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Characterization of novel glucose oxysilane sol–gel electrochemical biosensors with copper hexacyanoferrate mediator

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Abstract

New sol–gel encapsulated glucose oxidase (GOx) enzyme electrodes, constructed from carbon film resistor electrodes and chemically deposited copper hexacyanoferrate as mediator, have been developed and characterized using cyclic voltammetry and electrochemical impedance spectroscopy (EIS). The sol–gel was prepared using three different oxysilanes: 3-aminopropyltriethoxysilane (APTOS), 3-glycidoxypropyltrimethoxysilane (GOPMOS) and the well-known tetraethoxysilane (TEOS). The sol–gel composition was optimised for each oxysilane according to the gelation time of the sol–gel solution and response time of the enzyme biosensor to standard additions of glucose. Results obtained showed that APTOS and GOPMOS have similar electrochemical behaviour but differ from TEOS. As a glucose biosensor, the sensitivity can be described by the sequence: GOPMOS > APTOS > TEOS with detection limits down to 44 μM. The sensor lifetime was improved by elimination of ethanol from the hydrolysed oxysilanes by heating in a hot air stream after which APTOS-based sensors showed superior properties to GOPMOS, but with higher detection limit, and were sufficiently stable to be used for over several weeks.
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1. Introduction

Sol–gel has been shown to be an interesting and versatile way to prepare modified electrodes and solid electrolytes in recent years [1–3]. Most of the useful sol–gel solutions are organic orthosilicates having a solid and a gel phase, which is formed by gelation of a colloidal suspension and can be dried to form a xerogel, a dry porous silicate with controlled porous surface area [1,2]. For example, tetraethoxysilane (TEOS) in alcohol/water solution undergoes hydrolysis and forms a sol–gel:

$$\mathrm{Si(C_2H_5O)_4} + (4-x)\mathrm{H_2O} \rightarrow \mathrm{SiO}_x\mathrm{(OH)}_{(4-2x)} + 4\mathrm{C_2H_5OH} \quad (1)$$

where $0 < x < 2$.

The encapsulation of active biomolecules such as enzymes in sol–gel materials has been widely applied to the development of electrochemical [1–15] and optical [16–19] biosensors in the last decade. The oxysilanes most used for preparation of the sensors were tetramethyl-orthosilane [8,10,11,16,17,19,20], tetraethyloxysilane [7,12,13,18,21], 3-aminopropyltriethoxysilane [13,14], 2-(3,4-epoxycyclohexyl)-ethyltrimethoxysilane [2,14] and methyl-trimethoxysilane [1,5,13]. Enzyme encapsulation in sol–gel rather than in other matrices improves some of their properties, such as operational stability and activity compared to cross-linking with glutaraldehyde, and longer linear range [1–3]. Since such electrodes with encapsulated enzymes also showed interference problems due to high operating potentials at different electrode substrates, mediators were also applied in sol–gel biosensors, for example methyl viologen [22], ferrocene [2,6] and tetrathiafulvalene [2]. The electrode material used has varied, depending on the kind of sensor and particular application. Most were metals, such as platinum [6,9,10,14] and gold [11], or different types

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of carbon electrode, particularly graphite [5,22], carbon paste [5,8,23], glassy carbon [12] and carbon composite [24].

Recently, carbon thin film electrodes were introduced and suggested for use in electrochemical sensors and biosensors [25–29]. Carbon films are obtained by coating a ceramic substrate with a thin deposit of pyrolytic carbon [25,27,28] or by sputtering with graphite [26,29]. Electrodes made from carbon film electrical resistors [27] have been characterized in detail in different electrolyte solutions used in electroanalysis [30], have the anisotropic non-porous properties of glassy carbon, a large potential window after pre-treatment, are reproducible and do not need polishing before use. Such electrodes are inexpensive and offer an easy way for developing sensors and biosensors [31,32]. For a glucose biosensor, a high overpotential is required to detect hydrogen peroxide, which reduces the selectivity of the sensor [32]. To improve the selectivity, electrochemical biosensors have been modified with redox mediators, particularly with Prussian Blue analogues [33].

Electrochemical impedance spectroscopy (EIS) has not been widely used for the characterization of electrochemical biosensors due to deactivation of the enzymes at high frequency [34–37], especially with sol–gel-based biosensors [38]. However, it has been used to measure enzyme activity [39–41], cellular activity [42,43] and for the study of DNA-modified electrode biosensors [44]. A review of the use of EIS for probing biomolecular interactions has recently been published [45].

The objective of this work was to develop novel sol–gel glucose oxidase encapsulated glucose biosensors on a carbon film electrode substrate with copper hexacyanoferrate mediator and to electrochemically characterize them by cyclic voltammetry and electrochemical impedance spectroscopy. Two rarely explored oxysilanes were investigated for application in biosensors: 3-aminopropyltriethoxysilane (APTOS) and 3-glycidoxypropyltrimethoxysilane (GOPMOS), the latter being applied in biosensors for the first time and results were compared with the well-known tetraethoxysilane. Analytical parameters for the determination of glucose were determined for each biosensor assembly.

2. Experimental

2.1. Chemicals and solutions

Three different oxysilanes were used for enzyme encapsulation: APTOS, TEOS (Fluka, Switzerland) and GOPMOS (Aldrich, Germany), their structure is presented in Fig. 1. Glucose oxidase (GOx) from *Asperigillus niger*, anhydrous α-D-(+)-glucose crystals and bovine serum albumin (BSA) were obtained from Sigma (Germany). $CuCl_2{\cdot}2H_2O$, $K_3Fe(CN)_6$ and KCl were purchased from Merck (Germany).

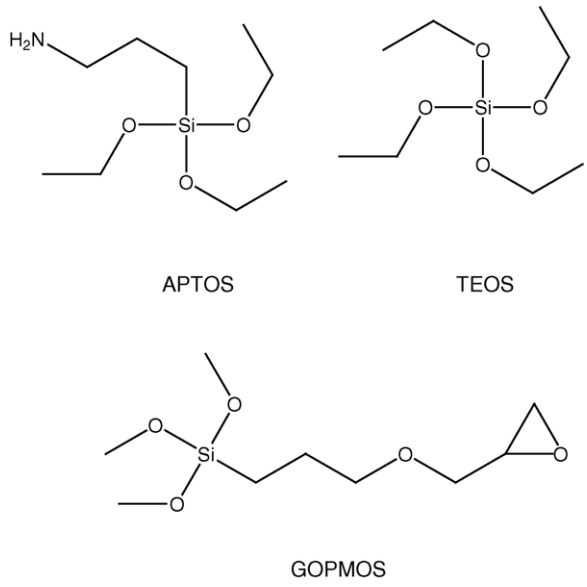

Fig. 1. Chemical structures of the sol–gel precursors: 3-aminopropyltriethoxysilane (APTOS), tetraethoxysilane (TEOS) and 3-glycidoxypropyltrimethoxysilane (GOPMOS).

Electrolyte solutions were 0.1 M phosphate buffer (PB) pH 5.5, prepared from sodium di-hydrogenphosphate and di-sodium hydrogenphosphate (Riedel-de-Haën), as well as 0.1 M phosphate buffer saline (PBS), where 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 resistance) as described previously [27,30]. 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, joined to thin conducting wire, was removed from one end of the resistor and the second one was sheathed in plastic tube gluing it with epoxy resin. In such way, the exposed electrode geometric area was ∼0.20 cm^2 (Fig. 2). Before use, electrodes were electrochemically pre-treated by potential cycling between 0.0 and +1.0 V versus SCE in 0.05 M KCl solution at scan rate 50 mV s^{-1} for at least five cycles, until stable cyclic voltammograms were obtained.

Copper hexacyanoferrate (CuHCF) was chemically deposited by direct immersion of the electrodes for 50 min in a solution containing 10 mM Cu^{2+}, 10 mM $K_3Fe(CN)_6$ and 100 mM KCl, the optimum procedure for forming CuHCF films on this carbon film substrate [46]. After this, the electrodes were dried with hot air for 3 min and left for 24 h to stabilize.

Sol–gel solution was prepared by mixing the chosen oxysilane and 0.1 M PB solution, pH 5.5 in different

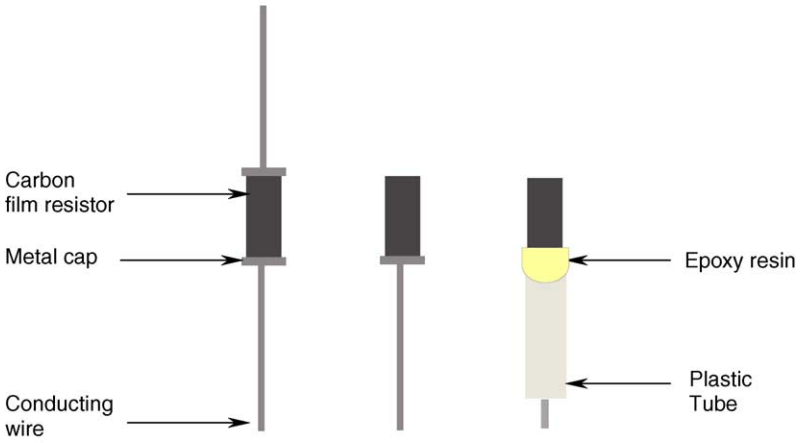

Fig. 2. Scheme of the preparation of electrode from carbon film resistor.

ratios: APTOS:PB 220:580 μl; GOPMOS:PB 200:600 μl and TEOS:PB 180:620 μl. The mixture was intensively mixed for 15 min and left for an hour at room temperature. Following this, 50 μl of each solution was mixed with 15 μl of GOx (10%)–BSA (10%) solution in 0.1 M PBS solution pH 7.0 and left for 1 day for gelation to begin. CuHCF-coated carbon film electrodes were then immersed in the sol–gel enzyme solution for 5 min, removed and left for sol–gel formation to occur at 4 °C for 2 days. Nafion solution (5% solution in alcohols, Sigma) was sometimes then applied to the biosensor. Electrodes were stored at 4 °C when not in use.

In some experiments, after formation of the oxysilane-PB sol–gel, the mixture was heated in a hot air stream (∼70 °C) for 1 h to evaporate the ethanol formed during hydrolysis before adding GOx and BSA, together with 3.5 μl of glycerol to improve homogeneity, as in Ref. [47].

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 (SCE) as reference. Measurements were performed using a computer-controlled μ-Autolab Type II potentiostat/galvanostat with GPES 4.9 software (Eco Chemie, The 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 of rms 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.

3. Results and discussion

3.1. Optimisation of sol–gel formation conditions

All the experimental results to be discussed concerned optimisation of the sol–gel-based enzyme biosensor, using glucose oxidase as test enzyme with copper hexacyanoferrate as mediator.

The sol–gel enzyme mixture was deposited onto the surface of the carbon film resistor, previously chemically coated with copper hexacyanoferrate, by dipping the electrode into sol–gel solution, as described in Section 2. Sol–gel formation conditions were optimised separately for each oxysilane because composition plays an important role for the enzyme activity [2]. The parameters varied were: sol–gel formation time, influence on GOx activity and response time to glucose. To avoid denaturation of the enzyme, no alcohol was added to the sol–gel solutions and only 0.1 M PB pH 5.5 was used, as in Refs. [4,9]. Since sonication of the precursor salts (without enzyme) gave a slightly lower or the same response to the analyte as an intensively mixed one, the latter was chosen for homogenisation of the sol–gel solution.

Results obtained are presented in Table 1. APTOS and GOPMOS show quite similar behaviour and led to a good environment for the enzyme at any of the sol–gel compositions tested. The response time to glucose decreases with an increase in oxysilane concentration and above a certain ratio, which is different for each precursor, it starts to increase again. It seems that the sol–gel is not properly formed at lower oxysilane concentrations and probably enzyme is leaching out. At the higher APTOS, GOPMOS or TEOS concentrations, the porosity is too low and there is poor contact between enzyme and substrate.

APTOS sol–gel was most different in its behaviour because it reacted with the mediator, the mediator layer degraded fast (observed from cyclic voltammetry, when the peak current significantly decreased with each cycle) and

Table 1
Optimisation of the composition of the sol–gel solution

Sol–gel PB volumes (μl)	Gelation time (days)	Interaction with GOx	$\tau^{95\%}_{glucose}$ (s)
APTOS			
40 + 760	1	No	200
60 + 740	1	No	200
80 + 720	1	No	100
100 + 700	2	No	80
150 + 650	2	No	70
180 + 620	2	No	50
200 + 600	2	No	30
220 + 580	2	No	20
250 + 550	3	No	50
280 + 520	3	No	100
GOPMOS			
100 + 700	2	No	60
150 + 650	2	No	50
180 + 620	2	No	30
200 + 600	2	No	15
220 + 580	2	No	50
250 + 550	3	No	100
TEOS			
100 + 700	3	No	80
150 + 650	3	No	80
180 + 620	3	No	50
200 + 600	3	Yes	100
220 + 580	4	Yes	180
250 + 550	4	Yes	250

Glucose (2 mM) was determined amperometrically in 0.1 M PBS solution pH 7.0 at +0.05 V vs. SCE.

also interacted with the PBS solution due to hydrophilic sol–gel formed with APTOS [13]. In previous studies with APTOS, Wang et al. [13] used sol–gel with this oxysilane as the most suitable precursor for the determination of phenols, but they worked in non-aqueous solution, while Couto et al. [14] developed a sol–gel glucose biosensor, which successfully worked in PBS pH 7.4, but they used mixed precursors, APTOS as a hydrophilic precursor together with the relatively hydrophobic 2-(3,4-epoxycyclohexyl)-ethyltrimethoxysilane. To reduce these disadvantages, Nafion was applied between the copper hexacyanoferrate layer and APTOS-GOx and also on the top of the sol–gel film to prevent any slow dissolution of the sol–gel layer.

The other two oxysilanes formed a stable sol–gel, which was compatible with the mediator and was not dissolved in PBS solution, so that Nafion was not necessary.

3.2. Voltammetric behaviour of the sol–gel enzyme biosensor

Fig. 3 shows cyclic voltammograms (CVs) in 0.1 M PBS solution at bare carbon film electrodes, and coated with CuHCF, the peaks corresponding to the Fe^{II}/Fe^{III} transition. Fig. 4 illustrates the behaviour obtained for the three different sol–gel coatings. CuHCF was deposited chemically as described in Section 2 and left for 1 day in air for stabilization. The reproducibility of the electrochemical response of the CuHCF layer at different carbon film resistor electrodes was demonstrated by cyclic voltammetry in 0.05 M KCl solution (not shown) comparing the peak currents and was found to be 96 ± 2% (nine electrodes). CuHCF exhibited reversible behaviour even in pH 7.0 PBS solution (Fig. 3, solid curve) although the peak shape and height was not as regular as in KCl solution and the peak separation was greater, i.e. 85 mV in PBS solution while in KCl solution, it was 61 mV.

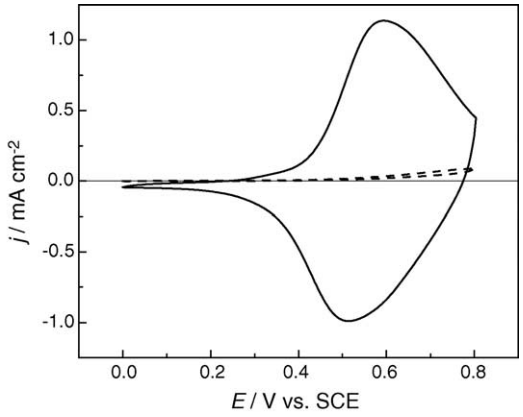

Fig. 3. Cyclic voltammograms, scan rate 50 mV s^{-1}, in 0.1 M PBS at bare carbon film electrode (- - -) and carbon film chemically coated with CuHCF (—).

Deposition of sol–gel encapsulated enzyme on the mediator changes the electrochemical behaviour of CuHCF, the peak current decreases almost 80% in the case of APTOS (Fig. 4A, dashed curve). As mentioned above, APTOS is not compatible with CuHCF and even with the intervening Nafion layer, it negatively affects the mediator more than other sol–gels. The sol–gel formed using GOPMOS leads to a current decrease of 70%, although the peak separation was much higher, i.e. the reversibility of the mediator was much poorer than that without sol–gel (Fig. 4B, dashed curve). However, the current of the CuHCF redox couple did not change during potential cycling with the GOPMOS GOx encapsulated sol–gel layer, contrary to what was observed for APTOS. The TEOS-based sol–gel caused less change to CuHCF's electrochemical behaviour; the current was reduced by 56% and redox peak separation and shape stayed the same as at bare CuHCF (Fig. 4C, dashed curve).

Fig. 4 also presents the electrochemical behaviour of these three biosensors after addition of 10 mM glucose to the PBS solution. In all cases, changes caused by the addition of glucose were the same, i.e. the peak current decreased and the oxidation current from 0.0 to +0.2 V became closer to zero, but no additional peak appeared for glucose/H_2O_2 as presented in Ref. [48], where glucose was determined at non-mediated ceramic–carbon composite electrode with GOx encapsulated by the sol–gel technique. The shape of the voltammograms suggests that the effect seen is due to reaction of hydrogen peroxide after diffusing to the CuHCF electrode surface.

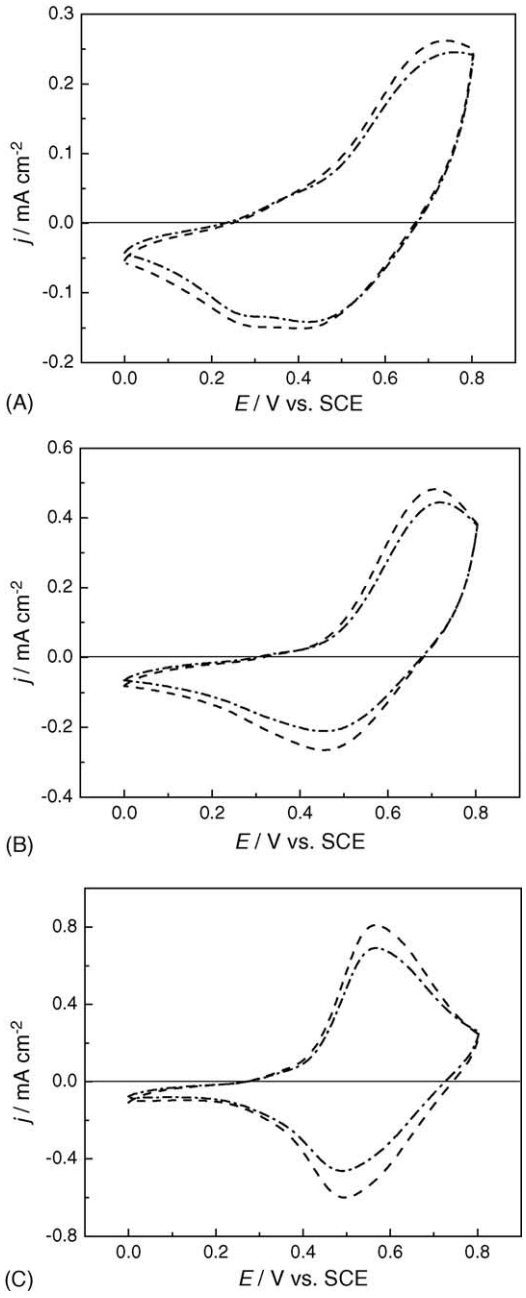

Fig. 4. Cyclic voltammograms, scan rate 50 mV s^{-1}, in 0.1 M PBS of sol–gel encapsulated glucose oxidase film applied on top of CuHCF mediator layer (- - -) and after addition of 10 mM glucose to the buffer solution (–·–·–). Sol–gel precursors: (A) APTOS, (B) GOPMOS and (C) TEOS.

3.3. Characterization of sol–gel electrodes by EIS

Complex plane plots of electrochemical impedance obtained at all three sol–gel sensors are shown in Fig. 5, covered with a Nafion layer to prevent damage to the enzyme during measurements. The impedance spectra were recorded at +0.05 V versus SCE, the operating potential of the biosensors. Fig. 5A shows spectra at bare carbon film and CuHCF-modified electrodes, evidencing some reduction in

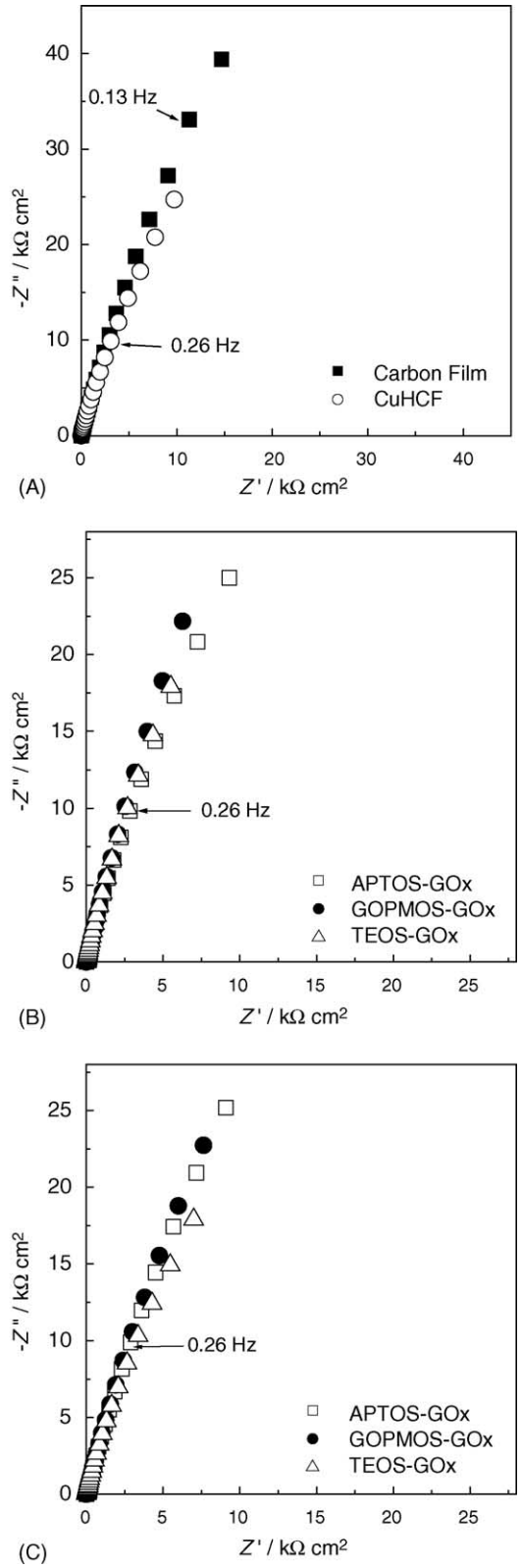

Fig. 5. Complex plane impedance spectra at bare carbon film electrodes, carbon film chemically coated with CuHCF (A), and at sol–gel layer based on APTOS, GOPMOS and TEOS deposited on top of mediator layer, without (B) and with addition of 10 mM glucose (C) to 0.1 M PBS solution, pH 7.0 and with at +0.05 V vs. SCE.

Table 2
Analysis of the electrochemical impedance data at modified carbon film electrodes

	R (kΩ cm^2)	C (μF cm^{-2})	α
Carbon film	128	23.3	0.88
+CuHCF	156	74.4	0.86
+Sol–gel			
APTOS	613	57.0	0.84
GOPMOS	660	65.0	0.86
TEOS	183	78.4	0.84
+10 mM glucose			
APTOS	613	57.1	0.82
GOPMOS	538	62.5	0.84
TEOS	117	73.4	0.86

the impedance at the modified surface, corresponding to a higher capacitance, due to limitations of charge movement through the hexacyanoferrate film. Only slight differences were observed at +0.05 V between CuHCF-modified electrodes without (Fig. 5A) and with (Fig. 5B) sol–gel encapsulated GOx, all these kinds of electrode exhibited close to straight-line spectra in the complex plane and only slight differences between them.

Addition of 10 mmol l^{-1} of glucose solution did not lead to any changes in impedance spectra at the APTOS-based sol–gel electrode compared to the spectrum without glucose at the same electrode (Fig. 5B and C), while small differences occurred with the two other sol–gel electrodes.

The same equivalent circuit model was used for fitting all spectra, consisting of the cell resistance, R_Ω, and a parallel combination of a constant phase element, CPE, modelled as a non-ideal capacitance, according to

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

and a charge transfer resistance, R. Values of R_Ω vary from 7.0 to 8.5 Ω cm^2. The fitted equivalent circuit of R, C and α values obtained are shown in Table 2. The charge transfer resistance values are very high, since there are no electroactive species at the applied potential of 0.05 V. They are between 100 and 200 kΩ cm^2 for the bare carbon film, CuHCF-coated electrode and with TEOS sol–gel with and without glucose. However, with the APTOS and GOPMOS sol–gels, the resistance values increase significantly to between 500 and 700 kΩ cm^2 showing that the charge transfer is much more difficult. Interestingly, the capacitance values remain very similar for all the assemblies studied except the bare carbon film; changes between the sol–gels can be ascribed to structural differences. The values of the roughness exponent, α, were always around 0.85, as expected for this type of system [30]. Addition of glucose to the buffer solution did not change the capacitance values significantly at any of the sol–gel biosensors. The main conclusion from the EIS data analysis is that APTOS and GOPMOS are similar to each other and show differences with respect to TEOS. This is the same deduction made from cyclic voltammetry.

The differences between the various sol–gels prepared from various oxysilanes could be caused not only by their different structure (Fig. 1) but also to the different composition of sol–gels. It is difficult to compare our results with data from the literature because most sol–gel electrodes previously studied by EIS were either developed as corrosion protection layers or for zeolites or batteries, although similar results were obtained by Szu and Lin at copper-doped silica glass [49].

3.4. 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 characterization, 0.1 M PBS pH 7.0. Calibration curves are presented in Fig. 6 and data from analysis of the curves are given in Table 3. The response time at APTOS- and GOPMOS-based biosensors was 20–30 s, depending on the concentration of the analyte and at TEOS-based biosensors was 50 s.

Highest sensitivity was observed at the biosensor with encapsulated GOx using GOPMOS as sol–gel precursor. It also had the lowest limit of detection while the linear range was the same as at a biosensor based on the APTOS precursor. The lowest sensitivity and highest limit of detection, but the biggest linear range, were observed at the widely used TEOS-based sol–gel. The relative standard deviation was found to be ∼3% (n = 5) in the case of APTOS and GOPMOS and 5.2% (n = 5) for TEOS. A higher sensitivity and lower detection limit were reported in Ref. [8] for hydrogen peroxide using horseradish peroxidase encapsulated in a TMOS-based biosensor with ferrocene as mediator. Wang and Dong [12] also found better sensing properties at TMOS sol–gel encapsulated tyrosinase for the determination of phenols in organic media.

The Michaelis–Menten constant was 3.2 mM at the electrodes with APTOS and GOPMOS and in the case of TEOS, the reaction is ∼2 times slower than at two other electrodes

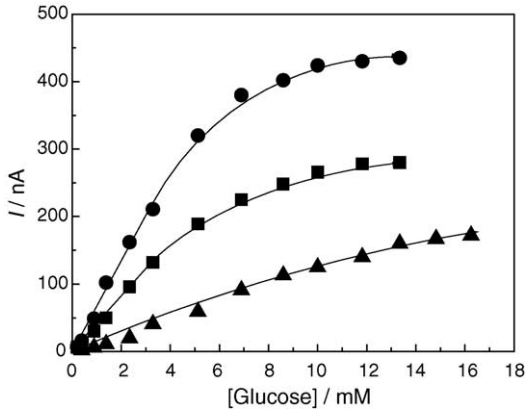

Fig. 6. Calibration curves for glucose at different sol–gel encapsulated GOx electrodes: APTOS (■), GOPMOS (●) and TEOS (▲), in PBS pH 7.0. Applied potential +0.05 V vs. SCE.

Table 3
Data calculated from calibration curves in Fig. 6

Sol–gel	Linear range (mM)	Sensitivity (nA μM^{-1})	Intercept (nA)	Correlation coefficient (R^2)	Limit of detection (μM)
APTOS	0.2–5.1	38.47	−1.56	0.994	78.0
GOPMOS	0.2–5.1	67.68	−5.03	0.996	44.3
TEOS	0.4–10.0	13.50	−6.36	0.994	216.3

and in this case, the Michaelis–Menten constant was 6.3 ± 0.4 mM ($n = 4$).

Without use, biosensor stability is at least 4 months (100% response), when stored dry at 4 °C. In order to investigate long-term stability with periodic measurements, 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 sensitivity, i.e. GOPMOS > APTOS > TEOS. In the case of GOPMOS, the sensor was stable for 1 week (when the signal for glucose decreased to 50% of the initial value), while sensors based on other sol–gel precursors had still lower continuous-use stability. Electrodes were stored dry at +4 °C, when not in use, and probably the drying of the sol–gel decreased the activity of the enzyme [1], since the electrochemical properties of CuHCF change only very slowly with time. The lifetime was shorter when electrodes were stored in buffer solution because of the release of the enzyme entrapped within the sol–gel layer [9], despite the presence of Nafion to reduce this effect.

The reduction in response with time could also be caused by lack of homogeneity and ethanol release during the sol–gel formation process (see Eq. (1)), which could deactivate the enzyme [50]. To attempt to minimise this latter problem, sol–gel solutions were heated in a hot air stream for 1 h to evaporate ethanol before enzyme addition, similar to Ref. [47] (except that in Ref. [47], a rotavapour method was used). A longer heating time than 1 h led to prompt gelation of solutions and at shorter times, the pH was still too low for all the HCl to have reacted. The solution was then left to cool for a few hours and then mixed with enzyme as described in Section 2. Glycerol was added, 5% by volume, to the solution obtained in order to increase the homogeneity of the sol–gel solution. These conditions were applied for two precursors, APTOS and GOPMOS, and gave very different effects: the stability of the APTOS sensor increased notably and after 2 weeks of measurements, the signal decreased by only 20%, but in the case of GOPMOS, the electrode was inactive. Possibly, during heating, the GOPMOS structure (Fig. 1) was changed owing to the existence of the epoxy group. Additionally, after this extra pre-treatment, the APTOS-based sol–gel film did not interact with the mediator and dissolution of the sol–gel layer did not occur, even without Nafion. Thus, the best enzyme biosensor assemblies contain hydrolysed APTOS with the hot air pre-treatment to remove ethanol or GOPMOS without the hot air treatment, which has a lower detection limit.

4. Conclusions

Sol–gel encapsulated glucose oxidase electrodes based on carbon film resistors with chemically deposited CuHCF as a mediator were developed and characterized using cyclic voltammetry and electrochemical impedance spectroscopy. Sol–gel was prepared using three different oxysilanes: APTOS, GOPMOS and the well-known TEOS. The sol–gel composition, without any addition of alcohol, was optimised for each oxysilane according to the response time of the enzyme biosensor to glucose. The best composition was found to be around 1:3 (oxysilane:phosphate buffer pH 5.5). It was shown that APTOS and GOPMOS exhibit similar electrochemical properties, but they differ from those with TEOS, which partly explains the glucose sensitivity parameters, which are in the order TEOS < APTOS < GOPMOS and the fact that sensors based on APTOS and GOPMOS sol–gels have a similar stability. Accelerated ethanol elimination by pre-treatment with hot air leads to the APTOS-based biosensor having a much longer lifetime, but GOPMOS-based sensors have a lower detection limit.

Further improvement of the biosensing properties of these new sol–gel encapsulated biosensors is in progress through optimisation of the sol–gel process.

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