifetime of glucose biosensor. The activity of an immobilized enzyme is governed by the kinetics of the enzymatic reaction. The reaction rate becomes faster with the increase in working temperature. The response sensitivity for glucose biosensor on exposure to 0.20 mM glucose at various temperatures was thus studied. The sensitivity of biosensor increases with the increase in temperature as depicted in Fig. S3. There was a significant increase in the sensitivity from 10 to 25 °C and reached the highest at 35 °C. However, further increase in temperature (>35 °C) would cause a decrease in

Table 1
Comparison of analytical performance of some recent biosensors based on natural biomaterials.

Immobilized enzyme	Eggshell membrane			Bamboo inner shell membrane	Onion inner membrane
	Uricase	Glucose oxidase	Alcohol oxidase	Glucose oxidase	Glucose oxidase
Gelating agent	Glutaraldehyde	Glutaraldehyde	Chitosan	Glutaraldehyde	O-HTCC NP
Response time	<100 s	100 s	60 s	–	70 s
Linear range	4.0 μM–0.64 mM	10 μM–1.3 mM	60 μM–0.80 mM	0.0–0.60 mM	0.0–0.60 mM
Detection limit	2.0 μM	–	30 μM	58 μM	50 μM
Reproducibility (between membranes)	3.1% (n = 7)	3.3% (n = 7)	3.2% (n = 11)	3.0% (n = 10)	3.2% (n = 10)
Ref.	Zhang et al. (2007)	Wu et al. (2004)	Wen et al. (2007)	Yang et al. (2006)	Present work

sensitivity. In general, the sensitivity changed little in the range of 25–35 °C. Even though O_2 has lower solubility in solution at higher temperatures, the immobilized enzyme can still maintain higher activities at higher temperatures. The decrease in sensitivity at above 35 °C is possibly attributing to denature of the enzyme and leakage of the enzyme molecules from the membrane. At elevated temperature, the tertiary structure of the protein is disrupted and the enzyme essentially unravels and is inactive (Johnson, 2002). In addition, the enzyme molecules leach out of the membrane as the electrostatic interaction between the enzyme molecules and O-HTTC is weakened (Rauf et al., 2006). Thus, for practical reasons room temperature is recommended to prolong the lifetime of the biosensor (Choi, 2005).

3.8. Effect of buffer concentration

Most biosensors with immobilized enzyme are influenced negatively in a higher ionic strength medium (Wang et al., 2008). So the response sensitivity of the biosensor may vary with different buffer concentrations. The effect of buffer concentration (10–500 mM) at pH 7.4 on the sensitivity of the biosensor was studied upon exposure to 0.20 mM glucose and the results are depicted in Fig. S4. The sensitivity increased with the increase in buffer concentration and reached the highest value at 200 mM. Further increase in buffer would cause a decrease in sensitivity. It is possible that too high buffer concentration can cause disruption of the electrostatic interaction between GO_x and O-HTCC by charge screening effect. As such, some enzyme molecules leached out from the GO_x/O-HTCC membrane. Another possible reason is the decrease in enzyme activity in high ionic strength medium. In essence, the optimal phosphate buffer concentration was chosen as 200 mM for this study.

3.9. Analytical figures of merit of glucose biosensor

The response time (t_{95}) is defined as the time taken to obtain a 95% steady-state signal when a biosensor is exposed to a given concentration of standard solution (Wu et al., 2005). In this work, the glucose biosensor could obtain 95% of the steady signal within 70 s. The inset of Fig. 2 shows the linear response range of the biosensor from 0.00 to 0.60 mM glucose with the regression equation: decrease in dissolved O_2 (mg/L) = 12.15 [glucose] (mM) + 0.2488 with r^2 = 0.9920. In addition, the detection limit of the biosensor was determined as 50 μM at a signal-to-noise ratio of 3.

The reproducibility of fabricating the enzyme-immobilized onion membranes was assessed. Ten enzyme-immobilized membranes were prepared and each was employed to determine the response to 0.20 mM glucose solution. The relative standard deviation (RSD) was 3.2% (n = 10), demonstrating that the biosensor fabrication process is highly reproducible. In addition, it was found that the enzyme activity of a GO_x/O-HTCC NP membrane could maintain 90% of its initial activity after 100 repeat uses. It can say that the reusability of GO_x/O-HTCC NP onion membrane is better than synthetic polymers (Kumar and D'Souza, 2008).

Table 1 summarizes the analytical performance of the recent biosensors using some biomaterials. Our GO_x/O-HTCC onion membrane shows comparable performance with other biomaterials. However, it is observed that enzymes using glutaraldehyde as gelating agent will produce longer response time as compared to chitosan. In brief, the glucose biosensor has the promising features of good reproducibility, reusability, low cost, simple design and ease of operation.

3.10. Interference study

The interference tests were carried out with some common potential interfering substances in real samples by exposing the biosensor to interferents in 200 mM phosphate buffer at pH 7.4. The decrease in the O_2 level was calculated as glucose concentration equivalence, *i.e.*, the concentration of glucose that can produce the same decrease as the interferents. The results are displayed in Table 2. Acetic acid, lactic acid, propionic acid, butyric acid, folic acid, methanol, glycine, DL-α-alanine and DL-cysteine caused only slight interferences on the response of the biosensor. Ascorbic acid, however, could produce more significant interference since it is a reducing agent which can be easily oxidized by dissolved O_2 in the sample. But this effect can be minimized by air-saturating the sample solution for 2 h before analysis.

3.11. Sample analysis

The glucose biosensor was employed to determine the glucose concentration in real samples including orange juice, red wine and tea drink. The samples were diluted 100–200-fold by a phosphate buffer (pH 7.4) to yield the test sample solutions whose glucose concentrations fall in the working range of the calibration curve. Dilution of samples can also lower down the concentration of any interferents in the samples. Moreover, the glucose contents of the samples were simultaneously analyzed by a standard spectrophotometric method (Sigma Diagnostics, 1995). All the results are

Table 2
Effect of potential interferents on the response sensitivity of glucose biosensor.

Interferent	Concentration (mM)	Decrease in oxygen level equivalent to glucose (mM)
Acetic acid	2.0	0.023
Ascorbic acid	2.0	0.243
Lactic acid	2.0	−0.018
Propionic acid	2.0	0.042
Butyric acid	2.0	−0.021
Folic acid	2.0	0.042
Citric acid	2.0	0.051
Methanol	0.1	0.054
Glycine	2.0	0.034
DL-α-Alanine	2.0	−0.078
DL-Cysteine	2.0	0.059

Table 3
Determination of glucose in real samples and the recovery test using the proposed glucose biosensor and a spectrophotometric method.

Sample	Concentration of glucose (mM)		Concentration of glucose added (mM)	Concentration of glucose found[a] (mM)	Recovery (%)	RSD (%)
	Proposed method[a]	Spectrophotometric method[a]				
Red wine	42.6 ± 1.3	40.2 ± 1.1	0.100	0.103	103	1.35
			0.200	0.194	97	1.27
			0.300	0.311	104	1.63
Orange juice	112.2 ± 6.8	115.4 ± 5.3	0.100	0.098	98	0.98
			0.200	0.219	110	2.03
			0.300	0.317	106	1.52
Tea drink	21.8 ± 0.9	23.5 ± 0.6	0.100	0.104	104	1.92
			0.200	0.203	102	1.33
			0.300	0.307	102	1.25

[a] Average value from triplicate.

summarized in Table 3. There is no significant difference between the values obtained from these two methods.

The recovery tests for glucose were performed by adding various known amounts of glucose in the sample solutions. The amounts of added glucose were then evaluated by using the proposed glucose biosensor. The results of recovery test are displayed in Table 3. These results demonstrate that the proposed glucose biosensor offers an excellent, accurate and precise method for the determination of glucose in real samples.

4. Conclusion

A glucose biosensor based on a GO_x/O-HTCC NP-immobilized onion membrane has been successfully constructed and applied to determine the concentration of glucose in real samples. The scanning electron micrographs reveal the microstructure of the onion inner membrane and show that GO_x/O-HTCC NP is successfully immobilized onto the onion inner membrane. The promising features of the biosensor are good reproducibility, repeatability, reusability, low cost, simple design, and ease of operation. In addition, it exhibits good stability with shelf-life of at least 3 weeks. Our work proves that onion inner membrane material is a promising enzyme immobilization platform for the fabrication of biosensor and O-HTCC NP is an effective material for entrapment of enzyme.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.02.033.

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