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Evaluation of a silver-based electrocatalyst for the determination of hydrogen peroxide formed via enzymatic oxidation

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ABSTRACT

Silver paste electrodes modified with lyotropic phases formed from dodecyl benzenesulphonic acid and KCl were used as the reductant in the determination of the hydrogen peroxide released from the enzymatic reaction of glucose oxidase with glucose and oxygen. The response of the modified electrode to hydrogen peroxide reduction (−0.1 V vs. Ag/AgCl) was shown to suffer from interference resulting from co-localization of enzyme and substrate at the electrode surface. This interference was eradicated by the introduction of a perm-selective membrane in the form of cellulose acetate. This further facilitated immobilization of the enzyme while allowing diffusion of the generated peroxide to the electrode. The resulting configuration was shown to be capable of the analytical determination of glucose.

1. Introduction

Electrochemical biosensors are self-contained integrated devices, which are capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element [1]. Although many different transducer types have been used in biosensor fabrication, such as optical, piezoelectric, thermal or electrochemical, the latter has been the most commercially successful because of its suitable sensitivity, reproducibility and mass production capability at low cost [2].

Many enzymatic reactions produce hydrogen peroxide (H_2O_2) as an end-product, so its concentration may be used as an indicator in the progress of the reaction [3,4]. The most notable example of this is, of course the glucose biosensor with glucose oxidase (GOx) as the biospecific reagent. GOx acts by oxidizing glucose to gluconolactone, accepting electrons in the process and thereby changing to an inactivated state. The enzyme is normally returned to the actively oxidized state by transferring these electrons to molecular oxygen, resulting in the production of hydrogen peroxide (H_2O_2), as is shown [2,5,6]:

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{Gluconic acid} + H_2O_2 \quad (1)$$

However, H_2O_2 has not become a popular target for measurement in such enzyme biosensors. Although capable of acting as an oxidizing or a reducing agent, its electrocatalysis at metallic electrodes such as Pt is kinetically slow and requires high applied potentials with consequent problems with interferences and sensitivity [2,7]. Thus, oxidase sensing has been largely achieved using soluble synthetic mediators such as ferrocene and ferricyanide as well as conducting organic salts or quinine compounds as a replacement for oxygen as the terminal electron acceptor [2,8–11]. In an attempt to utilize H_2O_2 as the signaling molecule, others have used systems employing horseradish peroxidase, which have been shown to have good sensitivity, but increase the complexity of the device with two enzymatic steps [12–14]. Others have continued to investigate ways of enhancing the electrocatalytic reduction of the liberated H_2O_2 which has allowed shifting the detection potential to the optimal region (0.0 to –0.2 V vs. Ag/AgCl) where most unwanted reactions are negligible [8,15]. In recent times, there has been a resurgence of methods and materials for enhancing this process [15–25]. However, such materials and fabrication methods must also meet additional challenges of cost and mass-producibility. Indeed, electrochemical sensor manufacture has been dominated by printed strip production for almost 20 years now [2,8,22] and so emerging technologies need to be compatible with such processes.

Silver is a well-established catalyst for the reduction of hydrogen peroxide. However, it is not comparable to Pt in this regard [3,26,27]. More recently, silver screen printed electrodes have shown a remarkable enhancement in their catalytic activity towards H_2O_2 reduction after exposure to a mixed surfactant/salt

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solution. The electrodes were modified with a dodecyl benzenesulphonic acid and KCl solution, exhibiting up to 80-fold higher responses when H_2O_2 was measured at -0.1 V vs. Ag/AgCl [28]. Although the mechanism remains unclear, it is believed that the formation of lyotropic phases at the electrode surface enhances the electrochemically-coupled reduction of hydrogen peroxide.

In the present work, this material was assessed for its ability to couple this electrocatalysis to the reduction of hydrogen peroxide generated from the oxidation of glucose by glucose oxidase. Notable was the fact that both enzyme and substrate present at the modified electrode led to a loss of catalytic activity, but that deposition of a membrane and immobilization of enzyme prevented this loss of activity. The device was shown to be capable of the quantitative measurement of glucose.

2. Materials and methods

2.1. Materials

Dodecylbenzenesulfonic acid (DBSA–D0989) was purchased from TCI Europe. Sodium and potassium chloride (NaCl, KCl), potassium dihydrogen phosphate (KH_2PO_4), D−(+)-glucose, cellulose acetate (CA), chitosan, hexamethylenediamine (HMDA), acetone and glucose oxidase (GOx, Type II-S: from *Aspergillus niger*, 20% protein) were purchased from Sigma-Aldrich. Disodium hydrogen phosphate (Na_2HPO_4) was purchased from Riedel-de Haen. 30% (v/v) hydrogen peroxide solution was purchased from Merck. Glutaraldehyde (GA) and Nafion® 117 solution (~5% in a mixture of lower aliphatic alcohols and water) were purchased from Fluka Chemika. Acetic acid glacial was purchased from Fisher Scientific. Silver conductive ink (Electrodag® PF-410) was purchased from Henkel (Scheemda, The Netherlands). Poly(ethylene) terephthalate substrates were Melinex® (pre-shrunk) films obtained from HiFi Industrial Film Ltd. (Dublin, Ireland). Polyester pressure sensitive adhesive (PSA, ARcare® 92712) was purchased from Adhesives Research Ireland Ltd. (Limerick, Ireland). All the solutions were prepared using 18 MΩ Milli-Q water.

2.2. Buffers and solutions

Unless otherwise stated, all electrochemical measurements were carried out in phosphate buffered saline solution (PBS). The buffer solution was 10^{-1} M phosphate, 1.37×10^{-1} M NaCl and 2.7×10^{-3} M KCl. This was prepared by mixing solution 1 (10^{-1} M Na_2HPO_4, 1.37×10^{-1} M NaCl and 2.7×10^{-3} M KCl) and solution 2 (10^{-1} M KH_2PO_4, 1.37×10^{-1} M NaCl and 2.7×10^{-3} M KCl) to a pH of 6.8.

Unless otherwise stated, GOx enzyme solution was prepared in PBS pH 5.0. Such buffer was prepared in the same way but adjusting the final pH to 5.0.

0.2 M glucose solution was prepared and left overnight to allow equilibration of the anomers to the stable ratio of α:β 36:64 [29–31].

2.3. Instrumentation

Silver paste electrodes were fabricated as already reported [28].

All electrochemical measurements were performed in a three-electrode electrochemical batch cell, using a Ag/AgCl/NaCl (saturated) electrode and a platinum mesh electrode as reference and auxiliary electrodes, respectively. Cyclic voltammetry and time-based amperometric measurements were carried out with a CHI601C electrochemical analyzer with CHI601C software (IJ Cambria Scientific Ltd., UK). Measurements were performed at room temperature, 18 ± 2 °C. Unless otherwise stated, all potential values are referenced to the Ag/AgCl/NaCl (saturated) electrode.

Scanning Electron Microscopy (SEM) using Secondary Electron (SE) detection was carried out with a Hitachi S-3400N. An acceleration voltage of 20 kV was used to obtain the surface images.

A Graphtec Robo Pro S (Model no. CE50000-4-CRP) cutting plotter and a Robo Master Pro software (Wrexham, UK) were used to prepare the PSA patterns for biosensor fabrication. Electrode patterns were drawn using AutoCAD and uploaded into the Robo Master software. A 3 cm × 12 cm PSA substrate was designed with 10 circular (0.4 cm diameter) patterns so 10 electrodes could be modified simultaneously, increasing the reproducibility of the devices.

2.4. Electrode modification and GOx immobilization

Silver screen printed electrodes were DBSA/KCl modified as previously reported [28]. Unless otherwise stated, the glucose biosensor was prepared following the next protocol adopted from Portaccio et al. [32]. The DBSA/KCl modified electrode was immersed in a CA solution (2×10^{-2}% CA in glacial acetic acid) for 3 s to create a thin and uniform layer of the polymer on the electrode. After the immersion, the electrode was placed for 10 min in cold deionised water to accelerate the polymer solidification phase. Activation of the CA layer was carried out by immersing the electrode into a 5% (w/v) HMDA aqueous solution for 20 min. After washing in deionised water, the electrode was immersed for 20 min in 2.5% (v/v) GA aqueous solution. After further rinsing with deionised water, the electrode was kept overnight at 4 °C in a 25 mg mL^{-1} GOx solution in PBS pH 5.0 for enzyme immobilization. DBSA/KCl modified silver screen printed electrodes with a cellulose acetate membrane will be referred to as Ag_DBSA/KCl_CA. Those electrodes with GOx immobilized on the surface will be referred to as Ag_DBSA/KCl_CA_GOx.

2.5. Electrochemical characterization

All the electrochemical measurements were carried out in a stirred batch system with a three-electrode configuration. Cyclic voltammograms were obtained in the potential range from -0.200 to 0.025 V (vs. Ag/AgCl) at a scan rate of 0.1 V s^{-1}. Amperometry was performed at -0.1 V (vs. Ag/AgCl). 1 M stock solution of hydrogen peroxide was prepared daily and then aliquots from this solution were added to the working cell during both cyclic voltammetric and amperometric measurements in order to characterize the sensor parameters.

3. Results and discussion

It has been shown that the electrocatalytic reduction of H_2O_2 can be significantly enhanced at a screen-printed, silver paste electrode through its modification with a lyotropic solution of surfactant and salt and was capable of achieving significant enhancement at quite moderate reduction potentials and under non-rigorous hydrodynamic conditions [28]. This material has also been shown to enhance catalysis on other metallic surfaces [33]. However, its usefulness beyond the direct measurement of hydrogen peroxide had not been evaluated. An obvious potentiality of such materials is in the measurement of H_2O_2 formed from important biochemical processes, particularly oxidase-based oxidation. The most well-known example of such a process is that of glucose oxidation via glucose oxidase.

Thus, the DBSA/KCl modified silver paste electrodes were initially evaluated for their ability to measure hydrogen peroxide formed from this process. Preliminary amperometric experiments in the presence of glucose and glucose oxidase appeared to demonstrate catalytic responses that were much lower than would be predicted for the amount of hydrogen peroxide generated. Control studies were performed in which the modified electrodes were tested for their direct catalysis towards hydrogen peroxide reduction before and after they had been used for glucose sensing to see if any loss in catalytic activity had occurred. Fig. 1A shows the typical response of the modified electrode to five injections of hydrogen peroxide of 1×10^{-3} M each before (a) and after (b) it had been used for the measurement of glucose by addition of 1×10^{-3} M injections of glucose in the presence of 1 mg mL^{-1} glucose oxidase (c). As might be expected, the response rate in (c) is much slower due to the rate of formation of hydrogen peroxide from the enzymatic reaction. However, the final response was only a fraction of that achieved for the stoichiometrically equivalent H_2O_2 concentration determined in (a). When exposed again to a fresh solution of hydrogen peroxide (b), the catalytic rate had clearly been reduced by approximately 50%, indicating some irreversible loss in catalytic activity following glucose measurement. A similar experiment was performed, but in this case addition of glucose was omitted (Fig. 1B) and the sensor response to hydrogen peroxide before (a), during (b) and after (c) exposure to a solution of 1 mg mL^{-1} glucose oxidase showed no loss in catalytic activity. Further controls in the presence of glucose alone or in combination with gluconolactone or gluconic acid showed no reduction in the catalytic activity of the modified electrode. Therefore, the loss in catalytic activity appeared to be a consequence of both enzyme and either substrate or product coming in contact with the modified electrode. Thus a strategy that would maintain separation of enzyme and substrate/product might eradicate this phenomenon.

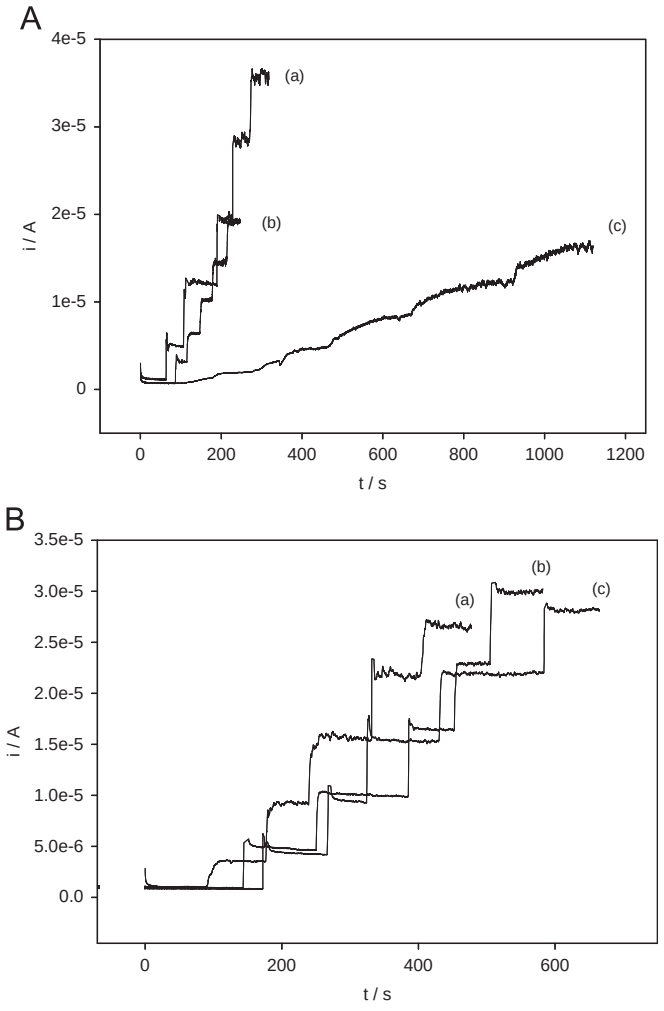

Fig. 1. (A) Amperometric responses of a DBSA/KCl modified silver paste electrode to H_2O_2 concentrations from 1 to 5×10^{-3} M measured at -0.1 V (vs. Ag/AgCl) in PBS pH 6.8: (a) before, and (b) after glucose sensing in (c) with 1 mg mL^{-1} GOx in solution, at glucose concentration from 1 to 5×10^{-3} M, and (B) a similar electrode at H_2O_2 concentrations from 1 to 5×10^{-3} M measured: (a) prior to exposure; (b) in the presence of and (c) following exposure to 1 mg mL^{-1} GOx.

The employment of a membrane and the subsequent immobilization of the enzyme were then investigated as a means to stabilize and control enzyme concentration, keep it close to the electrode surface, while also prevent it from coming in contact with the catalytic layer.

Several types of membrane have been used in the literature for the fabrication of glucose biosensors, e.g. cellulose acetate (CA) [32,34,35], chitosan [10,36], and Nafion [25,37]. In this work, these three materials were evaluated based on the residual catalytic response to H_2O_2 of the DBSA/KCl modified electrodes after their deposition. Preliminary work showed that all of them led to good retention of catalytic activity after their immobilization. Thus, the electrode modified with chitosan displayed a cathodic current in the presence of 5×10^{-3} M H_2O_2 of 2.5×10^{-5} A which equates to retention of 75% of the initial activity (3.3×10^{-5} A). For the Nafion membrane, the remaining catalytic activity to H_2O_2 of the electrode after its immobilization was 81% whereas for CA it was 93%. Therefore, CA seemed to result in the highest residual activity following membrane deposition. In addition, preliminary studies showed good immobilization of GOx to the CA membrane. Therefore, CA was chosen as the membrane of choice for more detailed evaluation of its effect on the modified electrode and the immobilization of enzyme. GOx was then immobilized following the adopted protocol (Section 2.4), which has already proved to be a suitable procedure for GOx covalent attachment [32]. Briefly, modified silver screen printed electrodes with a CA membrane is activated by immersion first in HMDA solution and then in GA one. After rinsing, the electrodes were kept in the GOx solution overnight for enzyme immobilization. Subsequently, several parameters such as CA and GOx concentrations, immersion time in cellulose acetate solution during the formation of the membrane and other aspects of the immobilization protocols were optimized.

As was mentioned above, the main purpose of the membrane deposition was to create a high-quality CA layer which would enable the immobilization of an optimal concentration of enzyme to achieve maximum activity while not reducing H_2O_2 diffusion to inhibitory levels. Some DBSA/KCl modified silver screen printed electrodes were immersed in CA solutions of several concentrations (0.02–20% w/v) in acetic acid for 3 s. The electrodes were then immersed in cold water for 10 min and rinsed again before measuring. Thicker CA membranes might show decreases in the response to H_2O_2 due to diffusion limitation. Plot of the cathodic currents at 5×10^{-3} M H_2O_2 vs. log[CA] used for CA membrane deposition is shown in Fig. 2. Amperometric responses of DBSA/KCl modified electrodes with and without CA membranes of different concentration solutions are shown in the inset of Fig. 2.

Electrodes modified with CA membranes generated from 0.02% (w/v) and 0.2% (w/v) CA solutions seemed to maintain full catalytic activity towards H_2O_2 reduction, although the quality of signal produced was poor compared to that in the absence of membrane. Thus, the cathodic currents obtained in the presence of 5×10^{-3} M H_2O_2 were 2.9×10^{-5} A and 3.1×10^{-5} A for 0.02% (w/v) and 0.2% (w/v) CA concentrations, respectively, whereas the reduction current for a DBSA/KCl modified electrode before CA

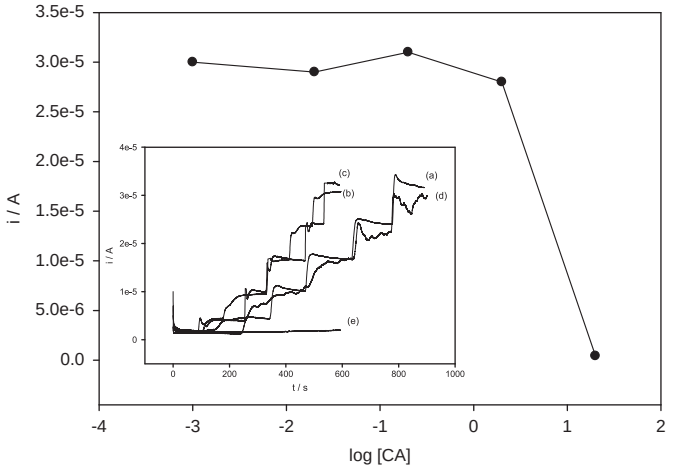

Fig. 2. Plot of catalytic currents at 5×10^{-3} M H_2O_2 vs. log[CA]. Data at log[CA] = −3 corresponding to the cathodic current of the electrode without CA membrane. Inset: amperometric responses of the reduction of 1–5×10^{-3} M H_2O_2 at DBSA/KCl modified silver paste electrodes measured at −0.1 V (vs. Ag/AgCl) in PBS pH 6.8: CA concentrations of (a) 0%; (b) 0.02%; (c) 0.2%; (d) 2% and (e) 20% (w/v).

deposition was 3.0×10^{-5} A. The DBSA/KCl modified electrode treated with a 2% (w/v) CA solution provided a similar cathodic current of 2.8×10^{-5} A. However, the quality of the amperometric responses was increasingly noisy at these higher CA concentrations, which might be a result of a thicker layer that may have formed on the electrode surface. At 20% (w/v) CA the current response was significantly reduced to approx. 4.2×10^{-7} A. This electrode also showed a significant increase in response time. Both effects were assumed to relate to the formation of a thick, dense polymer film on the electrode which decreased the H_2O_2 diffusion rate from the solution to the electrode surface. These data suggested that films prepared from CA concentrations of the order of 1% or 2% (w/v) would not significantly impact diffusion of substrate to the electrode surface. The cathodic currents obtained with these electrodes in the presence of 5×10^{-3} M H_2O_2 were similar to those obtained for the modified electrode before CA deposition, despite the increase in the noise and response time. Generally, CA concentrations around 1–2% (w/v) have been used in the literature for the deposition of CA membranes for the fabrication of glucose biosensors [35,38,39]. Initially, the standard protocol adopted here for enzyme attachment employed 20% (w/v) CA solution for membrane formation [32]. However, it was shown above that such a high concentration was not optimal in the present system as it hindered substrate diffusion and brought about a significant increase in response time. Therefore, 1% (w/v) CA in acetic acid was selected as the optimum concentration for the deposition of a CA membrane as it maintained high catalytic activity on H_2O_2 reduction and provided a suitable platform for GOx immobilization. Lower CA concentrations led to faster and less noisy responses which might imply the formation of CA layers not thick enough for our purposes.

The time when DBSA/KCl modified silver screen printed electrodes were immersed in the CA solution for the membrane deposition was subsequently studied. Several DBSA/KCl modified electrodes were immersed into 1% (w/v) CA solutions for different periods of time, from 5 to 20 s. The electrodes were then rinsed and the CA membranes were evaluated by amperometry of the glucose biosensors fabricated from them. The sensitivity for glucose determination as well as the remaining activity towards H_2O_2 reduction after enzyme immobilization for each electrode is shown in Table 1.

As can be observed, the catalytic activity towards H_2O_2 reduction after GOx immobilization decreased remarkably as

Table 1
Effect of membrane formation time on H_2O_2 response in 1% (w/v) CA solution.

Modification time/s	Before GOx i_1[a]/10^{-6} A	After GOx i_2[b]/10^{-6} A	% Ratio i_1/i_2	$i_{1\ \mathrm{mM\ glucose}}$/$\times10^{-9}$ A	Sensitivity (A M^{-1} cm^{-2})
5	3.49	2.93	84	9.0	1.3×10^{-4}
10	4.02	1.71	43	8.3	1.7×10^{-4}
15	4.29	1.66	39	11.0	1.2×10^{-4}
20	4.61	1.81	39	6.3	8.7×10^{-5}

[a] Cathodic current in the presence of 1×10^{-3} M H_2O_2 before GOx immobilization.
[b] Cathodic current in the presence of 1×10^{-3} M H_2O_2 after GOx immobilization.

the immersion time in CA solution increased. Moreover, the sensitivity of the glucose biosensor displayed a similar tendency with the immersion time. Ten seconds seemed to be the optimum immersion time, providing biosensors with the highest sensitivity towards glucose determination. These results suggested that longer exposures to CA solutions and the subsequent GOx immobilization resulted in the formation of thicker, denser enzyme layers, hindering both H_2O_2 and glucose catalytic processes. The longer exposures to CA solutions would lead to the formation of thicker CA membranes, providing a higher number of bonding sites for enzyme immobilization on the electrode surface. The thicker enzyme membranes might impede both H_2O_2 and glucose diffusion, which would explain the decrease in both catalytic signals with increasing CA deposition time after GOx immobilization. It is worth remarking here that the 10 s modification time followed by washing might result in significant fabrication process variability. The obvious alternative then would be to reduce concentration and increase time. However, longer exposure to solvent (glacial acetic acid) was found to be damaging to the surface modification, so exposure times were kept short. Further studies about such solvent effects will be performed and commented upon later in this section.

The next step in the enzyme immobilization protocol includes the immersions in 5% HMDA and 2.5% GA solution. Several DBSA/KCl modified silver paste electrodes with CA membranes on top (Ag_DBSA/KCl_CA) were immersed in 5% HMDA aqueous solution for different periods of time, from 1 to 20 min. After intensive washing with distilled water, the electrodes were placed in PBS pH 6.8 and amperometry at −0.1 V (vs. Ag/AgCl) was carried out. Varying the time of exposure to 5% HMDA aqueous solution did not seem to bring about noticeable changes in the cathodic currents in the presence of 5×10^{-3} M H_2O_2. In this way, 7.8×10^{-6} A was the cathodic current at that concentration shown by a Ag_DBSA/KCl_CA electrode after 1 min immersion in HMDA solution whereas an electrode immersed for 20 min provided 8.2×10^{-6} A. The currents exhibited by electrodes immersed in 5 and 10 min in HMDA solution were 8.1×10^{-6} A and 6.7×10^{-6} A, respectively. However, those currents were approx. 4-fold lower than that obtained by a DBSA/KCl modified silver screen printed electrodes in the presence of 5×10^{-3} M H_2O_2, being 3.5×10^{-5} A.

Further studies varying immersion times in 2.5% GA aqueous solutions did not lead to any improvement in reproducibility or sensitivity of the glucose biosensor whereas the cathodic current provided for the catalytic reduction of H_2O_2 were even lower. Thus, Ag_DBSA/KCl_CA electrodes immersed in 2.5% GA solution for 1, 5, 10 and 20 min showed cathodic currents of 3.7×10^{-6} A, 3.1×10^{-6} A, 3.8×10^{-6} A and 3.1×10^{-6} A, in the presence of 5×10^{-3} M H_2O_2 respectively.

As is well-known, CA is relatively inert and does not interact with or impede the movement of proteins, making it a useful

component for a support medium. The solubility of CA depends on the degree of substitution (DS), e.g. the most common form of CA has an acetate group on approx. 2–2.5 of every three hydroxyls ($DS=2$–2.5). This type is soluble in acetone, dioxane and methyl acetate; higher acetylated types are soluble in dichloromethane. Acetic acid is generally a good solvent for CAs with *DS* greater than 0.8 [40]. CA used in the present work presented a *DS* of approx. 1.2, therefore, glacial acetic acid was first used as a solvent. Acetic acid is a weak acid that in its pure, water-free formulation (glacial acetic acid) is quite corrosive. Therefore, it might interact with the silver paste electrode surface, removing the binder from the ink and causing further surface modifications. In order to evaluate its effect, amperometric responses at -0.1 V (vs. Ag/AgCl) in PBS pH 6.8 were performed for a DBSA/KCl modified screen printed electrode before and after 10 s immersion in glacial acetic acid. A significant negative impact of the acetic acid on H_2O_2 catalysis from modified electrodes was observed. The cathodic current shown by the electrode at 5×10^{-3} M H_2O_2 after immersion in acetic acid was approx. 6.8×10^{-6} A, almost one order of magnitude lower than that shown by the same electrode before acetic acid treatment, 4.9×10^{-5} A. Therefore, a different solvent for the preparation of CA solutions was required.

Acetone is a colorless, flammable liquid, miscible with water and used as an important solvent for many industrial applications. As was mentioned above, CA is readily soluble in acetone, so this solvent was evaluated as a suitable alternative to glacial acetic acid for cellulose membrane formation. In order to check the effect of CA/acetone on DBSA/KCl modification, amperometric responses and SEM images of surfactant-modified electrodes were taken before and after their immersion in an acetone-based solution. A DBSA/KCl modified silver screen printed electrode was first measured in PBS pH 6.8 in the presence of 1–5×10^{-3} M H_2O_2 by amperometry at –0.1 V (vs. Ag/AgCl). After that, the electrode was dipped into a 2% (w/v) CA in acetone solution for 1 min. After washing thoroughly with distilled water, the electrode was placed in a cell containing PBS, pH 6.8 and amperometry was again performed in the presence of H_2O_2. The effect of just acetone on DBSA/KCl modification was also checked by the immersion of the electrode in acetone for 10 s. DBSA/KCl modified electrode before immersion in CA solution exhibited a cathodic current of approx. 6.4×10^{-5} A in the presence of 5×10^{-3} M H_2O_2 whereas the same electrode after CA membrane deposition showed approx. 2.3×10^{-5} A. When immersed only in acetone for 10 s the cathodic current was 5.0×10^{-6} A. Therefore, the decrease in the catalytic current shown by the electrode after the immersion in CA/acetone could be attributed to the etching effect of the acetone diminished by the presence of CA. The etching effect of acetone on the electrode surface was also observed in the SEM images shown in Fig. 3.

Surface areas from electrodes after treatment in acetone-based solution alternated silver paste areas with blurred ones, as can be observed in Fig. 3B and C. Again, the presence of CA might have diminished the etching effect of the solvent, as can be observed by the higher catalytic responses towards H_2O_2 of the CA-modified electrodes with respect to those just exposed to acetone. However, the CA membrane is not conductive, which made its detection by SEM microscopy without any metal sputtered on top difficult. Nevertheless, the negative effect of acetone on DBSA/KCl modification was evident from the amperometric responses and the SEM images. Such effects have been previously reported in the literature. Thus, Polan et al. [39] developed a glucose

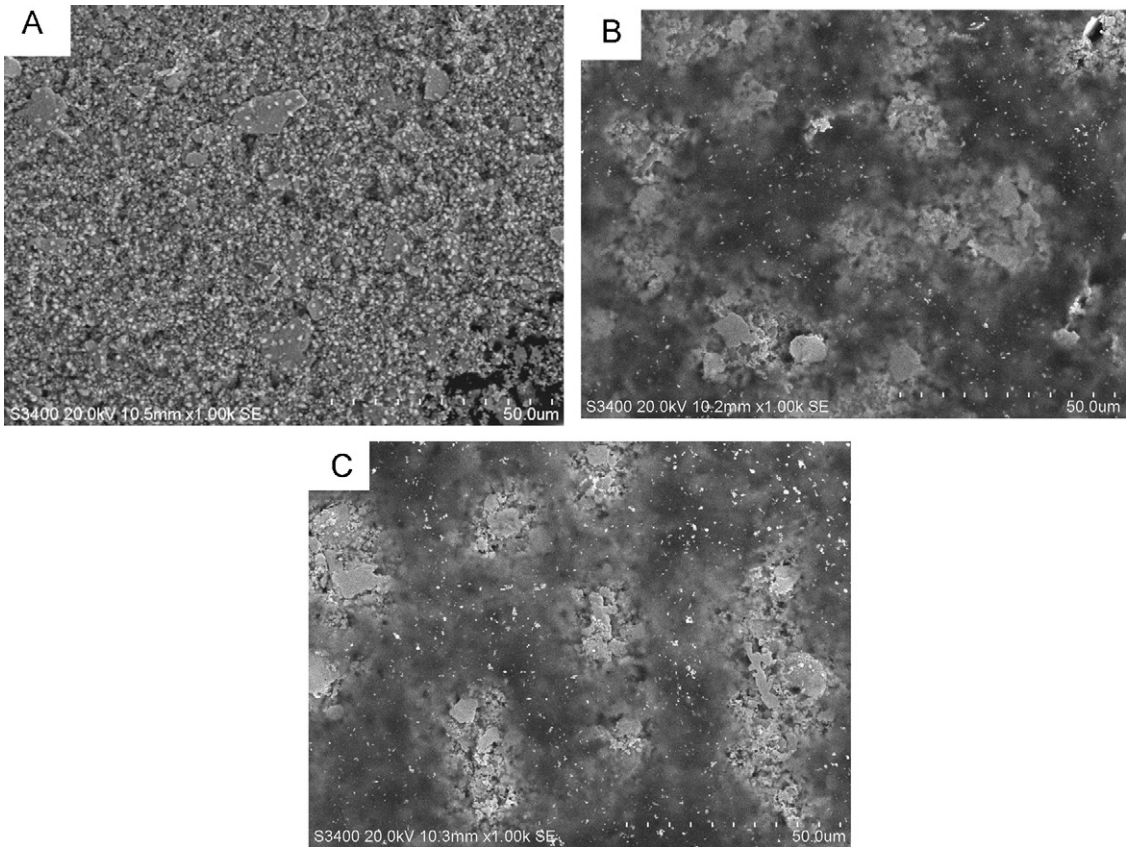

Fig. 3. SEM images of DBSA/KCl modified electrodes (A) without any further modification, (B) after 1 min immersion in 2% (w/v) CA in acetone and (C) after 10 s immersion in acetone. Accelerating voltage of 20 kV. (1.0 k × magnification).

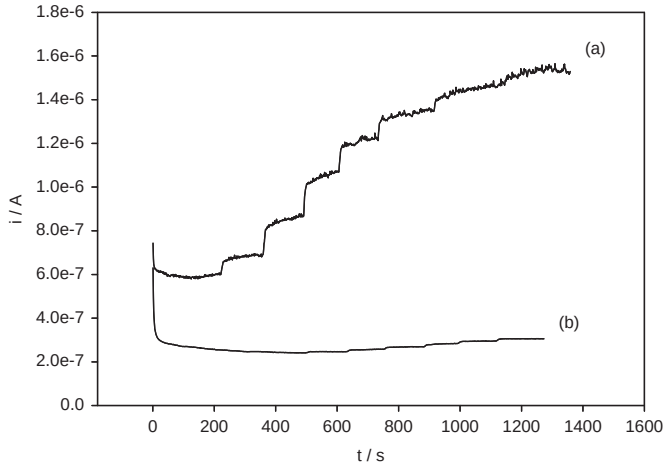

Fig. 4. Amperometric responses of Ag_DBSA/KCl_CA_GOx electrodes measured at –0.1 V (vs. Ag/AgCl) in PBS pH 6.8, at glucose concentration from 1 to 6×10^{-3} M. Ag_DBSA/KCl_CA_GOx electrodes were prepared by immersion in: (a) 2% (w/v) CA solution in acetone for 1 min and (b) 1% (w/v) CA in glacial acetic acid solution for 10 s.

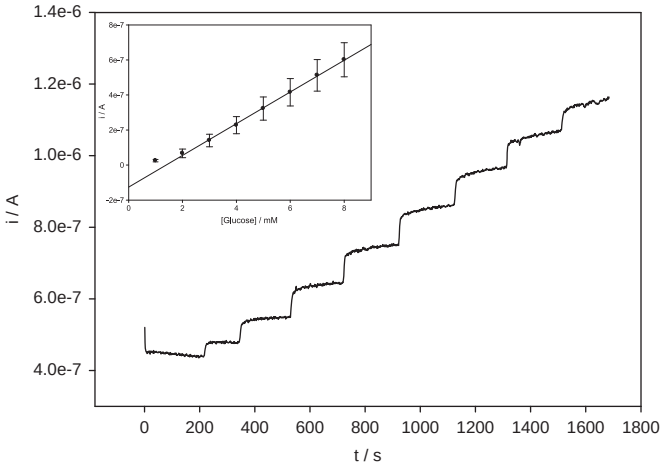

Fig. 5. Amperometric responses of a Ag_DBSA/KCl_CA_GOx electrode in 1×10^{-3} M aliquots of glucose ($1-8 \times 10^{-3}$ M) at –0.1 V (vs. Ag/AgCl) in PBS pH 6.8. Insert: plot of average and standard deviation of the cathodic current vs. glucose concentration at –0.1 V (vs. Ag/AgCl) in PBS pH 6.8. (slope = 9.05×10^{-8} A mM^{-1}).

biosensor by immobilizing GOx onto carbon screen printed electrodes with CA. Solutions at concentrations from 0.05% to 3% of CA in acetone were used. They realized that acetone dissolved the binder in the carbon ink (of a resin type), which caused partial washing of the electrode. In this case, that could be a further evidence for the presence of the binder playing a role in the H_2O_2 catalysis observed after DBSA/KCl modification. The etching effect of the acetone might remove the binder with the consequent loss of the DBSA/KCl modification.

Despite the observed effect of the acetone, a glucose biosensor was fabricated using acetone as a solvent for CA. Amperometry at –0.1 V (vs. Ag/AgCl) in PBS pH 6.8 was carried out at glucose concentrations from 1 to 6×10^{-3} M. Varying the solvent from CA solution during GOx immobilization procedure did alter the catalytic response towards glucose determination shown by the biosensor devices, as is illustrated in Fig. 4. CA dissolved in acetone seemed to provide better membranes on the DBSA/KCl modified electrodes compared to acetic acid. The cathodic current shown by the former was approx. 8.6×10^{-7} A in the presence of 6×10^{-3} M glucose whereas the latter exhibited approx. 6.6×10^{-8} A. A catalytic enhancement of the reduction current using acetone as solvent for CA was observed. Therefore, acetone was selected as the solvent in the CA solution in further.

3.1. Analytical response to glucose detection

Subsequently, the response of the Ag_DBSA/KCl_CA_GOx electrodes to glucose was investigated and LOD, sensitivity and reproducibility studies were performed. Six DBSA/KCl modified electrodes were treated according to the adopted standard protocol. Amperometric responses to glucose from 1 to 8×10^{-3} M were measured. Fig. 5 shows an example of the amperometric response obtained with one of the electrodes. The input in Fig. 5 shows the averages of the cathodic currents and the standard deviation obtained for the six modified electrodes. Current data corresponding to 1×10^{-3} M glucose were not considered for the regression line. The average LOD and sensitivity obtained for these electrodes were found to be 1.55×10^{-4} M (5% r.s.d.) and 7.2×10^{-4} A M^{-1} cm^{-2} (30% r.s.d.). As can be observed in Table 2, these values were in the same order of magnitude as other LODs and sensitivities obtained with biosensors based on screen-printed electrodes in the literature.

As previously stated in this section, the responses derived from this electrocatalytic sensor are based on the rate of electrocatalytic reduction of H_2O_2. Glucose responses obtained by the Ag_DBSA/KCl_CA_GOx electrodes were in part dependent on the initial catalytic activity of those electrodes towards H_2O_2 reduction. Therefore, the variability of the catalysis of the DBSA/KCl-modified electrode surface might be a contribution to the variability of the biosensor devices. To assess this, data corresponding to glucose determination were ratioed with respect to the response of each electrode to 1×10^{-3} M H_2O_2. Averages and standard deviation for the normalized data were determined and are shown in Fig. 6. Current data corresponding to 1 and 2×10^{-3} M glucose were not considered for the regression line. As can be observed, low glucose concentrations led to higher standard errors in the cathodic currents than high glucose concentrations. Thus, the variability of the sensors at glucose concentrations below 3×10^{-3} M was 20–30% whereas reproducibility values of 9–13% were obtained at higher glucose concentrations. However, the variability shown by the electrodes modified by CA in acetone was far lower than that previously observed when the electrodes were immersed in CA solution containing acetic acid. Standard error for acetone-based solution at 4×10^{-3} M glucose was approx. 17% whereas the error for the electrodes modified with the acetic acid-based solution was 43%. However, the variability shown by the former was still high in comparison with other systems in the literature [34,45]. DBSA/KCl modified electrodes had undergone several modification steps during the GOx immobilization process, which might partially reduce the catalytic activity towards H_2O_2 reduction.

The use of acetone as a CA solvent might also be responsible of the relatively poor reproducibility of the biosensors. Polan et al. [39] reported the uneven distribution of CA onto carbon screen-printed electrodes when acetone was used as a solvent. They attributed that effect to the volatility of the acetone, which vaporized very quickly while CA was spread onto the electrode surface. In the present work, the membrane was formed by immersion in a CA solution and subsequently in cold distilled water. However, the period of time employed to move the electrodes from one solution to the next one might be enough to introduce a relative variability in the membrane deposition.

Although the biosensors fabricated following the established protocol exhibited acceptable analytical performance parameters for determination of glucose compared to other devices in the

Table 2
Comparison of analytical parameters of electrochemical glucose biosensors based on screen printed electrodes.

Sensor surface	E_{app} vs. Ag/AgCl (V)	LOD (M)	Sensitivity (A M^{-1} cm^{-2})	Reproducibility	Linear range (mM)	Ref.
GOx–PB/C SPE	0	2.5×10^{-5}	5.4×10^{-2}	7% ($n=5$)	0.025–1	[25]
GOx/Co-PC/C SPE	0.4	2.7×10–4	–	6.2–10.7%	0.27–2	[41]
GOx/PVA/SiO_2/AgNP–PB/C SPE	−0.05	–	2.0×10^{-2}	7.6% ($n=12$)	0.0125–2.56	[42]
GOx/MWCNT–C SPE	0.5	–	4×10^{-3} A M^{-1}	4% ($n=5$)	0–4	[43]
LBL GOx–MWCNT–PVI-Os–C SPE	0.3	1.0×10^{-4}	1.64×10^{-2}	–	0.5–6	[44]
GOx–Ferri/COs–C SPE	0.3	1.4×10^{-3}	6.77×10^{-4} A M^{-1}	–	0–33.3	[31]
GOx–CA–DBSA/KCl–Ag SPE	−0.1	1.55×10^{-4}	7.2×10^{-4}	30% ($n=6$)	1–8	This work

Note: Gox: Glucose oxidase; PB: Prussian Blue; C SPE: carbon screen printed electrode; Co-PC :cobalt phthalocyanine; PVA: polyvinyl alcohol; Ag NP: silver nanoparticles; MWCNT: multiwalled carbon nanotubes; LbL: layer-by-layer; PVI-Os: Poly(1-vinylimidazole)-osmio redox polymer; Cos: chitosan oligomers.

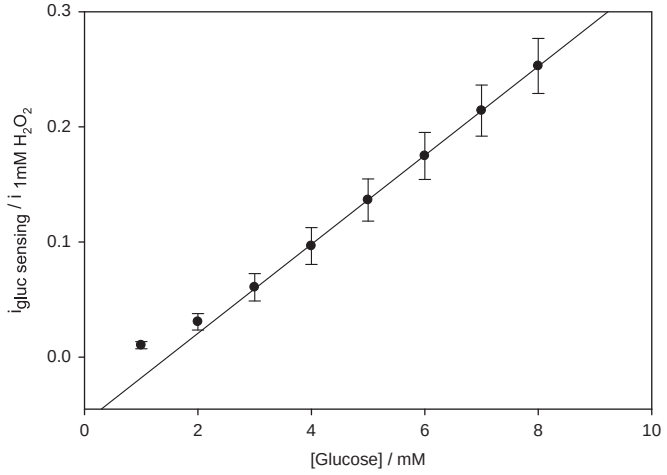

Fig. 6. Plot of average and standard deviation of the ratios glucose/1×10^{-3} M H_2O_2 responses vs. glucose concentration (1–8×10^{-3} M) for Ag_DBSA/KCl_CA_GOx electrodes ($n=6$). Data were obtained by amperometry at −0.1 V (vs. Ag/AgCl) in PBS pH 6.8. CA membrane deposited after 1 min immersion in 2% (w/v) CA solution in acetone. (slope$=3.9\times10^{-2}$ mM^{-1}).

literature, further studies should be carried out to obtain a more amenable GOx immobilization procedure.

4. Conclusions

DBSA/KCl modified silver screen printed electrodes as have been used as a platform for the fabrication of a glucose biosensor. Previous investigations had shown the enhancement of the catalytic activity towards H_2O_2 undergone by silver paste electrodes after surfactant-based modification. This phenomenon was employed here for the construction of an enzymatic device for glucose determination. The catalytic activity of the electrodes to H_2O_2 reduction seemed to be affected by the enzymatic reaction of the glucose. Therefore, a protective membrane was required to avoid any damages on DBSA/KCl modification and simultaneously to facilitate GOx immobilization. Cellulose acetate (CA) was selected as the isolating layer and a glucose biosensor was built by covalent attachment of GOx using hexamethylenediamine (HMDA) and glutaraldehyde (GA). Several parameters such as CA concentrations and appropriate solvent for its solutions, modification times in HMDA and GA were further studied. One minute in 2% (w/v) CA in acetone, 20 min in 5% (w/v) HMDA aqueous solution and 20 min in 2.5% GA aqueous solution turned to be the optima parameters for the biosensor. An average LOD of 1.55×10^{-4} M and sensitivity of 7.2×10^{-4} A M^{-1} cm^{-2} were obtained when glucose concentration ranged from 1 to 8×10^{-3} M. The cathodic currents corresponding to glucose sensing were normalized with respect to the catalytic response to 1×10^{-3} M H_2O_2 and a R.S.D. of up to 9% was obtained by six electrodes when glucose concentration was 8×10^{-3} M.

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