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Use of the ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate in the optimization of the performance of amperometric biosensors

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ABSTRACT

Cholesterol and Glucose biosensors were prepared by deposition of cholesterol oxidase and glucose oxidase respectively entrapped within a U.V. crosslinked poly (2-hydroxyethyl methacrylate), [p(HEMA)] hydrogel containing tetraethyleneglycol diacrylate onto a platinum electrode. The performance characteristics for the Pt/p(HEMA)/ChOx biosensor were as follows: Response time of 50 seconds at all cholesterol concentrations investigated, detection limit of 3.0×10^{-6} M, liner range of $3 \times 10^{-5} - 1.6 \times 10^{-2}$ M of cholesterol, sensitivity of 56510μA/M, working potential of +450mV with a regression value of 0.986 for the calibration curve (y = 5.651x). The Pt/p(HEMA)/GOx optimized sensor parameters were: an enzyme loading of 1365 units GOx/cm^2; a HEMA:TEGDA ratio of 2.75:1% vol.; a film thickness of 0.2mm; a stirring speed of 450 r.p.m, and a temperature optimum of 50°C, a linear response over a glucose concentration range of 1×10^{-5} to 1×10^{-3} mM (r = 0.994), with a detection limit of 6.0×10^{-6} M, with a response time of 45 seconds at all glucose concentrations investigated. These biosensors retained an average of 90% of initial enzyme activity when stored in the ionic liquid, [bmim][BF_4], at 4 °C after six months.

KEYWORDS

Cholesterol biosensor;
Amperometric detection;
Ionic liquids;
Glucose oxidase;
Cholesterol oxidase;
Poly[HEMA].

INTRODUCTION

As part of an ongoing regimen into investigations of the stabilization of enzymes in ionic liquid solvent media, the preparation of oxidoreductase based amperometric biosensors using the ionic liquid [bmim][BF_4] as the substrate matrices were executed. Such sensors have found wide use in clinical analyses due to their increased sensitivity, selectivity, stability, reproducibility and most significantly, their compact size[1]. Previous work by[2] has demonstrated the stabilization of oxidoreductase enzymes in sensors possessing a novel amino-acid squarate mediator. This current body of work investigates the stabilization and enhancement of activity of such enzymes without the presence of a mediator, in an ionic liquid substrate matrix.

The increasing incidences of coronary heart disease (CHD) are a major cause of death amongst the population, and two of the most important risk factors identified are hypercholesterolemia and elevated blood glucose levels[3]. The regular estimation of cholesterol and glucose levels in blood is vital for the management and prevention of CHD. Advances in biosensor design are appearing at a high rate as these devices play increasingly important roles in our daily lives.

A precise and rapid determination of cholesterol and glucose concentrations in clinical, biological and chemical samples is of considerable interest to the clinical, biological as well as the chemical communities[4]. The two techniques widely employed for this encompass spectroscopic (fluorescence and colorimetry) and electrochemical methods of analysis. The mode of operation of these sensors is based on electron transfer between an electrode and the immobilized oxidoreductase enzyme[5], wherein the enzyme recognizes target molecules with the direct transduction of the reaction rate into a current.

Various enzymatic biosensors based on immobilized cholesterol oxidase (ChOx) have been reported in the literature[6]. Cholesterol can be analyzed indirectly by monitoring hydrogen peroxide generated in enzymatic reactions using voltammetric and amperometric methods. However, various issues arise due to the effect of interferents such as ascorbic acid, uric acid and acetaminophen, which are co-oxidized along with cholesterol. In addition, the high working potential of +600mV causes a decrease in the sensitivity of such cholesterol sensors. Mediators or coenzymes such as ferrocyanide, ferrocene derivatives, and quinones have been used to alleviate this problem[7], but excessive diffusion may result in mediator loss from the film to the bulk solution, especially if it has a small molecular weight[8]. The most commercialized and popular biosensors employ a mediator compound for the detection of blood sugar levels of diabetic patients on a daily basis[9]. However, the sensitivity and application of this approach has been limited to some extent due to the lack of a simple approach to immobilize and stabilize the enzyme. Furthermore the difficulty of direct electron transfer between a redox enzyme and an electrode, due to the thick insulating protein layer surrounding the active centre[4] of the enzyme, severely limits this type of sensor.

Consequently there remains a need to develop cholesterol and glucose biosensors that take advantage of the chemical properties of the enzyme.

One approach to circumvent this property of the enzyme has been to use "redox hydrogels", which consist of a highly hydrophilic polymer backbone. Such gels enable the current densities to substantially increase resulting in reduced response times of the resultant biosensors. Additionally the film properties can be modulated by adjusting the polymerization parameters and these can be achieved changing the monomer concentration, applied potential, potential profile, temperature, counter anion and the nature of the solvent[10]. As a result it is beneficial to develop biosensors which are capable of producing high current responses, with a minimal response time, whist bearing the additional advantages of ease of preparation, reproducibility and high stability, selectivity and sensitivity. The development of redox hydrogel based electrochemical biosensors has been extensively explored[11]. Many of these sensors have also incorporated the inclusion of electrically conducting polymers, such as poly(pyrrole), in the matrix that suppress the biological interferents, ascorbic acid and urea, allowing enhancement of the sensitivity of the biosensors[11].

Mindful of these requirements we considered the use of ionic liquids on the basis of their good electrochemical properties, which include high conductivity and a wide electrochemical window. In particular, the wide electrochemical window promised the potential use of these ionic liquids as solvents for electrochemical investigation. At the outset we utilized 1,3-dialkylimidazolium salts $[RR'Im]^+X^-$ for this study, as they are amongst the most stable and conductive ionic liquids[12]. Furthermore there had been few literature reports[13] that detail the use of ionic liquids for the development of amperometric biosensors.

Informed by these findings we embarked on an investigation of biosensors that were based on Pt/[p(HEMA)]/GOx as well as a Pt/[p(HEMA)]/CHOx that utilized 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF_4]), as the solvent for D-glucose and cholesterol quantitation respectively.

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EXPERIMENTAL

Materials

Cholesterol oxidase (ChOx, E.C. 1.1.3.6 from Pseudomonas fluorescens, 4.8 units/mg), Glucose oxidase (GOx), (Type VII, E.C. 1.1.3.4. from *aspergillus niger*, 128,000 units/g solid), cholesterol, glucose, and 1-butyl-3-methylimidazolium tetrafluoroborate were obtained from Sigma Chemical Co. (St. Louis, MO.). Hydroxyethyl methacrylate (HEMA) was obtained from Polysciences Inc. (Warrington, PA.); the crosslinking agent tetraethyleneglycol diacrylate (TEGDA), inhibitor remover columns (250ml capacity; packed with alumina to remove phenolic inhibitor), platinum foil (0.1mm thick), platinum wire (0.1mm diameter), and dimethoxyphenyl acetophenone (DMPA) were all purchased from Aldrich Chemical Co. (Milwaukee, WI.). The HEMA monomer was vacuum distilled (1.3mmHg, 80°C) before use. All other reagents used were of the analytical reagent grade (and distilled or recrystallized as necessary prior to use) and were obtained from BDH (Poole, UK.). Cyclic voltammetry and time based electrochemical experiments were carried out using the BAS 100B Electrochemical Analyzer.

Cleaning and preparation of pt/p(HEMA)/CHOx electrode

Platinum electrodes (10 x 15mm) were cleaned using the following standard protocol[14]: washing for one minute each in boiling trichloroethylene, then boiling acetone, followed by sonication (1 min.) in propan-2-ol and finally in deionized (DI) water. Subsequently cathodic cleaning (-1.2V to -2.0V vs. SCE for ten cycles (ten seconds per cycle) in 0.1 M phosphate buffer (NaH_2PO_4) containing KCl (0.01 M), pH = 7.2) was carried out followed by immersion in the cleaning agent, NH_4OH:H_2O_2:H_2O/ 1:1:5 $^{v}/_{v}$, for ten seconds at 60ºC. The electrode was then dried for thirty minutes at 60ºC. Activation of the cleaned electrode was carried out by: immersion in 10mL of a 1%w/v sodium dithionite solution, for 30 minutes at 56ºC, followed by immersion in 10mL of a 2.5% solution of $K_2Cr_2O_7$ in 15% HNO_3 for 30 minutes at 56ºC then rinsed with DI water (25 ml) and oven dried (30 minutes) at 60ºC.

Following this, an area (0.25cm^2) was demarcated on the clean electrode surfaces using transparent scotch tape. The cleaned Pt electrode surfaces were treated with 5mL of a 1% 3-aminopropyltrimethoxysilane (γ-APS) in dry toluene, (30 mins.) with agitation, and subsequently cured at 110°C for 10 minutes. The counter (platinum) electrode was cleaned via flaming until red hot and then washing with acetone (10 ml) and isopropanol (10 ml) successively.

Preparation of the pt/p(HEMA)/CHOx sensor

Cholesterol oxidase, **(**40 mg), was dissolved in inhibitor free HEMA (55µL) and TEGDA (20µL). The photoinitiator, DMPA (0.5mg), was added to the mixture and purged with dry argon for 5 mins. The polymerization chamber was also degassed for thirty minutes with argon. Depending on the desired area of the exposed electroactive window, the requisite volume of the glucose oxidase/monomer mixture was applied to the exposed platinum window. The functionalized windowed electrodes were placed flat in the UV polymerizing chamber and irradiated for 45 minutes at 366nm under an argon atmosphere. The resultant polymeric hydrogel film was washed using the ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate (10 ml) and stored in this same ionic liquid at 4°C.

Calibration of the pt/p(HEMA)/CHOx biosensor using cholesterol

To evaluate this sensor in terms of its cholesterol response, the electrode was made into the working electrode in a three-compartment cell with a platinum coil counter electrode and a Ag/AgCl reference electrode in 5mL of the ionic liquid [bmim][BF_4] . The response of this sensor to cholesterol concentrations in the range 0.01-16 mM were investigated and current responses obtained. A calibration plot was generated utilizing the standard addition method.

Storage stability studies

The performance of sensors stored under three different conditions was investigated. Sensors were stored in the ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate at 4°C, phosphate buffer (0.05M, pH 7.0) at 4°C**,** and in a desiccator at 4°C. The amperometric response of these electrodes was monitored once per week (using the ionic liquid as the reaction medium) for a period of six months at two D-glucose, (2mM and 16mM), concentrations.

Characterization of the working pt electrode

The working platinum electrode was characterized at each step of its construction in order to ensure that the current response generated was due to the electrochemical breakdown of the hydrogen peroxide produced as a result of the reaction between the enzyme and the substrate D-glucose and not due to any other component such as the HEMA, crosslinker or the bare platinum surface.

The sensor was monitored by cyclic voltammetry at all of the stages of its preparation by sweeping through a potential range of -1000mV to 1000mV. The stages encompassed the bare platinum surface, the Pt surface containing HEMA and crosslinker, and the Pt surface containing HEMA and crosslinker with immobilized enzyme, glucose oxidase. The cyclic voltammograms were recorded in the neat ionic liquid in addition to the ionic liquid containing varying concentrations of glucose (2-10mM). The electrode was made into the working electrode in a one-compartment cell with a platinum coil counter electrode and a Ag/AgCl reference electrode in 5mL of the ionic liquid.

Optimization of the prepared pt/p(HEMA)/GOx electrode

The Pt/p(HEMA)/GOx biosensor constructed (following the same procedure as for the Pt/p(HEMA)/CHOx sensor, except replacing cholesterol oxidase with glucose oxidase) was optimized, separately, with respect to the following parameters: enzyme loading, polymer film composition, film thickness, temperature and stirring speed.

Optimization of enzyme loading

The amount of enzyme dissolved in the formulation in preparation of the gel was varied. Four sets of gels were prepared, each containing 13.75μL of HEMA, 5μL of TEGDA and 0.125mg of DMPA. To each batch was added 2.5, 5.0, 10.0 and 12.5mg of GOx respectively (resulting in electrodes bearing 341, 683, 1365 and 1707 units of enzyme/cm^2). The resultant formulations (5μL) was offered to defined electroactive windows of four different Pt electrodes, and polymerization carried out as before. Each electrode was made into the working electrode in a one-compartment cell with a platinum coil counter electrode and a Ag/AgCl reference electrode in 5mL of ionic liquid. A constant potential of +750mV was applied and a blank current reading obtained in order to compensate for background voltage. For each working electrode, glucose calibration curves were generated over the concentration range of 2-12mM.

Optimization of polymer film composition

The percentage of crosslinker incorporated into the gel for preparation of the working p(HEMA)/GOx electrode was varied. Glucose oxidase (10 mg, 128,000 units/g solid) was dissolved into each of four monomer formulations–HEMA (50, 55, 65 or 70μL) and TEGDA (25, 20, 10 or 5μL) with 0.5mg DMPA, such that the final volume of solution was 75μL.

Polymerization of each formulation onto demarcated areas on the working electrode surface was subsequently effected. Each biosensor formulation was analyzed in terms of its amperometric response to glucose by generation of glucose calibration curves over the concentration range of 2-12mM, using the standard addition method. A constant potential of +750mV was applied and a blank current reading obtained in order to compensate for any background signals that may be generated.

Optimization of polymer film thickness

The variation in sensor film thickness was achieved by adjusting the volume of the formulation applied to the platinum sensor window. To the defined electroactive areas of 0.25cm^2 , 1.0, 2.5, 5.0 or 7.0μL, of the prepared formulation was applied, resulting in electrodes with film thicknesses of 0.04, 0.1, 0.2 and 0.3mm respectively. These film thicknesses were calculated by dividing the volume of the applied gel by the surface area of the electroactive portion of the platinum. A constant potential of +750mV was applied and a blank current reading obtained in order to compensate for background current. For each working electrode, glucose calibration curves were determined over the concentration range of 2-12mM.

Optimization of temperature

The temperature of the system (ionic liquid containing glucose, as well as the working, counter and reference electrode) was varied over the temperature range 10ºC to 60ºC at 5 ºC intervals using a

thermostated circulating water bath.

Fixed concentrations of substrate (2 and 16mM) were added to 5mL of the ionic liquid, and the subsequent oxidation at +750mV was monitored amperometrically at each temperature interval in ascending and then descending order, and average values calculated.

Stirring speeds study

The response of the optimized Pt/p(HEMA)/GOx biosensor to two concentrations of glucose, $1x10^{-5}$ M and 2×10^{-3} M, at three different stirring speeds of 200, 450 and 800 r.p.m was investigated. Graphs of current vs. time were plotted for each speed and compared with the unstirred solution.

RESULTS AND DISCUSSION

Performance of the pt/p(HEMA)/ChOx biosensor

The performance characteristics of this sensor are summarized in TABLE 1. Brahim[15] described the amperometric determination of cholesterol utilizing a biosensor of cholesterol oxidase contained within a polypyrrole-hydrogel membrane, with a 0.1M, pH 7.0 phosphate buffer solvent medium. A linear range of 5×10^{-4} to 1.5×10^{-2} M cholesterol, detection limit of 120μM and a response time of 30 seconds were obtained for this formulated sensor. In our study, which uses an ionic liquid as the solvent medium, a larger linear range of 3×10^{-5} - 1.6×10^{-2} M with a detection limit of 3.0×10^{-6} M was achieved. The response time however, was increased to 50 seconds (TABLE 2). The successful use of [bmim][BF_4] as a solvent medium in the amperometric detection of cholesterol indicates its ability to function successfully as a non-aqueous solvent in electrochemical systems.

Characterization of the working GOx/Pt electrode

Cyclic voltammograms of the bare Pt electrode, the Pt/p(HEMA) and the Pt/p(HEMA)/GOx electrodes in the ionic liquid, [bmim][BF_4], in the absence and presence of the substrate glucose are shown in Figures 1-3.

From the characterization studies (Figures. 1-3), it was concluded that:

Once a background current is noted and corrections applied to the current values generated, it can be confidently assumed that the enzyme is the only com-

TABLE 1 : Performance characteristics of the Pt/p(HEMA)/ChOx electrode using [bmim][BF_4] as the substrate medium

Regression Equation	Response time/s	Detection limit	Linear Range	Solvent medium	Sensitivity	R^2	Working Potential/ mV
y = 5.651x	50	3.0×10^{-6} M	3×10^{-5} - 1.6×10^{-2} M	[bmim][BF_4]	5651.0μA/M	0.986	+450

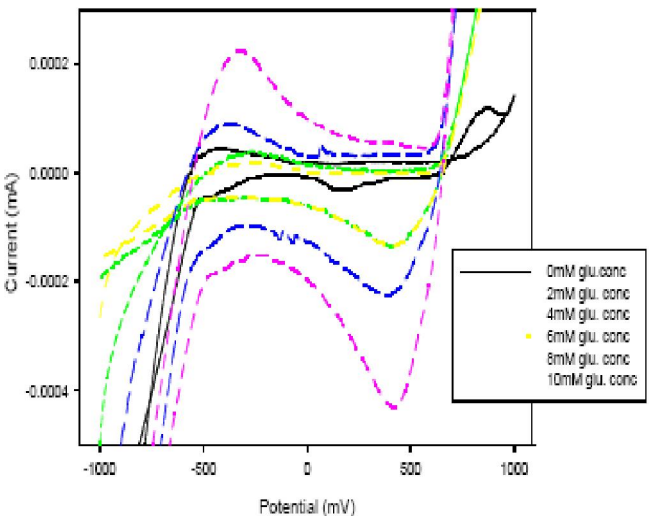

Figure 1 : CV's for bare Pt electrode at verying glucose concertration @ 25°C.

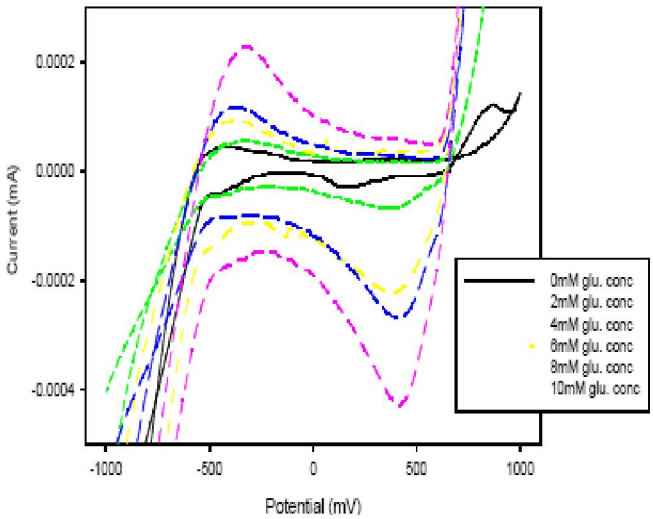

Figure 2 : CV's for Pt electrode bearing HEMA and TEGDA at verying glucose concertration @ 25°C.

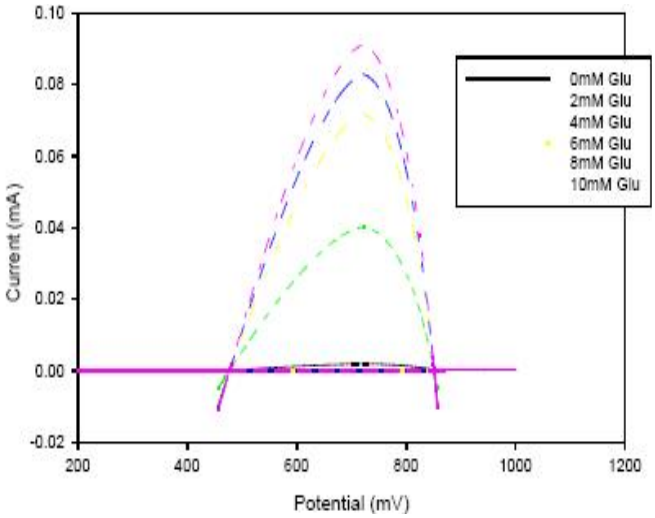

Figure 3 : CV's for complete working electrode (Pt/p(HEMA)/ GOx)- at verying glucose concertration @ 25C.

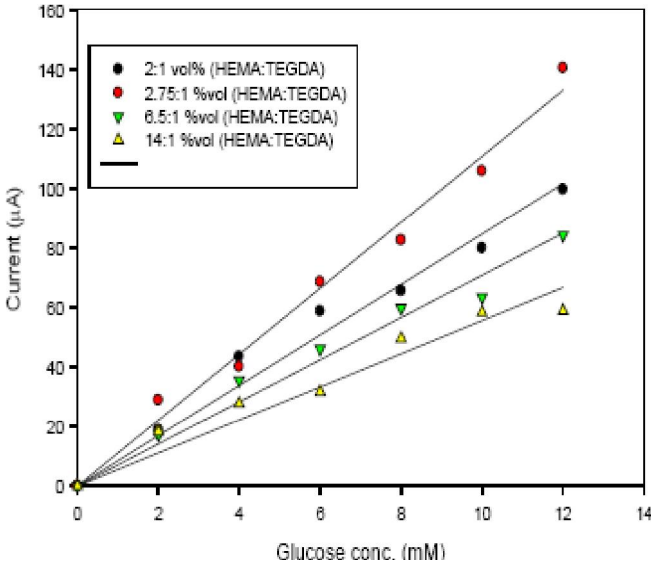

Figure 4 : Calibration plots for the monomer formulation used in the gel preparation over verying glucose concertration @ 25°C.

ponent contributing to the evolution of hydrogen peroxide.

The optimal potential at which the decomposition of hydrogen peroxide is monitored is +750mV.

Any small peaks generated other than that arising from the decomposition of hydrogen peroxide are simply a function of the system (ionic liquid, metal e.t.c.) and do not affect the oxidation current.

At the offset, the behavior of the platinum electrode was monitored as above, only this time in aqueous phosphate buffer, and no significant differences were noticed in the shape of the resulting CVs, save for the magnitude of the current responses generated (which was ten times higher in the ionic liquid using the complete working electrode).

Optimization of the monomer formulation

Four different ratios of HEMA:TEGDA (2:1, 2.75:1, 6.5:1 and 14:1% vol) were used in the preparation of the glucose sensors, giving calibration equations of $y = 0.0085x$, $r^2 = 0.9735$; $y = 0.0111x$, $r^2 = 0.9876$; $y = 0.0071x$, $r^2 = 0.9719$; and $y = 0.0056x$, $r^2 = 0.9381$ respectively, Figure 4. From these calibration plots generated, it was found that 2.75:1% vol. of HEMA:TEGDA gave the widest linear response to glucose standards (2-12mM), along with the greatest sensitivity (11.1μA/mM) and fastest response times of 45 seconds, (where response time is defined as the time to achieve ninety-five percent of equilibration current), compared to the other three biosensors constructed.

As the amount of TEGDA in the monomer formulation increased, the response time generally increased as did the magnitude of the current generated. The chosen formulation was a compromise between linear range, response time, current magnitude and physical properties of the gel.

Increased amount of the crosslinker (TEGDA) in the formulation gave a higher degree of crosslinking of the gel matrix. The current response obtained from these biosensors reflected their physical properties in that small amounts of the crosslinker (14:1 % vol. HEMA:TEGDA) caused the gel to be runny resulting in poor adhesion and uniformity on the metal surface. Consequently the responses of these three electrodes were slightly erratic. On the other hand, too high amounts of the crosslinker (2:1 % vol. HEMA:TEGDA) caused the gel to be very rigid and tough, also affording poor adhesion to the metal and ready detachment from the surface. Also the high viscosity of such gels resulted in localization and access to the active site of the immobilized enzyme was restricted and gave an inconsistent response.

On the basis of these studies we determined the best formulation as having a composition of 2.75:1% vol. of HEMA:TEGDA

Optimization of enzyme loading

Four different enzyme loadings (341, 683, 1365

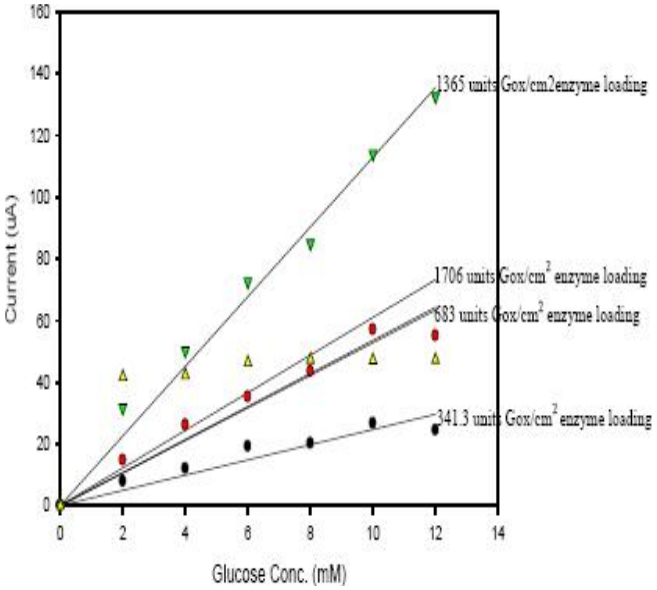

Figure 5 : Glucose calibration for sensors prepared using the varying enzyme loadings incorporated into gel formulation @ 25°C, unstirred in [bmim][BF4].

and 1706 GOx/cm^2) were used in the preparation of glucose sensors, giving calibration equations of y = 0.0025x, r^2 = 0.8862; y = 0.0053x, r^2 = 0.9463; y = 0.0113x, r^2 = 0.9869; and y = 0.0052x, r^2 = -0.182 respectively, Figure 5. We found that 1365 units GOx/ cm^2 afforded the optimal enzyme loading resulting in the widest linear response to glucose standards (2-12mM), as well as the greatest sensitivity (11.3μA/mM) and fastest response times of 45 seconds compared to the other three biosensors that were constructed. The linear range of this biosensor (1365 units GOx/cm^2) covers the physiological glucose levels found in non-diabetic individuals (4.5–6.0mM). As enzyme loading increases from 341 to 683 then to 1365 Gox/cm^2, the current responses increase as well; a higher loading of 1706 GOx/cm^2 demonstrated a marked decrease in magnitude of the current responses, as well as loss of linearity in response. The response times of the sensor decreased as enzyme loading is increased, from 60 seconds to 45 seconds. However, enzyme loading above 1365 GOx/cm^2 caused an increase in the response time, reaching up to seventy seconds as the loading reaches 1706 units. Increasing the amount of immobilized enzyme per unit area on the biosensor increased the rate of substrate binding and product production. This allowed the biosensor to detect small changes in [S], as well displaying increased reproducibility of the results. At loadings greater than 1365 units GOx/ cm^2 of the enzyme resulted in sensors which gave a non-linear response and a marked low precision. This maybe due to the formation of multi layers of enzyme stacked upon each other on the electrode surfaces that are readily desorbed. Furthermore the resulting steric hindrance may prevent binding at the active site. Consequently 1365 units GOx/ cm^2 were employed in the gel formulation, which is comparable to other glucose biosensors employing GOx[16].

Optimization of polymer film thickness

Deposition of polymer films (0.04, 0.10, 0.20 and 0.40mm, thick) onto the electrode surface gave biosensors which produced the following calibration equations on analysis: y = 0.002x, r^2 = 0.9442; y = 0.0045x, r^2 = 0.7521; y = 0.0113x, r^2 = 0.9852; and y = 0.0011x, r^2 = -0.1606 respectively, Figure 6. This data enabled us to determine that the best working film was 0.2mm thick and resulted in a biosensor that displayed the best responses, with a sensitivity of 11.3 μA/mM, the widest linear range (2-12mM) and fastest response time of 45s. The response time of the biosensor was dependant on the film thickness as this controlled the rate of diffusion of hydrogen peroxide and the transfer of substrate from the ionic liquid to the entrapped enzyme active site. As the film thickness increased, the response time of the sensor also increased,

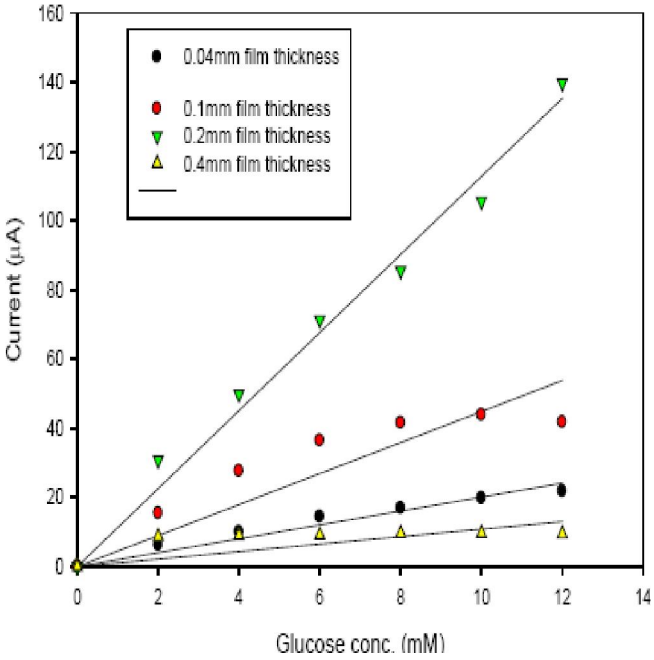

Figure 6 : Glucose calibration plots for sensors of varying polymer film thickness.

from 30s to 70s. A thickness of 0.2mm gave a response time of 45 seconds, with the largest linear range and highest current responses. As a result, a compromise was made in choosing the thickness of the deposited film. Generally, response times in aqueous buffer have been reported to be lower, by about 10 seconds[17], possibly due to the lower viscosity of the medium. However, the enhanced current responses in the ionic liquid compensated for this limitation in buffer, as the magnitude of the current responses in ionic liquid were up to ten times higher than those in aqueous buffer.

Temperature studies

The activity of the thus far optimized biosensors was investigated as a function of temperature (10-60°C). Amperometric responses were obtained at two concentrations of D-glucose (2 and 16mM), whilst increasing **and** decreasing the temperature, and average values utilized, Figure 7. We observed that there were no statistical significant differences (95% C.L.) between the current values obtained on increasing the temperature and then decreasing the temperature.

The optimum temperature was found to be 50°C, compared to the free enzyme optimum[18] of 35°C. This optimum is higher than what is reported by other workers in this field[19]. As the temperature of the system exceeded optimum, there was a gradual decrease in the response of the biosensor. This may be a reflection of the inherent resistance of [p(HEMA)] hydrogels to high temperatures[18], thus providing high retention of biocatalytic activity of immobilized enzymes. Additionally the ionic liquid provided a highly stable microenvironment for the enzyme. This was demonstrated by two key observations:

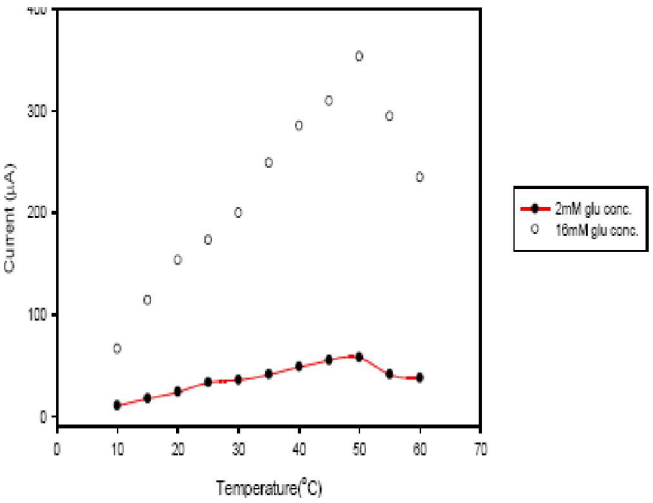

Figure 7 : Temperature profiles for complete working Pt/p(HEMA)/GOx biosensor Conditions were – monomer composition of 2.75:1%vol (HEMA: TEGDA), film thickness = 0.2mm, with an enzyme loading of 1365 units/cm2

The current values generated upon increase of temperature and then upon decrease of temperature bore no significant differences, at both glucose concentrations investigated. This demonstrated retention of configuration and that permanent denaturation had not occurred typified by an aqueous system.

The observed optimum temperature was well above the normal value in aqueous system possibly due to the ionic liquid stabilizing the three dimensional configuration of the enzyme, resulting in a wider window of temperature ranges within which the enzyme remains active. Denaturation, which normally arises from the conformational changes due to increased temperature, was apparently prevented.

Stirring speeds study

In order to determine the diffusion limits for the operation of the GOx-hydrogel biosensor we investigated its response at two glucose concentrations and at stirring speeds of 200, 400, and 800 rpm, TABLE 2. The results of this study led us to conclude that there was little to no differences in the response times amongst the unstirred and stirred solutions at all three speeds investigated for both the high and low glucose concentrations. These findings suggest that the response time

TABLE 2 : Current values generated at varying stirring speeds and corresponding equilibration times for two substrate concentrations

		STEADY STATE (CORRECTED) CURRENT ($x10^{-3}$ mA)			
GLUCOSE CONC (M)		$1x10^{-5}$		$2x10^{-3}$	
CONDITION		stirred	unstirred	stirred	unstirred
Stirring Speed (r.p.m.)	200	0.087	0.089	31.09	31.11
	Response Time(s)	46	46	45	45
	450	0.089	0.088	32.18	31.99
	Response Time(s)	47	45	45	44
	800	0.088	0.091	31.04	30.19
	Response Time(s)	44	43	43	44

is not limited by bulk transfer of substrate to enzyme, but rather by the breakdown of the [ES] complex. The observed response time of about 45s was comparable to that reported by other workers in the area[20].

Calibration of optimized biosensor using D-Glucose

The optimized Pt/p(HEMA)/GOx biosensor was calibrated with respect to its amperometric current response on addition of glucose standards, over a concentration range of 1 x 10^{-5} to 16.0 M, Figure 8. The biosensor gave a linear response within this concentration range, with a calibration equation y = 8.698x, r^2 = 0.994. The dynamic linear range of the optimized sensor was found to be 0.01 to 16mM, with a sensitivity of 8698.0μA/mM, and a detection limit of 6.0 x 10^{-6} M (detection limit being defined here as the concentration corresponding to a current three times the noise level of the background). The electrode responded quickly to changes in glucose concentrations, with response times of 45s. The optimized glucose biosensor, detailed above, employing the ionic liquid [bmim][BF_4], afforded a larger linear range, greater sensitivity, shorter response times and greater stability when compared to literature reports[21], which describe glucose sensors in aqueous media. The low detection limit, coupled with a high sensitivity to glucose, makes the optimized biosensor effective and precise.

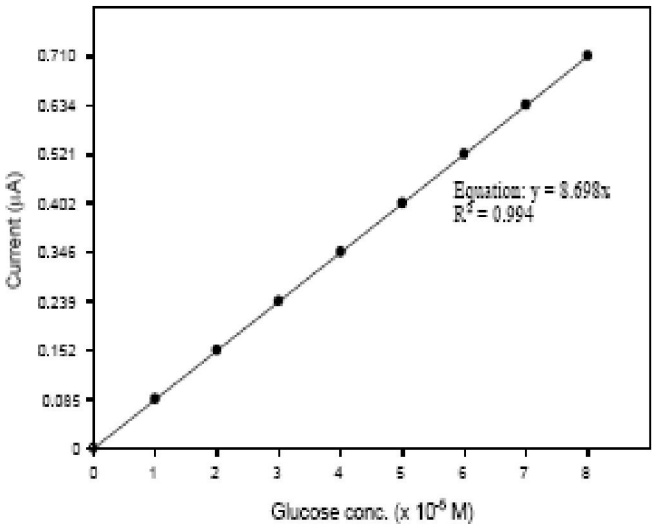

Figure 8 : Calibration for optimized Pt/p(HEMA)/GOx electrode Conditions were - Film thickness = 0.2mm, enzyme loading of 1365 units/cm2 , monomer composition of 2.75:1%vol (HEMA: TEGDA), @ 25 °C, unstirred

Storage stability

Furthermore the optimized glucose biosensor possesses a greater stability, Figure 9, when stored in the ionic liquid, [bmim][BF_4] at 4°C. We found that after twenty weeks, the sensor retained 97% of initial activity on storage in the ionic liquid at 4°C, whilst storage in air at 4°C and in aqueous phosphate buffer at 4°C, resulted in a decrease of 10% and 65% in activity respectively, demonstrating that the optimized sensor has a longer shelf life, when stored in [bmim][BF_4], compared to previously reported glucose sensors[22] in aqueous buffer.

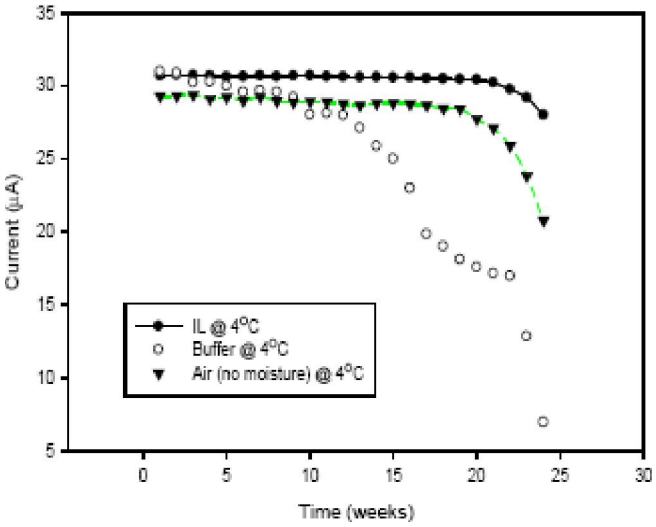

Figure 9 : Storage stability studies over a period of six months for the optimized glucose biosensor for [S] of 2mM, under three different storage regimes.

CONCLUSION

We have successfully prepared amperometric cholesterol and glucose biosensors that uses the ionic liquid, [bmim][BF_4] as the solvent for the CHO_x and GO_x enzymes. This optimized glucose biosensor displays superior characteristics in terms of stability, reproducibility and sensitivity. The use of an ionic liquid as the solvent medium for substrate detection resulted in enhanced detection and stability of the glucose biosensor compared to previously reported glucose biosensors[18-20] that utilized aqueous buffer as the solvent medium. The prepared cholesterol biosensor demonstrated comparable performance characteristics to similar sensors described in the literature[22].

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