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Short communication

Direct electron transfer of glucose oxidase immobilized in an ionic liquid reconstituted cellulose–carbon nanotube matrix

Xuee Wu [a,*], Feng Zhao [a], John R. Varcoe [a], Alfred E. Thumser [b], Claudio Avignone-Rossa [c], Robert C.T. Slade [a,*]

[a] Chemical Sciences, University of Surrey, Guildford, GU2 7XH, United Kingdom
[b] Biological Sciences, University of Surrey, Guildford, GU2 7XH, United Kingdom
[c] Microbial Sciences, University of Surrey, Guildford, GU2 7XH, United Kingdom

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ABSTRACT

Conductive cellulose-multiwalled carbon nanotube (MWCNT) matrix with a porous structure and good biocompatibility has been prepared using a room temperature ionic liquid (1-ethyl-3-methylimidazolium acetate) as solvent. Glucose oxidase (GOx) was encapsulated in this matrix and thereby immobilized on a glassy carbon surface. The direct electron transfer and electrocatalysis of the encapsulated GOx has been investigated using cyclic voltammetry and chronoamperometry. The GOx exhibited a pair of stable, well defined and nearly symmetric reversible redox peaks. The experimental results also demonstrate that the immobilized GOx retains its biocatalytic activity toward the oxidation of glucose and therefore can be employed in a glucose biosensor. The results show that the bioelectrode modified by the cellulose-MWCNT matrix has potential for use in biosensors and other bioelectronics devices.

1. Introduction

The investigation of direct electron transfer (DET) between redox enzymes and electrodes is important in the development of electroanalytical applications and bioelectrocatalytic devices. Such bioelectronic devices require an optimized procedure for enzyme immobilization e.g., operational simplicity, low fabrication expense and long-term and high enzyme activity. Electrode materials with optimal physical and chemical properties, biocompatibility and low cost are important considerations for the immobilization of enzymes [1]. Enzymes are typically immobilized using a range of different strategies involving materials such as inorganic supports and synthetic polymers e.g., Fe_2O_3- or Au-nanoparticles, Nafion-nanotube composites, self-assembled material layers, and conducting polymers [2,3]. These synthetic materials are easily processed, but they are less suitable for enzyme immobilization, as the undesirable surface characteristics and low biocompatibility increase the denaturation of the proteins [4]. In contrast, naturally occurring macromolecular materials are pertinent to enzyme immobilization technologies owing to their biocompatibility. Cellulose, which is present in the cell walls of plants, is the most abundant and renewable biopolymer on earth and has many advantages when used as an enzyme immobilization material, including its biocompatibility, chemical stability, mechanical and physical properties, low cost and availability as renewable natural resources [5]. Cellulose derivatives, such as cellulose nitrate, cellulose acetate, carboxymethyl cellulose, have been used as carriers for immobilized enzymes [6–8]. However, the application of natural cellulose has been hindered by its poor solubility due to the presence of strong inter- and intra-molecular hydrogen bonding.

Recent studies have showed that room temperature ionic liquids (RTILs), such as 1-ethyl-3-methylimidazolium acetate ([EMIM][CH_3COO]) and 1-butyl-3-methylimidazolium chloride (BMIMCl), exhibit good dissolution power for cellulose, which can then be reconstituted into a variety of forms such as membranes, beads and hollow fibres [9,10]. Turner et al. have developed cellulose-RTIL composite materials for the immobilization of laccase with the retention of catalytic activity using BMIMCl [11]. In the field of bioelectrochemistry, however, RTIL reconstituted cellulose materials have not been given the appropriate level of attention.

Due to their outstanding physicochemical properties, carbon nanotubes (CNTs) are attracting considerable attention for the development of bioelectrochemical devices. However, the poor dispersibility of carbon nanotubes has traditionally been a major technical barrier to their application. Researchers have shown that RTILs can assist in the dispersion of carbon nanotubes and impart useful properties in their application in bioelectronics [12–17].

In this communication we report a simple method to immobilize enzymes in a cellulose-multiwalled carbon nanotube (MWCNT) matrix reconstituted using [EMIM][CH_3COO], which is an enzyme-friendly co-solvent for organic reactions [18], and subsequent use of this matrix to modify electrodes with glucose oxidase (GOx) as a model enzyme. The direct electron transfer between the active site of GOx and the electrode has been achieved, and its behaviour as a biosensor and enzyme stability were investigated by electrochemical techniques.

* Corresponding authors. Tel.: +44 1483 682588; fax: +44 1483 686851.
E-mail addresses: Xuee.wu@surrey.ac.uk (X. Wu), R.Slade@surrey.ac.uk (R.C.T. Slade).

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2. Experimental section

2.1. Chemicals, reagents and pretreatments

Glucose oxidase (GOx, EC 1.1.3.4, 200 U mg^{-1}, from *Aspergillus niger*), microcrystalline cellulose and 1-ethyl-3-methylimidazolium acetate ([EMIM][CH_3COO]) were purchased from Sigma-Aldrich and used with no additional purification. Multiwalled carbon nanotubes (MWCNT, Nanocyl-3100 series with an average diameter of 10 nm) were refluxed in HNO_3 (aq, 2.6 M) for 10 h, followed by precipitation, rinsing with distilled water to eliminate the residual HNO_3, and drying in a vacuum oven at 80 °C for 12 h [19]. D-glucose solutions were prepared the day before use and allowed to stand overnight to allow equilibration of the monomers. All solutions were prepared with ultrapure water (18.2 MΩ cm^{-1}) from a Select Fusion system (Purite Corporation).

2.2. Electrode modification

Glassy carbon (GC, 3 mm diameter) was sequentially polished with 1.0 and 0.5 μm alumina slurry and then washed ultrasonically in ultrapure water for a few minutes. The cellulose-MWCNT-GOx modified GC electrodes were prepared as follows: the cellulose (3.0 wt.%)-[EMIM][CH_3COO] solution was obtained by thoroughly mixing cellulose and [EMIM][CH_3COO] and then heating to 70 °C for 1 h in an ultrasound bath until an optically clear solution was obtained. The MWCNT (1.0 wt.%) were then suspended in [EMIM][CH_3COO]-cellulose solution by grinding in an agate mortar for 15 min under high purity nitrogen to prevent the [EMIM][CH_3COO] from absorbing moisture. GOx (~0.3 wt.%) was finally added to the resulting mixture to afford the target GOx-cellulose-MWCNT-RTIL dispersion. A 10 μL aliquot of this solution was evenly spread on the GC surface and the modified electrode was then immersed in deionized water, to remove the [EMIM][CH_3COO] by dissolution, leaving the cellulose-MWCNT matrix with encapsulated GOx on the surface; the [EMIM][CH_3COO] can be recovered by distillation. The electrode was dried at room temperature and stored dry in air at 4 °C until required.

2.3. Apparatus and electrochemical measurements

A Hitachi S2300 SEM was used to characterize the modified electrode, with an acceleration voltage of 5.0 kV. Electrochemical measurements were carried out using an Autolab PGSTAT (EcoChemie, Netherlands) in a three-electrode cell with 25 mL volume. The cellulose-MWCNT-GOx modified GC was used as a working electrode, a Pt-wire served as a counter electrode and the reference electrode was an Ag/AgCl type (3 M NaCl, +0.196 V vs. SHE at 298 K). The electrolyte was aqueous citrate phosphate buffer (0.2 M), which was purged with either high purity nitrogen or air for testing. All electrochemical experiments were carried out at 22 ± 1 °C.

3. Results and discussion

3.1. Characteristics of the cellulose–MWCNT matrix

Fig. 1 shows a SEM image of the RTIL reconstituted cellulose–MWCNT matrix which has a porous and three-dimensional structure. This electrically conductive, porous and biocompatible microenvironment represents a suitable host for enzyme immobilization, which may enhance direct electron transfer between the active site of enzyme and the stability of bioelectrode.

3.2. Direct electron transfer by GOx

GOx is of considerable commercial importance and has wide-ranging applications in the food and fermentation industry and clinical analysis; it is a flavin enzyme with a molecular weight of 160 kDa,

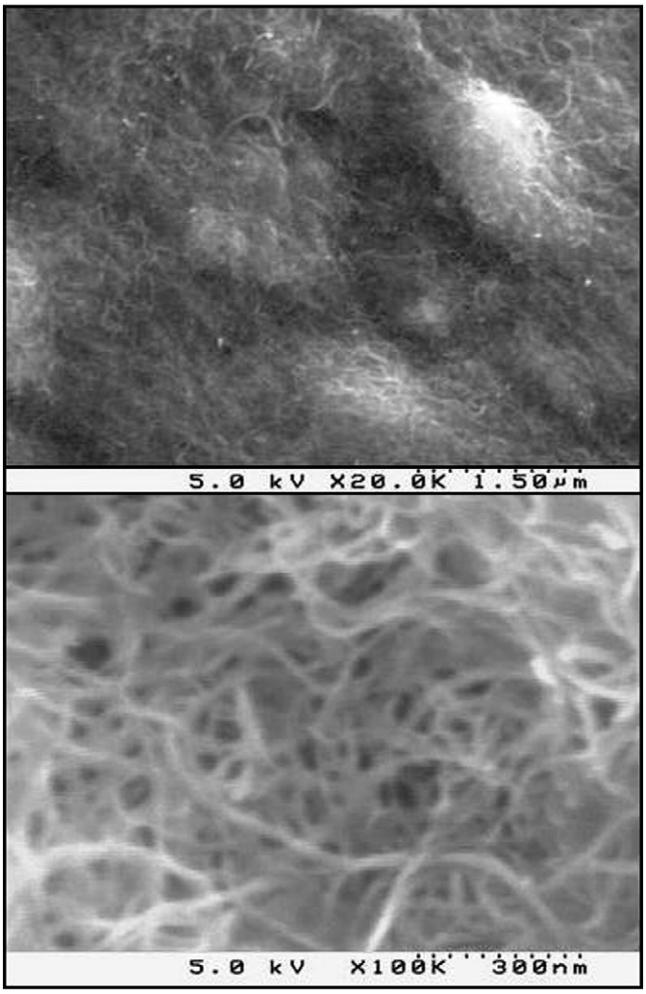

Fig. 1. SEM images of the cellulose–MWCNT matrix.

containing one tightly bound flavin adenine dinucleotide (FAD) per monomer as the redox prosthetic group and can catalyze the oxidation of β-D-glucose by molecular oxygen [20–23]. GOx is especially suitable for application in bioelectronic devices due to its high catalytic activity and the negative redox potential of the enzyme active site. The FAD redox center of GOx, however, is embedded within a protective protein shell, making electron-transfer to the electrode extremely difficult [24]. Attempts to reduce the electron tunneling distance between GOx and the electrode have been investigated by using mediators or by incorporating the enzyme into an electrically conductive matrix.

Fig. 2A shows the cyclic voltammograms (CVs) of a cellulose–MWCNT–GOx modified electrode in a N_2-saturated buffer at increasing scan rates. The anodic and cathodic peak potentials at a scan rate of 100 mV s^{-1} are located at −357 and −386 mV respectively (see Fig. 2B). The peaks are attributable to the reduction and oxidation of the FAD/$FADH_2$ electroactive centre of the GOx enzyme by a direct electron transfer process (reaction 1) [25]; no redox peaks were observed with a enzyme-free cellulose–MWCNT modified electrode in the potential range −650 to 0 mV.

$$GOx(FAD) + 2e^- + 2H^+ \rightleftharpoons GOx(FADH_2) \quad (1)$$

The redox potentials do not vary at the different scan rates, and the separation of the cathodic and anodic peak potentials for each rate is 29 ± 3 mV, which implies that the reaction is a reversible two-electron reversible redox process (reaction 1). The anodic and cathodic currents were both linearly proportional to the scan rate (see inset of Fig. 2A), indicating that the electrode reaction is a surface confined process [26].

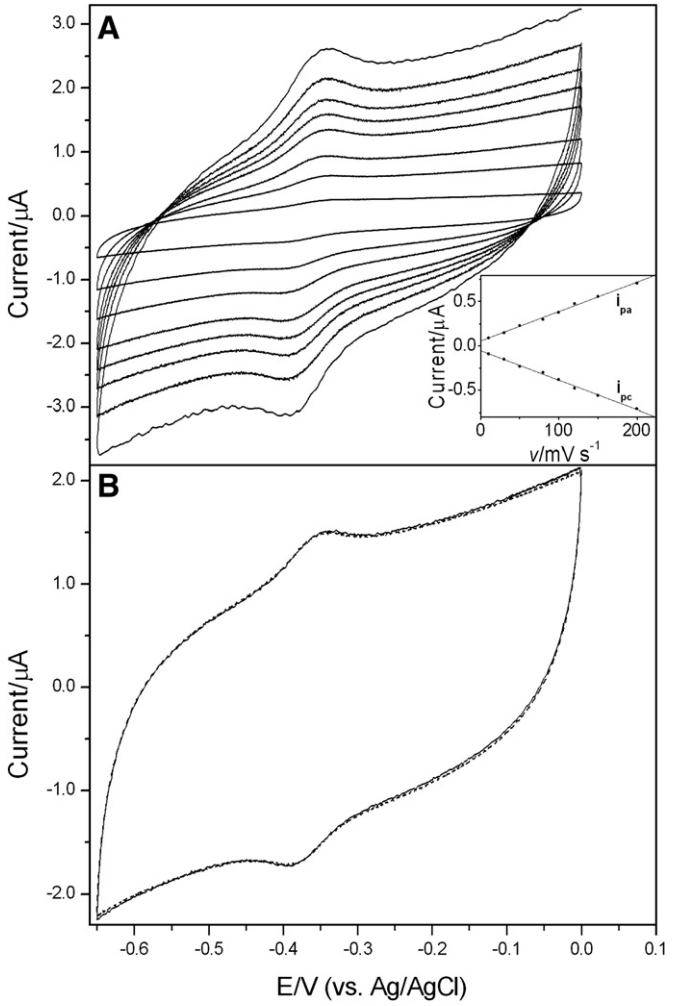

Fig. 2. (A) Cyclic voltammograms of a cellulose–MWCNT–GOx modified electrode in anaerobotic buffer (0.2 M) at pH 6.0. The scan rates were 10, 30, 50, 80, 100, 120, 150, and 200 mV s^{-1} (inner to outer CV). The inset shows the relationship between scan rate and the cathodic and anodic peak currents. (B) Cyclic voltammograms of a cellulose–MWCNT–GOx modified electrode at pH 6.0 and a scan rate of 100 mV s^{-1} under anaerobic condition. The solid line represents the first scan cycle and the dashed line represents the CV after 200 continuous cycles.

3.3. Effect of solution pH on the modified electrode

A series of experiments was performed to study the dependence of potential response on the solution pH. Fig. 3 shows that an increase of pH causes a negative shift in both the reduction and oxidation peak potentials. The linear regression is $E^{o\prime}/V = -0.032–0.058\text{pH}$, where $E^{o\prime}$ is the formal potential $[E^{o\prime} = (E_{pa} + E_{pc})/2]$ and the linear regression coefficient $R = 0.99$. The slope of -58 mV pH^{-1} (Fig. 2, inset) is very close to the theoretical value of -58.5 mV pH^{-1} expected at 22 °C for a reversible, 2-proton and 2-electron transfer process according to the reaction 1 above.

3.4. Biocatalytic activity of the modified electrode

Compared with N_2-saturated buffer (Fig. 4A, curve a), a pair of well defined GOx redox peak was also observed in air-saturated buffer (Fig. 4A, curve c) in the absence of glucose, with an increased reduction peak current and a decreased oxidation peak current. After the addition of glucose (0.5 mM) into the buffer, the reduction peak current decreased significantly (Fig. 4, curve b); this is because the dissolved oxygen and GOx take part in the oxidation of glucose. This

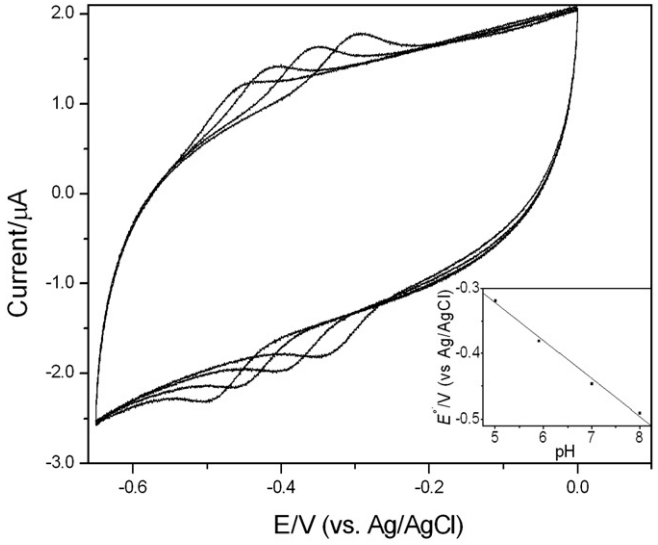

Fig. 3. Cyclic voltammograms of a cellulose–MWCNT–GOx modified electrode in aqueous citrate phosphate buffer (0.2 M) of pH 5.0, 6.0, 7.0 and 8.0 (from left to right) under anaerobic conditions. The scan rate was 100 mV s^{-1}. The inset shows the dependence of $E^{o\prime}$ of GOx on pH.

phenomenon can be explained by the following previously proposed mechanism [27,28]:

$$\text{GOx}(\text{FADH}_2) + \text{O}_2 \rightarrow \text{GOx}(\text{FAD}) + \text{H}_2\text{O}_2 \quad (2)$$

$$\text{GOx}(\text{FAD}) + \text{Glucose} \rightarrow \text{GOx}(\text{FADH}_2) + \text{Gluconolactone} \quad (3)$$

In the presence of oxygen, the reduced enzyme $\text{GOx}(\text{FADH}_2)$ is rapidly oxidized to the oxidized form GOx(FAD) via reaction (2). The electrocatalytic regeneration of the oxidized enzyme GOx(FAD) via electrochemistry reaction (1) causes the loss of reversibility and the increase in size of the cathodic peak current. As the substrate of GOx, the presence of glucose may result in an enzyme-catalyzed reaction according to reaction (3) which will decrease the concentration of the oxidized form of GOx on the electrode surface, leading to the decrease of reduction peak current. These results provide evidence that the GOx,

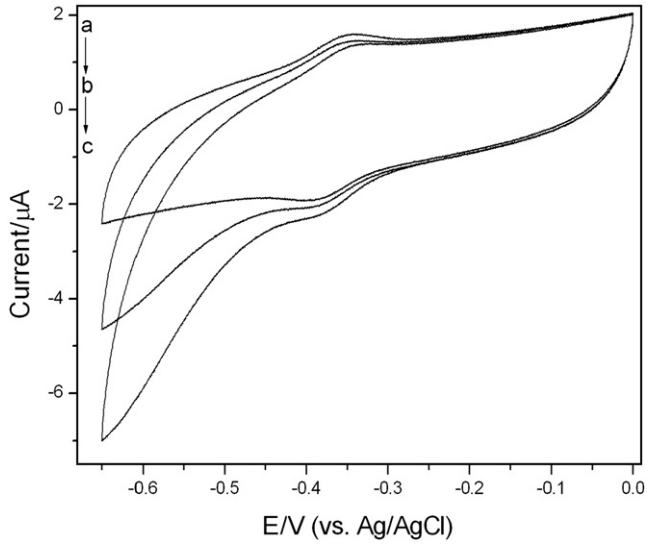

Fig. 4. Cyclic voltammograms of a cellulose–MWCNT–GOx modified electrode in buffer (0.2 M) at pH 6.0 and a scan rate of 100 mV s^{-1} in: (a) anaerobic aqueous citrate phosphate buffer, (b) aerobic buffer with the addition of 0.5 mM glucose and (c) aerobic aqueous citrate phosphate buffer.

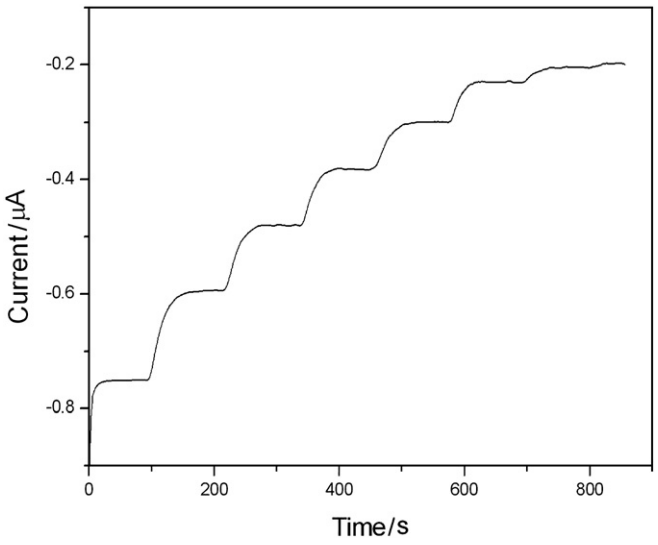

Fig. 5. Current–time curves obtained at the cellulose–MWCNT–GOx modified electrode on successively increasing the glucose concentration in 0.2 mM steps in air-saturated buffer at pH 6.0. The electrode was held at a potential −0.50 V vs. Ag/AgCl.

encapsulated by the RTIL formulated cellulose-MWCNT composite retains its biocatalytic activity towards glucose oxidation. The decrease of the reduction peak current can be employed to determine the glucose concentration.

Fig. 5 shows a typical current–time curve of the GOx electrode on successively increasing the glucose concentration in 0.2 mM steps when the electrode was poised at −500 mV vs Ag/AgCl in air-saturated solution. The current values change linearly with the concentration of glucose from 0.05 to 1.0 mM, with a linear regression equation of I_{pc} (μA) = 0.68–0.46$C_{glucose}$(mM) and a correlation coefficient $R = 0.99$. The sensitivity of the cellulose-MWCNT-GOx electrode is 6.57 μA mM^{-1} cm^{-2}, which is higher than the previously reported 3.3 μA mM^{-1} cm^{-2} on chitosan modified electrodes [26] and 3.47 μA mM^{-1} cm^{-2} on silica gel based electrodes [27].

The apparent Michaelis–Menten constant K_m of the GOx was determined using the Michaelis–Menten equation to be 1.10 mM. The lower value of K_m compared with reported values of 6.8 mM on carbon nanotubes–chitosan modified electrode [29] and 15.19 mM on boron-doped carbon nanotubes modified electrode [30] indicates enhanced enzyme affinity of GOx for glucose, which might be due to the biocompatible microenvironment of the cellulose-MWCNT matrix and a positive effect on protein structure and its biocatalytic activity.

3.5. Reproducibility and stability

The reproducibility and stability of the proposed GOx electrode have also been investigated. The fabrication reproducibility of five electrodes, made independently under the same conditions, showed an acceptable reproducibility for the current determined at 0.2 mM glucose concentrations. The stability of the electrode was tested by repeated CV scanning in a potential range from −650 to 0 mV vs Ag/AgCl at 100 mV s^{-1}; the modified electrode showed an unchanged peak current after 200 cycles (see Fig. 2B). The electrode was tested again after the bioelectrode was stored for 10 days and a decrease of only 11% in peak currents was observed.

These favorable results could be mainly attributed to two factors: firstly, cellulose, endowed with a large amount of –OH groups, provides a biocompatible environment for encapsulation of GOx; secondly, the cellulose–MWCNT matrix possesses a porous structure (Fig. 1) allowing a large amount of enzyme to be immobilized close to the electrode surface, where direct electron communication between active site of enzyme and the electrode is enabled.

4. Conclusions

This study developed a simple procedure for the preparation of a biocompatible cellulose–MWCNT matrix via the RTIL reconstitution process, and that the resulting porous matrix has been employed in the immobilization of GOx. The encapsulated GOx showed good bioelectrochemical activity, enhanced biological affinity as well as good stability and repeatability. The simple electrode fabrication methodology and the biocompatibility of the cellulose–MWCNT matrix mean that the immobilization matrix cannot only be used for GOx but can also be extended to other proteins (e.g., laccase and hydrogenase), thus providing a promising platform for further research and development of biosensors and other bioelectronics devices. Further investigations into electrochemical properties of the cellulose–MWCNT matrix and immobilization process with different enzymes, with the objective of application in enzymatic fuel cells, are underway.

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