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Electrochemical, EIS and AFM characterisation of biosensors: Trioxysilane sol–gel encapsulated glucose oxidase with two different redox mediators

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Abstract

Sol–gel encapsulated glucose oxidase (GOx) enzyme electrodes based on carbon film resistors with chemically deposited copper hexacyanoferrate (CuHCF) or poly(neutral red) (PNR), made by electrochemical polymerisation, as redox mediator have been developed and characterised using cyclic voltammetry, electrochemical impedance spectroscopy and atomic force microscopy. The sol–gel was prepared using three different trioxysilanes: 3-aminopropyl-triethoxysilane (APTOS), 3-glycidoxypropyl-trimethoxysilane (GOPMOS) and methyltrimethoxysilane (MTMOS), without alcohol addition, and alcohol formed during the hydrolysis of the precursor compounds was removed. The best sensitivity, $\sim$60 nA mM^{-1}, for glucose and limit of detection (2–40 μM, depending on the sol–gel precursor) were obtained when PNR was used as a mediator, but the linear range (50–600 μM) was two to four times lower than that at CuHCF mediated biosensors, using an operating potential of +0.05 V at CuHCF or −0.25 V versus saturated calomel electrode (SCE) at PNR mediated electrodes. The stability of the sensor depended on the sol–gel morphology and was 2 months testing the biosensor every day, while the storability was at least 4 months in the case of GOPMOS, the sensors being kept in buffer at +4 °C.

Keywords: Oxysilane sol–gel; Amperometric biosensor; Copper hexacyanoferrate; Poly(neutral red); Electrochemical impedance spectroscopy; Atomic force microscopy

1. Introduction

Sol–gel enzyme encapsulation has become a more and more attractive method for the development of biosensors during the past decade [1,2] in electrochemical [3–18] and optical biosensors [19–22]. Numerous sol–gel composites for glucose oxidase entrapment have been reported in the literature, e.g. [5,6,8,14–18]. The most used oxysilanes for the preparation of sensors are usually tetraoxysilanes [6–13,15,16,19,20,23,24], and trioxysilanes such as 3-aminopropyl-triethoxysilane [12,14], 2-(3,4-epoxycyclohexyl)-ethyltrimethoxysilane [13,14] and methyltrimethoxysilane [2,12,13,16,17]. Enzyme encapsulation in sol–gel rather than in other matrices improves some of their properties such as activity, sensitivity and longer linear response range to analyte, as compared to cross-linking with glutaraldehyde [1,2,13]. However, such electrodes with encapsulated enzymes have also shown interference problems due to high operating potentials at different electrode substrates. To decrease the operating potential, mediators were usually applied in sol–gel biosensors, for example, methylviologen [25], ferrocene [2,6], tetrathiafulvalene [3] and copper hexacyanoferrate [26].

Various electrode substrates have been used in the preparation of electrochemical sol–gel biosensors [1–3], but the majority are different types of carbon electrode, particularly graphite [27] including screen printed electrodes [28], carbon paste [5,7,29], glassy carbon [11] and carbon composite electrodes [30]. Recently carbon film electrodes have been introduced for use in biosensors [30–41]. Carbon films are obtained by coating a ceramic substrate by a thin deposit of pyrolytic carbon [34,35] or by sputtering of graphite [31,38]. Electrodes made from carbon film electrical resistors have the anisotropic non-

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porous properties of glassy carbon, a large potential window after pre-treatment, are reproducible and do not need polishing before use [34]. Such electrodes are inexpensive and offer an easy way for developing a glucose oxidase-based glucose biosensor, but require a high overpotential to oxidise hydrogen peroxide, which reduces sensor selectivity [37]. To improve the selectivity, electrochemical biosensors have been modified with redox mediators, particularly with Prussian Blue analogues [26,36] and methylviologen [41].

In this work trioxysilane-based sol–gel encapsulated glucose oxidase biosensors on a carbon film electrode substrate with copper hexacyanoferrate (CuHCF) or poly(neutral red) (PNR) mediator have been developed and characterised electrochemically by cyclic voltammetry and electrochemical impedance spectroscopy using an improved method for sol–gel enzyme layer formation compared to [26]; additionally, in [26] only copper hexacyanoferrate was investigated as mediator. Sol–gel layers from two of the oxysilane precursors: 3-aminopropyl-triethoxysilane and 3-glycidoxypropyl-trimethoxysilane which were applied in biosensors for the first time in [26], are compared here with the well-known sol–gel based on methyltrimethoxysilane with both CuHCF and the new PNR mediator.

2. Experimental

2.1. Chemicals and solutions

Three different trioxysilane solutions were used for enzyme encapsulation: 3-aminopropyl-triethoxysilane (APTOS) obtained from Fluka (Switzerland), 3-glycidoxypropyl-trimethoxysilane (GOPMOS) and methyltrimethoxysilane (MTMOS) from Aldrich (Germany). Glucose oxidase (GOx) from *Asperigillus niger* (EC 1.1.3.4), anhydrous α-D-(+)-glucose crystals, bovine serum albumin (BSA) and 5% Nafion® solution in alcohols were obtained from Sigma (Germany). $CuCl_2{\cdot}2H_2O$ and $K_3Fe(CN)_6$ were from Merck (Germany).

Electrolyte solution, 0.1 M phosphate buffer saline (PBS), pH 7.0, was prepared from sodium dihydrogenphosphate and disodium hydrogenphosphate (Riedel-de-Haën, Germany), to which 0.05 M NaCl was added. Millipore Milli-Q nanopure water (resistivity > 18 MΩ cm) was used for preparation of all solutions. Experiments were performed at room temperature, 25 ± 1 °C.

2.2. Electrode preparation

Electrodes were made from carbon film resistors (2 Ω nominal resistance) as described previously [33,34]. The resistors were fabricated from ceramic cylinders of external diameter 1.5 mm and length 6.0 mm by pyrolytic deposition of carbon. One of the tight fitting metal caps at each end, joined to thin conducting wires, was removed from the resistor and the second was sheathed in a plastic tube gluing it and the cap/cylinder contact with epoxy resin. In this way the exposed electrode geometric area was ∼0.20 cm^{-2}. Before use electrodes were electrochemically pre-treated by cycling between 0.0 and +1.0 V versus saturated calomel electrode (SCE) in 0.1 M KCl solution (in the case of chemical deposition of copper hexacyanoferrate mediator) or between −1.0 and +1.0 V versus SCE in 0.1 M KNO_3 solution (prior to electropolymerisation of neutral red) for not less than five cycles, until stable cyclic voltammograms were obtained.

Copper hexacyanoferrate was chemically deposited by immersing the electrodes for 50 min in a solution containing 10 mM Cu^{2+}, 10 mM $K_3Fe(CN)_6$ and 100 mM KCl. After this, the electrodes were dried in a hot air stream for 3 min and left for 24 h to stabilize.

Poly(neutral red) was prepared by polymerising electrochemically from 1 mM of its monomer (neutral red) aqueous solution in 0.05 M phosphate buffer, pH 5.5, plus 0.3 M KNO_3, cycling the applied potential from −1.0 to 1.0 V 10 times at a potential sweep rate of 50 mV s^{-1}; and they also were left for 1 day in air at room temperature before use.

Sol–gel solution was prepared by mixing one of the oxysilanes with water or, in the case of MTMOS, pH 5.5 0.1 M phosphate buffer in optimised ratios [26]—APTOS:H_2O, 220:580 μl; GOPMOS:H_2O, 200:600 μl; and MTMOS:H_2O, 180:620 μl. To each mixture 2 μl of 1 M HCl solution was added. The mixtures obtained were intensively stirred for a few minutes and then sonicated for 15 min. Following this, the solutions were heated (except MTMOS, since after heating for a few minutes the components of the mixture separated and there was rapid gelation) to evaporate the alcohol formed during hydrolysis of the oxysilanes [42] in a hot air stream (∼70 °C) for a short period of time until the solutions lost 40% of their volume. They were then left for an hour at room temperature to cool down and neutralized to pH 7.0 if necessary. A volume of 50 μl of each solution was then carefully mixed with 15 μl of GOx (10%) solution in 0.1 M PBS solution, pH 7.0, and left for 2 h to equilibrate. Then the CuHCF- or PNR-coated carbon film electrodes were immersed in the sol–gel enzyme solutions for 5 min, removed and left for sol–gel formation at 4 °C for 3 days. Electrodes were stored at 4 °C when not in use.

2.3. Methods and instruments

The three-electrode electrochemical cell contained a sol–gel encapsulated enzyme carbon film working electrode, a platinum foil as counter electrode and a saturated calomel electrode as reference. Measurements were performed using a computer-controlled μ-Autolab Type II potentiostat/galvanostat with GPES 4.9 software (Eco Chemie, Netherlands).

Electrochemical impedance measurements were carried out in the same electrochemical cell with a PC-controlled Solartron 1250 frequency response analyser coupled to a Solartron 1286 electrochemical interface using ZPlot 2.4 software (Solartron Analytical, UK). A sinusoidal voltage perturbation amplitude 10 mV was applied, scanning from 65 kHz to 0.1 Hz with 10 points per frequency decade, integration time 120 s. Fitting to equivalent circuits was performed with ZView 2.4 software.

AFM measurements were performed with a Pico SPM controlled by a MAC Mode module and interfaced with a PicoScan controller (Molecular Imaging, USA) and silicon type II MAClevers of 225 μm length, tip radius of curvature less

than 10 nm, 2.8 N m^{-1} spring constant and 60–90 kHz resonant frequencies in air (Molecular Imaging, USA) were used. HOPG, grade ZYH, from Advanced Ceramics (USA) was used as a substrate for sol–gel deposition. All images (256 samples line^{-1} × 256 lines) were taken at room temperature, with scan rates of 1.0–2.5 lines s^{-1}. The images were processed by flattening in order to remove the background slope, and the contrast and brightness were adjusted.

3. Results and discussion

3.1. Preparation and voltammetric characterisation of sol–gel enzyme biosensors with redox mediator

Biosensors were prepared by sol–gel enzyme encapsulation using the three different oxysilane precursors on top of a layer of CuHCF or PNR mediator, by the new sol–gel preparation procedure involving removal of the alcohol. They were then characterised in 0.1 M PBS solution, pH 7.0, using cyclic voltammetry.

3.1.1. Copper hexacyanoferrate

Fig. 1 shows the voltammetric behaviour of CuHCF in 0.1 M PBS before and after sol–gel coating. Prior to sol–gel enzyme layer deposition CuHCF was deposited chemically as described in Section 2. The reproducibility of the CuHCF layer at different carbon film resistor electrodes was examined by cyclic voltammetry in 0.1 M KCl solution by comparing the magnitude of the peak currents and was found to be 96 ± 2% (n = 9). CuHCF exhibited reversible behaviour even in pH 7.0 PBS solution although the peak shape and height were not as constant as in KCl solution, and the peak separation was larger, i.e. 85 mV in PBS solution, while in KCl solution it was 61 mV [43,44].

The voltammetric behaviour at the sensors prepared using the three sol–gel precursors with a CuHCF mediator layer differs slightly. This could be caused by the differences in the structure of the oxysilane precursors. MTMOS has the simplest structure of the three compounds with three methoxy groups

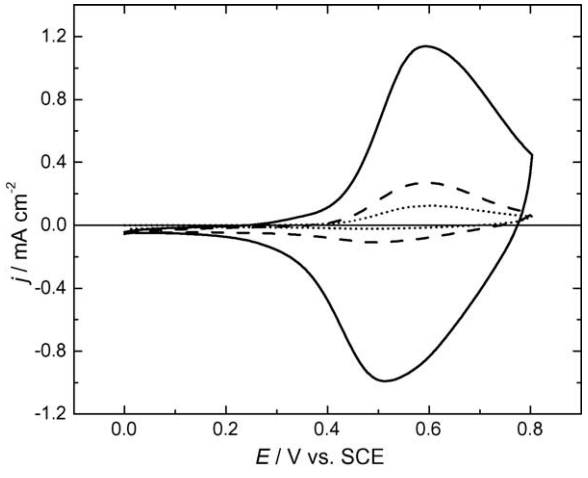

Fig. 1. Cyclic voltammograms, scan rate 50 mV s^{-1}, in 0.1 M PBS, pH 7.0, of: (—) CuHCF, (- - -) MTMOS-based sol–gel encapsulated GOx film applied on top of CuHCF mediator layer and (· · ·) after addition of 10 mM glucose to the buffer solution.

and one methyl group in tetrahedral positions around the Si atom. APTOS and GOPMOS have a more complex structure: APTOS has three ethoxy groups and the fourth position is the aminopropyl group which exhibits higher chemical activity than a methyl group. Since it was reported that this sol–gel can make strong covalent bonds with organic and inorganic species [45], it could be a good material for enzyme encapsulation. The most complex oxysilane used in this study, GOPMOS, has three ethoxy groups and a relatively long chain of three carbons and then the glycidoxy group which has an epoxy ring at the end. The epoxy group exhibits chemical activity and can react with amino groups in other compounds [46]. Both APTOS and GOPMOS can also be expected to lead to a more open polymeric structure than MTMOS due to the length of the aminopropyl and glycidoxypropyl groups, respectively, which may be better for enzyme encapsulation.

The APTOS sol–gel encapsulated enzyme layer was different from the other two in its behaviour, owing to its amino group, which probably reacts with the mediator forming copper complexes leading to slow dissolution of the mediator layer. The peak current due to oxidation and reduction of the CuHCF layer decreases initially by almost 80% and more so with each voltammetric cycle, and the peak separation is much higher, i.e. the reversibility of the mediator is much poorer than that without sol–gel, as in [26]. To reduce these effects, Nafion was applied between the CuHCF layer and APTOS–GOx to try to prevent CuHCF complexation. In addition, APTOS interacts with electrolyte solution due to hydrophilic sol–gel formed with this precursor [12]. In previous work with APTOS and to circumvent this problem, Wang et al. [12] used sol–gel with this oxysilane as the most suitable precursor for the determination of phenols, but they worked in non-aqueous solution. Additionally, in [14], a sol–gel glucose biosensor was developed, which successfully worked in PBS, pH 7.4, but mixed precursors were used, APTOS as a hydrophilic precursor together with the relatively hydrophobic 2-(3,4-epoxycyclohexyl)-ethyltrimethoxysilane. However, after heating of the hydrolysed sol–gel solution to evaporate the alcohol formed, the sol–gel was much less hydrophilic and did not dissolve into solution. APTOS is not compatible with CuHCF, and even with the intervening Nafion layer it negatively affects the mediator more than other sol–gels. It was found that the CuHCF redox peak current after sol–gel enzyme layer deposition decreased to 65% and still decreased slightly with each potential cycle.

The other two hydrophobic oxysilanes did not show any incompatibility with the CuHCF mediator, so did not dissolve in PBS solution.

The sol–gel formed using GOPMOS has a similar effect on cyclic voltammetric peak currents as APTOS with a decrease in peak current of 70% (not shown). However, the current corresponding to the CuHCF redox couple does not further decrease during repeated potential cycling with the GOPMOS GOx encapsulated sol–gel layer, contrary to what was observed for APTOS.

The MTMOS-based sol–gel, which has not been examined previously in these CuHCF sensor assemblies, was also compatible with CuHCF mediator and did not dissolve in solution

but caused a different change to CuHCF's electrochemical behaviour; see Fig. 1. Although the current was reduced by 75% and redox peak separation and shape remained the same as at bare CuHCF, the reduction peak decreased much more in size than the oxidation peak (Fig. 1, dashed curve). The smallest changes in the shape of the cyclic voltammograms of CuHCF on depositing the sol–gel layer were obtained using MTMOS as a sol–gel precursor, since it has a simple structure and forms a silane net at the electrode surface without any further chemical reaction with mediator or enzyme.

Fig. 1 also shows the electrochemical behaviour of the same biosensors after addition of 10 mM glucose to PBS. Changes caused by addition of glucose are the same for all three sol–gel types, i.e. the current decreased, but no additional peak appeared for glucose/H_2O_2 as in [47], where glucose was determined at a non-mediated ceramic–carbon composite electrode with GOx encapsulated by the sol–gel technique. In the case of CuHCF, when the sensor is used at neutral pH and CuHCF catalyses reduction of the hydrogen peroxide formed during enzymatic reaction, hydroxyl ions are formed:

$$H_2O_2 + 2e \rightarrow 2OH^- \quad (1)$$

which leads to a decrease in height of the redox peaks of CuHCF [26,43].

3.1.2. Poly(neutral red)

Very similar tendencies as for CuHCF were observed with poly(neutral red) films as mediators. Nevertheless, none of the three sol–gel precursors interacted chemically with it and so it was not necessary to apply Nafion between mediator and sol–gel film, representing a significant advantage for application as a biosensor.

Fig. 2 illustrates the voltammetric behaviour at the three different PNR mediated sol–gel biosensors in 0.1 M PBS solution, pH 7.0, together with that at an uncoated poly(neutral red) electrode. The first quasi-reversible oxidation peak of PNR at −0.4 V versus SCE is attributed to oxidation of polymer, and the last oxidation wave at +0.8 V is due to irreversible oxidation of the monomer. The redox couple in between these two peaks, i.e. between −0.1 and +0.4 V, is due to deprotonation of an ionogenic group [48,49]. The reproducibility of PNR at different modified carbon film electrodes was found to be similar to that with CuHCF mediator and was 95 ± 3% ($n = 5$).

Deposition of sol–gel-GOx on the top of the PNR layer does not change the shape of the voltammograms, but the current diminishes over the whole potential range. In the case of APTOS and GOPMOS, a very similar sol–gel effect was obtained, i.e. the current decreased by a factor of 5 compared to that at bare PNR (Fig. 2A and B). However, due to its high hydrophobicity, MTMOS deposition onto PNR led to a 10-fold decrease in current (Fig. 2C). The addition of an aliquot of 10 mM glucose to the buffer solution caused an increase in oxidation peak current of the polymer as would be expected and as occurred in the case of GOx immobilized by cross-linking with glutaraldehyde [49]. No additional specific peak for glucose oxidation was obtained.

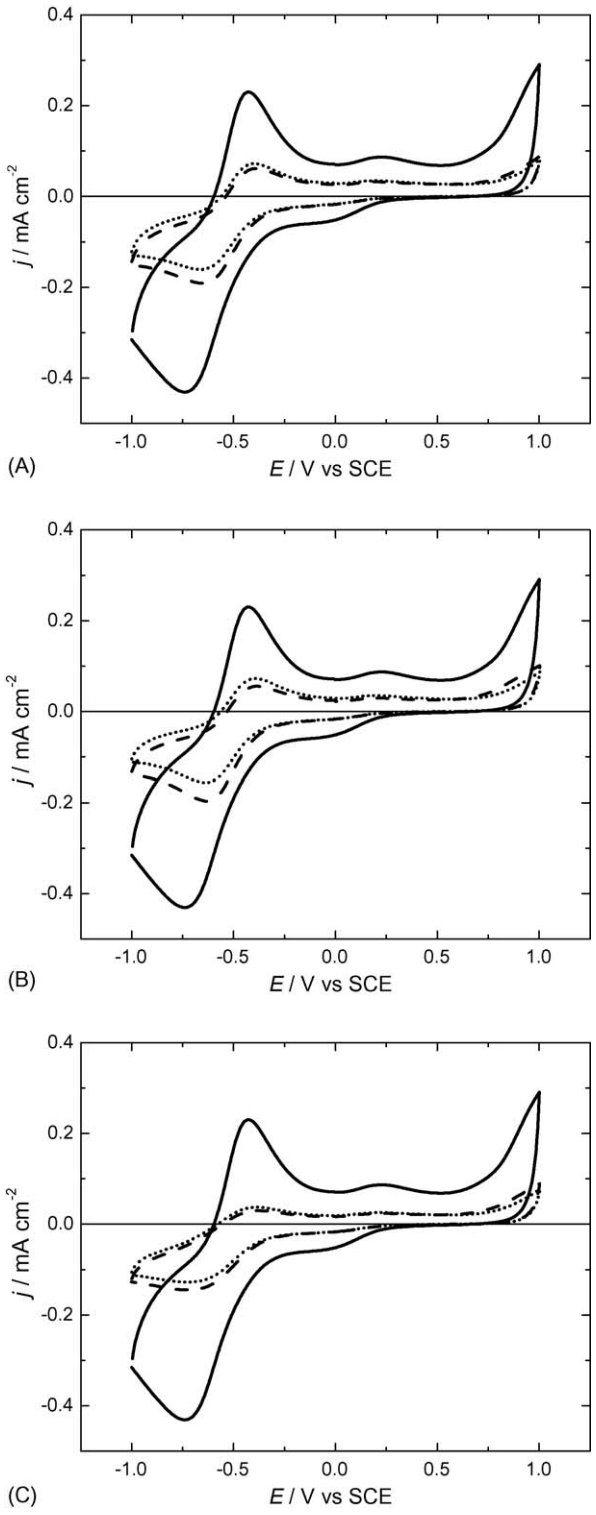

Fig. 2. Cyclic voltammograms, scan rate 50 mV s^{-1}, in 0.1 M PB, pH 5.5, of: (—) PNR, (- - -) sol–gel encapsulated GOx film applied on top of PNR mediator layer and (· · ·) after addition of 10 mM glucose to the buffer solution. Sol–gel precursors: (A) APTOS, (B) GOPMOS and (C) MTMOS.

3.2. Characterisation of sol–gel electrodes by EIS

Complex plane plots of electrochemical impedance obtained at all three sol–gel sensors, covered with a Nafion layer to pre-

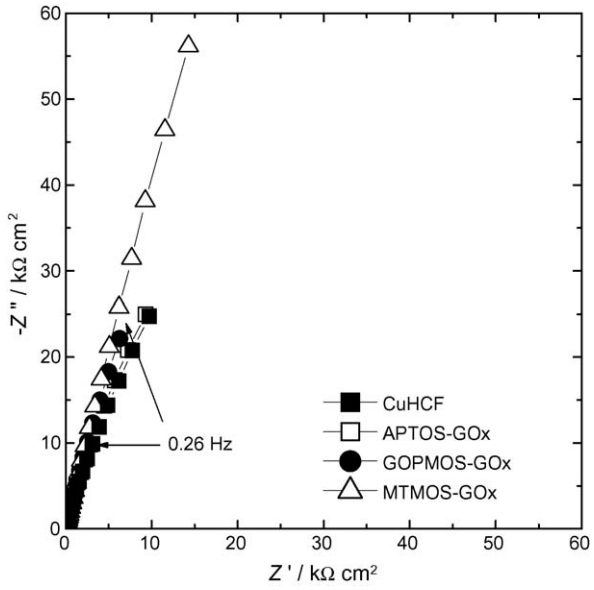

Fig. 3. Complex plane impedance spectra at a sol–gel enzyme layer based on APTOS, GOPMOS and MTMOS deposited on top of CuHCF mediator layer. Supporting electrolyte 0.1 M PBS solution, pH 7.0, at +0.05 V vs. SCE.

vent damage to the enzyme during measurements, are presented in Figs. 3 and 4. The impedance spectra were recorded at +0.05 V versus SCE for CuHCF mediator and −0.25 V versus SCE for PNR mediator, the operating potential of the biosensors. The same equivalent circuit model was used for fitting all spectra, consisting of the cell resistance, R_Ω, in series with a parallel combination of a charge transfer resistance, R_{ct}, and a constant phase element, CPE, modelled as a non-ideal capacitance, according to

$$\mathrm{CPE} = \frac{1}{(Ci\omega)^\alpha} \quad (2)$$

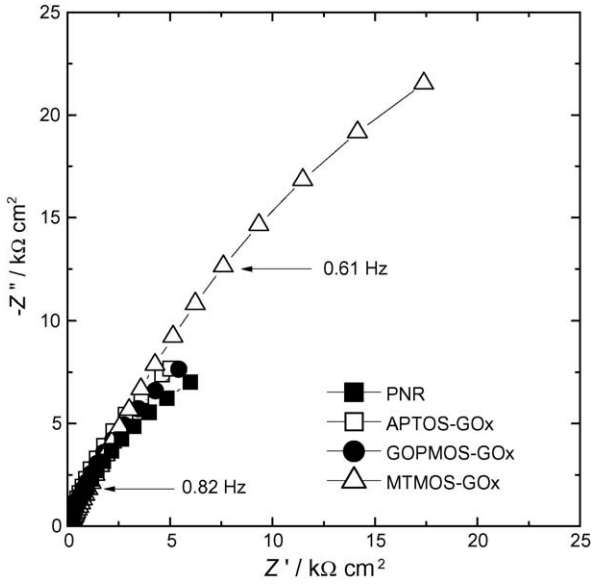

Fig. 4. Complex plane impedance spectra at a sol–gel enzyme layer based on APTOS, GOPMOS and MTMOS deposited on top of PNR mediator layer. Supporting electrolyte 0.1 M PBS solution, pH 7.0, at −0.25 V vs. SCE.

Table 1
Analysis of the electrochemical impedance data at carbon film electrodes

	R_{ct} (kΩ cm^2)	C (μF cm^{-2} s$^{\alpha-1}$)	α
+CuHCF	156	74.4	0.86
+Sol–gel			
APTOS	613	57.0	0.84
GOPMOS	660	65.0	0.86
MTMOS	571	26.8	0.87
+PNR	56	57.9	0.83
+Sol–gel			
APTOS	29	124	0.82
GOPMOS	28	134	0.81
MTMOS	20	101	0.84

Values of R_Ω vary from 7.0 to 8.5 Ω cm^2. The values of the other parameters are shown in Table 1. Values of α are approximately constant at around 0.85, as for the bare carbon film and as expected [31].

As was shown in [26], spectra at bare carbon film and CuHCF modified electrodes exhibit some reduction in the magnitude of the impedance at the modified surface, corresponding to a higher capacitance (23.3 μF cm^{-2} s$^{\alpha-1}$ at bare carbon film and 74.4 μF cm^{-2} s$^{\alpha-1}$ at CuHCF modified electrode), due to limitations of charge movement through the hexacyanoferrate film. The capacitance also increased at PNR modified electrodes compared to the bare carbon film to 57.9 μF cm^{-2} s$^{\alpha-1}$ and there was a large reduction in the value of R_{ct}. Only slight visible differences in the spectra were observed in the case of both CuHCF- and PNR modified electrodes without and with encapsulated GOx in sol–gel based on APTOS and GOPMOS, but in the case of MTMOS impedance values obtained were similar to those at the bare carbon film.

With respect to CuHCF mediated electrodes, values of R_{ct} with sol–gel were higher than at CuHCF only, similar for all three sol–gels, in agreement with the observed chemical interaction between the sol–gel layer and the CuHCF mediator layer. Values of C were lower with APTOS and GOPMOS sol–gels but significantly less with MTMOS (57.0, 65.0 and 26.8 μF cm^{-2} s$^{\alpha-1}$, respectively). Addition of glucose to the buffer solution did not change the capacitance value at APTOS-based sol–gel, but decreased a little at the GOPMOS sol–gel and increased at MTMOS-based biosensors.

A similar tendency was obtained at the sol–gel electrodes which had PNR mediator. PNR, polymerised on the top of the carbon film resistor electrodes, led to a significant decrease of the impedance value compared to the bare carbon film electrode. Moreover, with the PNR layer there was a big decrease in the charge transfer resistance on adding the sol–gel (Table 1), an opposite effect to that observed with CuHCF mediator. The reason for this difference and easier charge transfer can be attributed to the lack of any tendency for mediator degradation as well as some contribution from direct electron transfer to the enzyme and the different operating potential. Capacitance values were much higher than at the bare PNR mediator.

The differences between spectra for the sol–gels based on different oxysilanes could be caused not only by their differ-

ent structures but also by the different compositions of their solutions (see Section 2) [2]. Unfortunately, it is not easy to compare our results with the data from the literature because most sol–gel electrodes were studied before by EIS either as a corrosion protection layer or for developing zeolite-like structures or batteries. However, similar results were obtained by Szu and Lin at copper-doped silica glass [50].

3.3. Glucose determination with sol–gel biosensors

The amperometric response to glucose at different sol–gel biosensors was recorded in the same buffer solution as for characterisation—0.1 M PBS, pH 7.0. Calibration curves are shown in Fig. 5 and data from analysis of the curves are given in Table 2. The highest sensitivity and the lowest limit of detection were observed at the biosensor with encapsulated GOx using GOPMOS as sol–gel precursor and CuHCF as a mediator as found in [26], except that the sensitivity there was higher since a slightly different sol–gel preparation protocol was used. However, the biosensor in [26] had an inferior long-term stability. In

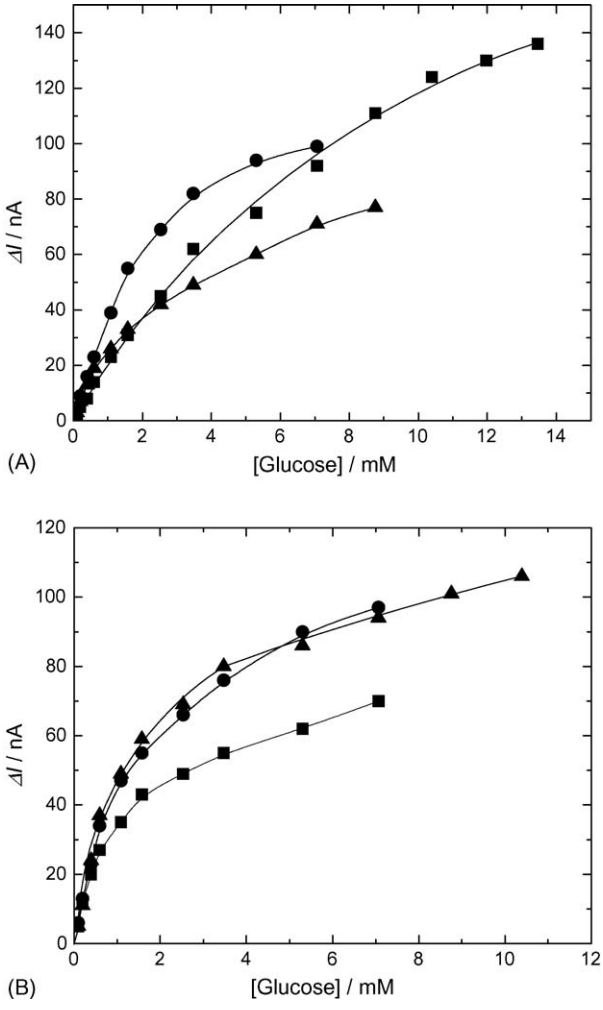

Fig. 5. Calibration curves for glucose at different sol–gel encapsulated GOx electrodes: (■) APTOS, (●) GOPMOS and (▲) MTMOS, in 0.1 M PBS, pH 7.0. (A) CuHCF mediator at +0.05 V vs. SCE and (B) PNR mediator at −0.25 V vs. SCE.

Table 2
Data calculated from calibration curves in Fig. 5

Sol–gel	Linear range (mM)	Sensitivity (nA μM^{-1})	K_M (mM)	Limit of detection (μM)
CuHCF				
APTOS	0.1–2.5	18.0 ± 0.4	3.7	38.0
GOPMOS	0.1–1.6	34.6 ± 0.8	1.4	22.3
MTMOS	0.05–1.5	32.1 ± 0.1	2.2	24.4
PNR				
APTOS	0.05–0.60	53.1 ± 0.2	1.0	38.7
GOPMOS	0.05–0.40	57.9 ± 1.0	1.0	37.8
MTMOS	0.05–0.60	64.8 ± 0.7	1.2	11.7

the case of PNR mediator, the sensitivity with GOPMOS sol–gel was a little bit lower than MTMOS and a little bit bigger than at APTOS-based biosensor (Table 2). A very high sensitivity for glucose was obtained using a PNR mediated system in [49] equal to 700.5 nA mM^{-1}, but the operating potential was more negative, −0.35 V versus Ag/AgCl (saturated KCl). The lowest sensitivity and limit of detection and the biggest linear range were observed at the widely used APTOS-based sol–gel, with CuHCF mediator. The relative standard deviation was found to be ∼3% ($n = 5$) in the case of all electrodes where CuHCF served as a mediator. The biosensors mediated by PNR had a slightly lower relative standard deviation value, i.e. ∼2% ($n = 5$), but the linear range was much lower as well as the limit of detection for glucose.

The Michelis–Menten constant obtained using Lineweaver–Burke linearisation (see Table 2) was higher at the electrodes with CuHCF and differed from one electrode to another depending on the sol–gel precursor, while it always remained around 1 mM at electrodes based on PNR. These analytical data show that the influence of the sol–gel precursor on the response to the analyte depends on the mediator and, most probably, on the mechanism of the mediated reaction. CuHCF catalysed reduction of product of the enzymatic reaction, H_2O_2, and PNR had a more complex mechanism. The reproducibility of the biosensors with CuHCF was slightly less than with PNR and it was from 89.2% (MTMOS) to 93% (GOPMOS) and from 91% (MTMOS) to 94% (GOPMOS) at CuHCF- and PNR-based biosensors, respectively.

In order to demonstrate long-term stability, the response to glucose was measured once per day. The stability of the sol–gel biosensors was found to be in the same sequence as the sensitivity, i.e. GOPMOS > APTOS > MTMOS, and under such conditions a GOPMOS-based biosensor was stable for 2 months; see Fig. 6. It is also of interest to note that in the case of CuHCF–MTMOS biosensors, maximum response is reached after a period of 1 day, which can be interpreted in terms of rearrangement of the polymer structure allowing easier access to the enzyme active site. All electrodes, when not in use, were kept in buffer at +4 °C and, under these conditions, were stable for at least 4 months. If electrodes were stored under dry conditions the sol–gel cracked after 1 week which decreased the activity of the enzyme as reported in [2]. However, stable electrodes were reported by Noguer et al. under dry storage conditions at

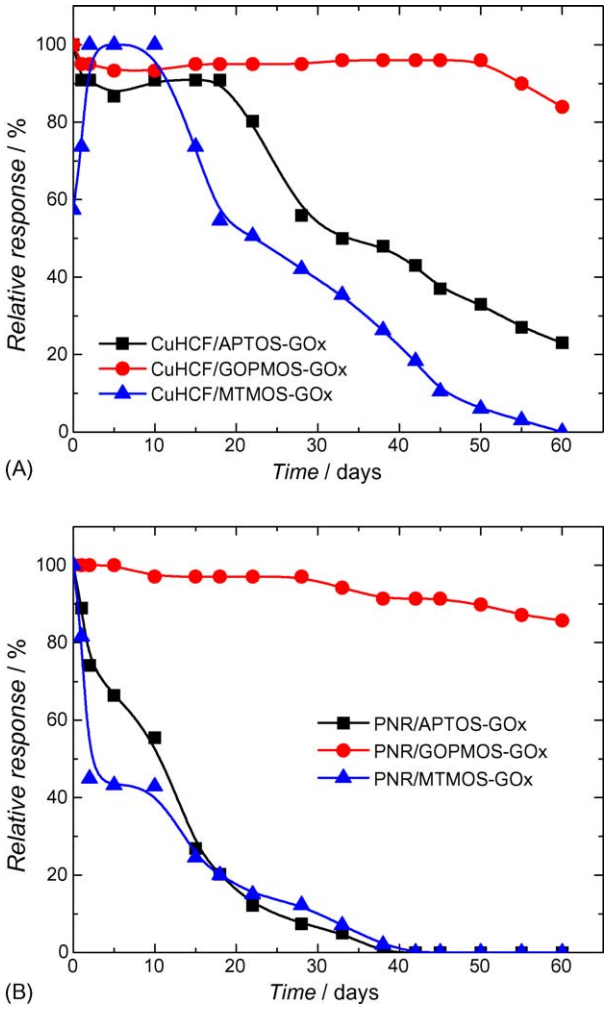

Fig. 6. Relative response to 0.4 mM glucose at different sol–gel encapsulated GOx electrodes: (■) APTOS, (●) GOPMOS and (▲) TEOS, vs. time with (A) CuHCF or (B) PNR redox mediators. Experimental conditions as in Fig. 5.

−18 °C [28]. Also the effect of the mediator on stability was examined—biosensors based on CuHCF were more stable than those with PNR.

Stability data can be explained with reference to the morphology of the sol–gel based on AFM studies. Fig. 7 shows typical AFM images of the sol–gel surface deposited onto highly oriented pyrolytic graphite (HOPG). MTMOS has both large pores (see Fig. 7A) of approximately 350 nm upper diameter and smaller pores of ∼60 nm in diameter, and the enzyme is probably leaching out through these larger pores. APTOS show many small pores of 30–60 nm in diameter and just a few very large pores with sizes between 600 and 800 nm in diameter (see Fig. 7B), which can also be the cause of instability in time due to enzyme leakage. The GOPMOS surface (Fig. 7C) which is much flatter than for MTMOS- and APTOS-based sol–gels (note the vertical scale) presents no big pores in the AFM images, just small ones of less than 50 nm diameter, that do not allow enzyme to escape but allow penetration through to the substrate. The decrease in signal that appears after 2 months is probably due to deactivation of the enzyme.

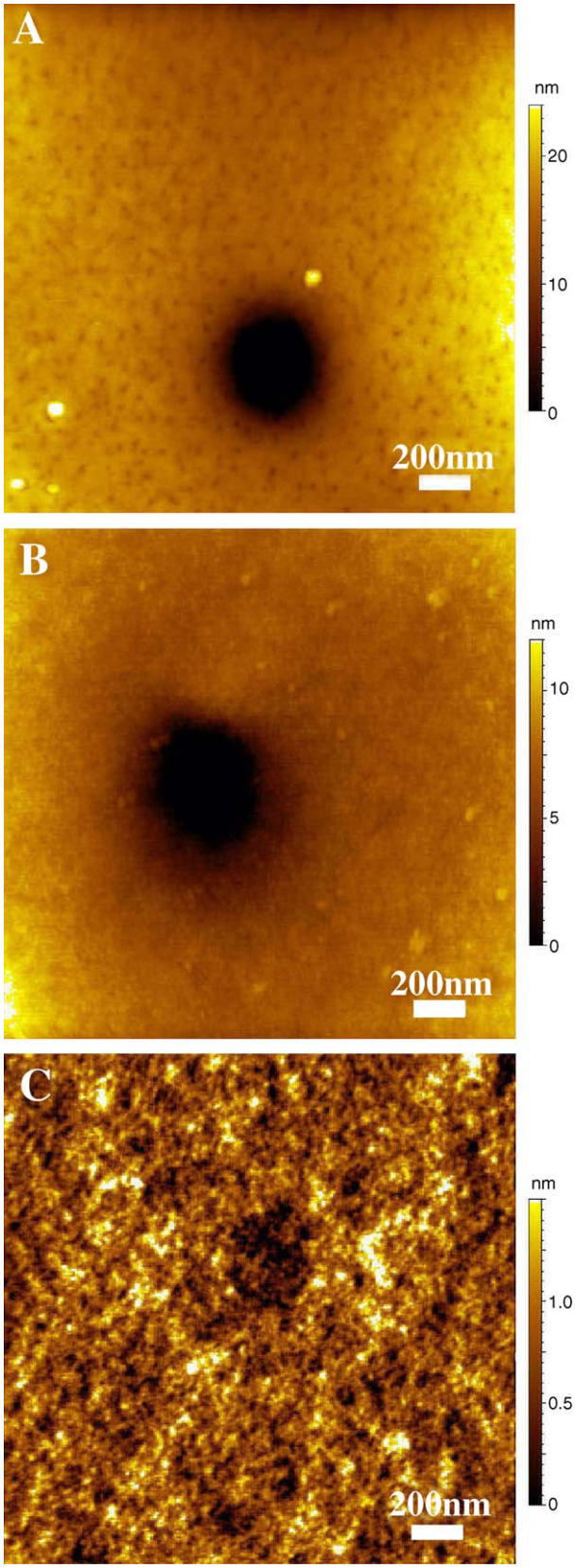

Fig. 7. MAC Mode AFM topographical images in air of sol–gel deposited onto HOPG: (A) APTOS, (B) GOPMOS and (C) MTMOS. Sol–gel solutions prepared using the protocol described in Section 2.

The GOPMOS-based glucose biosensor was applied to glucose determination in red wine using the standard addition method. Due to some interference from ascorbate [26,44], the response at CuHCF mediated electrodes was found to be higher (2.9 mM) than that obtained using the standard spectrophotometric enzyme assay (2.2 mM) [51]. At PNR mediated electrodes, there is good agreement between the two methods.

Other interference studies showed that sol–gel encapsulated GOx is not itself the cause of the interference by some other sugars or carboxylic acids—in particular, carboxylic acids decrease the glucose signal at PNR-based electrodes [49]. This problem, when the analyte is a neutral molecule such as glucose, could be solved by application of a permselective coating over the sol–gel enzyme layer.

4. Conclusions

Sol–gel encapsulated glucose oxidase enzyme electrodes based on carbon film resistors with chemically deposited CuHCF or PNR as a mediator were prepared and characterised using cyclic voltammetry, electrochemical impedance spectroscopy and atomic force microscopy. Sol–gel was prepared using three different oxysilanes: APTOS, GOPMOS and the well-known MTMOS, without any alcohol addition and with the additional step of removal of the alcohol formed to protect the enzyme from deactivation.

Results obtained showed that APTOS and GOPMOS exhibit similar electrochemical properties, but they differ from those obtained at MTMOS; impedance spectra clearly demonstrate that there are differences. The sensing properties of the sol–gel biosensors, particularly sensitivity and limit of detection, can be described by the sequence: APTOS < MTMOS < GOPMOS in the case of CuHCF mediator and GOPMOS < APTOS < MTMOS, with PNR mediator. GOPMOS-based sol–gel glucose biosensors are stable for about 2 months when stored in buffer solution at +4 °C; the stability of the biosensor was shown by AFM to be related to the sol–gel morphology. Finally, the linear range of the biosensor depends mainly on the mediator and was longer at CuHCF mediated electrodes.

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