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Talanta 56 (2002) 997–1003

Talanta

www.elsevier.com/locate/talanta

Application of amperometric sol–gel biosensor to flow injection determination of glucose

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Received 15 May 2001; received in revised form 1 October 2001; accepted 3 October 2001

Abstract

A glucose biosensor with enzyme immobilised by sol–gel technology was constructed and evaluated. The glucose biosensor reported is based on encapsulated GOX within a sol–gel glass, prepared with 3-aminopropyltriethoxy silane, 2-(3,4-epoxycyclohexyl)-ethyltrimetoxy silane and HCl. A flow system incorporating the amperometric biosensor constructed was developed for the determination of glucose in the 1×10^{-4}–5×10^{-3} mol l^{-1} range with a precision of 1.5%. The results obtained for the analysis of electrolytic solution for iv administration and human serum samples showed good agreement between the proposed method and the reference procedure, with relative error $< 5\%$.

Keywords: Flow injection analysis; Glucose oxidase; Sol–gel; Glucose biosensor

1. Introduction

Enzymes are catalysts with high selectivity toward a given substrate, increasing the rate of reaction by many orders of magnitude. For this reason, enzymes are widely used in analytical chemistry. The gradual replacement of classical wet chemistry techniques by enzyme-catalysed reaction is mainly evident in clinical analysis [1]. This replacement has been slow because of the undesirable properties associated with soluble enzyme reagents. In aqueous solutions, enzymatic catalytic activity is usually lost rather rapidly, because enzymes can suffer oxidation reactions or its tertiary structure can be destroyed at the air–water interface, making the use of enzyme reagents both expensive and complex. These problems can be minimised by enzyme immobilisation. By attachment to an inert support material, enzymes may be rendered insoluble, retaining cata-

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0039-9140/02/$ - see front matter
PII: S0039-9140(01)00587-2

lytic activity and thereby extending their useful life [1].

One of the main applications of sol–gel glasses is the development of sensors, particularly as an inert support of sensing material on the surface of physicochemical transducers, mainly those of optical and electrochemical origin [2]. The sol–gel process involves a low temperature production of ceramic materials through the hydrolysis of the alkoxide precursor and co-polymerisation of the hydroxylated monomers [2–4]. These alkoxysilanes in acidic medium generate a solid network whose physical structure is comparable to conventional glass, with a controlled porosity and thickness. The development of electrochemical biosensors can be obtained by doping organic or biological molecules in the porous sol–gel matrix, thus retaining their chemical stability and functional activity.

In the literature, several papers describe the development and construction of glucose biosensors based on sol–gel glass [3,5–16]. Some also report the use of co-immobilised mediators, mainly ferrocene [3,5–7,11,16,17], for operation at lower potential to avoid interference of the analyte matrix (i.e. ascorbic acid); others report the use of a multilayer 'sandwich' configuration [3,6,7,12,13].

The intercalation of GOX biosensors in flow injection analysis (FIA) systems has seldom been exploited [7,10,16,18–20]. In particular, few papers address the sol–gel biosensor approach [7,10]. Amongst the referred papers [7,10,16,18–20] only two [18,20] describe analytical applications, which are related to the determination of pentachlorophenol in contaminated soils [18] and control of industrial processes in food processing [20]. All others refer to assays of the process parameters for the fabrication of a glucose biosensor, the sol–gel matrix and the use of mediators [7,10,16,19].

In this paper, we report on an amperometric sol–gel glucose sensor incorporated into a single line FIA system. The reaction of β-D-glucose catalysed by *GOX* resulted in the formation of gluconic acid and hydrogen peroxide. The hydrogen peroxide that is produced was monitored electrochemically at ± 0.7 V vs Ag/AgCl. Influencing factors on the sensor response such as pH, buffer composition and immobilisation procedures are discussed. The applicability of the optimised glucose sensor incorporated in a FIA system for the determination of glucose in haemodialysis solutions and human serum was evaluated.

2. Experimental section

2.1. Reagents and solutions

Glucose oxidase type X-S from Aspergillus niger (50000 units) (GOx) was purchased from Sigma, while 3-aminopropyltriethoxy silane (3-APTMS), 2-(3,4-epoxycyclohexyl)-ethyltrimethoxy silane (EETMS) and polyethylene glycol 6000 (PEG 6000) were purchased from Fluka.

For the reference test a Boehringer–Mannheim test Kit was used, using two enzyme types, hexokinase (320 U) and G-6-P dehydrogenase (160 U).

All solutions were prepared with bideionized water (specific conductivity < 0.1 $\mu S\,cm^{-1}$) and with reagents of analytical grade without further purification.

2.2. Preparation of sol–gel glass biosensors

The electrode body for biosensor construction was made from Teflon containing a platinum base with a recessed depth of approximately 1.5 mm. A platinum disk (3 mm diameter) placed on a threaded aluminium rod was fitted in a hollow Teflon cylinder (5 mm diameter; 50 mm length) with its top exposed to the solution.

Before placing the sol–gel membrane into the platinum surface its surface was polished with aluminium oxide and washed with HNO_3 before being carefully dried.

The components of the sol–gel membrane, described in Table 1, were mixed thoroughly by ultrasonication for 10 min, and 20 μl of the homogenised solution was placed in the platinum base of the electrode body. Gelation took place at room temperature for ≈ 60 min. The GOx solution (20 mg ml^{-1}) was then added to the layer of

Table 1
Composition of sol–gel based glucose sensors

	Type A	Type B	Type C	Type D	Type E	Type F	Type G
3-APTMS	70	70	70	70	70	70	70
EETMS	20	20	20	20	20	20	20
H_2O	500	500	700	700	700	700	700
PEG 6000 (2 mg ml^{-1})	200	200	500	500	700	700	700
HCl (0.1 mol l^{-1})	7	7	7	7	7	7	7
GOx (20 mg ml^{-1})	7	20	7	20	7	20	–

3-APTMS (3-aminopropyltriethoxy silane), EETMS (2-(3,4-epoxycyclohexyl)-ethyltrimetoxy silane); PEG 6000 (polyethylene glicol 6000); GOx (glucose oxidase solution). *Volumes in μl.

the sol–gel glass before a 24-h incubation period at room temperature was observed.

The electrodes were washed with 0.01 mol l^{-1} phosphate buffer (pH 7.4) and stored in the same buffer at 4 °C when not in use.

A control sol–gel glass electrode without the enzyme was also prepared to evaluate the electrochemical response of this type of membrane (type G of Table 1).

2.3. Equipment for electrochemical measurements and flow injection system

The electrochemical measurements were performed with a PSTAT10 Echochemie/Autolab potentiostat with GPES software v3.2 connected to a PC. Measurements were made using a cell of the wall-jet type 656 VA Stand Metrohm electrochemical detector with three electrodes—glucose sol–gel sensor and Ag/AgCl, KCl sat and Au, as reference and auxiliary electrodes, respectively.

The amperometric response was operated at 0.70 V. The experiments were performed in phosphate buffer (0.01 mol l^{-1}, pH 7.4).

The flow system developed (Fig. 1) used a Gilson Minipuls 3 peristaltic pump and samples were inserted through a Rheodyne 5020 injection valve. The connection of all components of the FIA system was done with PTFE tubes (0.8 mm i.d).

2.4. Reference method

The reference UV method used for the determination of glucose was performed using a Boehringer–Mannheim test Kit. D-glucose is phosphorilated to D-glucose-6-P (G-6-P) in the presence of hexokinase and ATP. G-6-P is oxidised by NADP, in the presence of G-6-P dehydrogenase, to D-gluconate-6-P with the formation of NADPH. The NADPH increase is measured by UV spectrophotometry.

3. Results and discussion

3.1. Optimisation of the sol–gel glass based biosensor

The sol–gel process involves the development of an inorganic matrix through the formation of a colloidal suspension (sol) and gelation of the sol to form a wet gel, which upon drying forms a xerogel [13]. The substrate and oxygen diffusion through the sol–gel matrix are essential for the

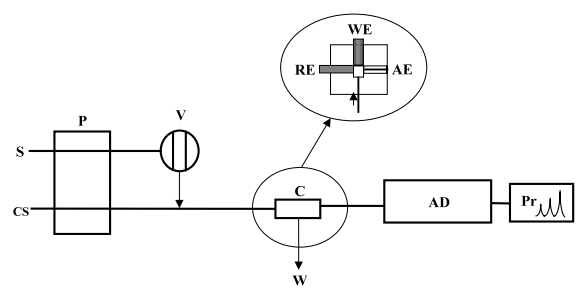

Fig. 1. FIA manifold for glucose determination. Legend: P, peristaltic pump; S, sample; CS, carrier solution; V, injection valve; C, wall-jet cell; W, waste; AD, amperometric detector; Pr, printer; RE, reference electrode; WE, working electrode; AE, auxiliary electrode.

performance of the GOX biosensor, as its response is a function of the formation of hydrogen peroxide and its subsequent electrochemical oxidation. The properties of the porous sol–gel matrix formed, namely the substrate and oxygen diffusion within the sol–gel matrix and its porous structure, and consequently its electrochemical performance, are affected by many factors, such as pH (type of catalyst), H_2O:PEG ratio and enzyme concentration. In the development of the sol–gel glass, the formulations described in Table 1 were assayed. The main precursors of the sol–gel were 3-APTMS (a relatively hydrophilic modifier) and EETMS (a relatively hydrophobic modifier), according to results previously described in the literature [3,5,21]. It has been reported that neither highly hydrophobic nor fully hydrophilic sol–gel glasses are desirable for sensing application [22].

It was decided to maintain 3-APTMS (70 μl), EETMS (20 μl) and HCl (7 μl) constant, according to results previously described [3,5,21], while varying the amount of H_2O (500–700 μl), PEG (200–700 μl) and enzyme (0–20 μl). The sensors obtained with the formulations indicated in Table 1 were found to be strongly attached and were only removed from the Pt surface through strong polishing (except type A and B). The best analytical response and lower value of end current intensity (≈ 20 nA) was verified in the formulations with greater amounts of H_2O and PEG (Type C and E). The more hydrophobic formulations gave rise to a brittle sol–gel surface that cracked after prolonged immersion in aqueous solutions (Type A and B). The analytical results obtained are in agreement with those described in the literature, which show that hydrophilic modifiers give rise to an increased conductive surface accessible to the solution, and an increased rate of oxygen diffusion [22] and consequently, a greater amplitude of amperometric response. Nevertheless, the parameter that exerted more influence in the amperometric response was the quantity of enzyme added. The colour of the enzyme immobilised sol–gel film was yellow unlike the white colour observed in the absence of GOX. It was verified that the electrodes with greater quantity of added enzyme (type B, D and F electrodes), for reasons possibly related to immobilisation on the sol–gel matrix or the fact that insufficient silane molecules were present in the sol–gel system to effectively couple the enzyme to the backbone of gel matrix, either did not show analytical response or alternatively, rapidly lost enzymatic activity. Furthermore, they were characterised by a long stabilisation period (≈ 30 min) and they presented a large response time, compared to type C and E, possibly related to slow substrate diffusion.

The smoother sol–gel film without the presence of cracks and giving improved performance together with greater durability when used as sensor was obtained with type E biosensors. These sensors, with sol–gel layer thickness of about 0.3 mm, presented a fast response time, both at low and higher substrate concentrations (< 30 s), a low intensity of end current (≈ 20 nA) and were those used in the determination of glucose in the analytical flow injection system shown in Fig. 1.

3.2. Optimisation of the flow injection analysis system

A single channel FIA manifold for glucose determination with amperometric detection incorporating the glucose biosensor developed was established (Fig. 1). The sample (75 μl) was inserted in a phosphate carrier solution (0.01 mol l^{-1} phosphate buffer, pH 7.4) and the amperometric current was monitored electrochemically using a three-electrode cell. Meanwhile, the working electrode was polarised at 0.70 V vs Ag/AgCl.

The influence of the different physicochemical and hydrodynamic parameters in enabling the measurements to be carried out within a wide concentration range, with good sensitivity and with an elevated sampling rate were evaluated.

The influence of the injected sample volume was assessed for values ranging from 25 to 125 μl. With an injection value of 25 μl, a low current intensity was obtained, which compromised the determination of analyte concentration lower than 5×10^{-4} mol l^{-1}. With volumes greater than 75 μl, the variation in current intensity was of minor significance. Therefore, an injection volume of 75 μl was selected since it provided a good sensitivity and reproducibility and avoided the necessity of

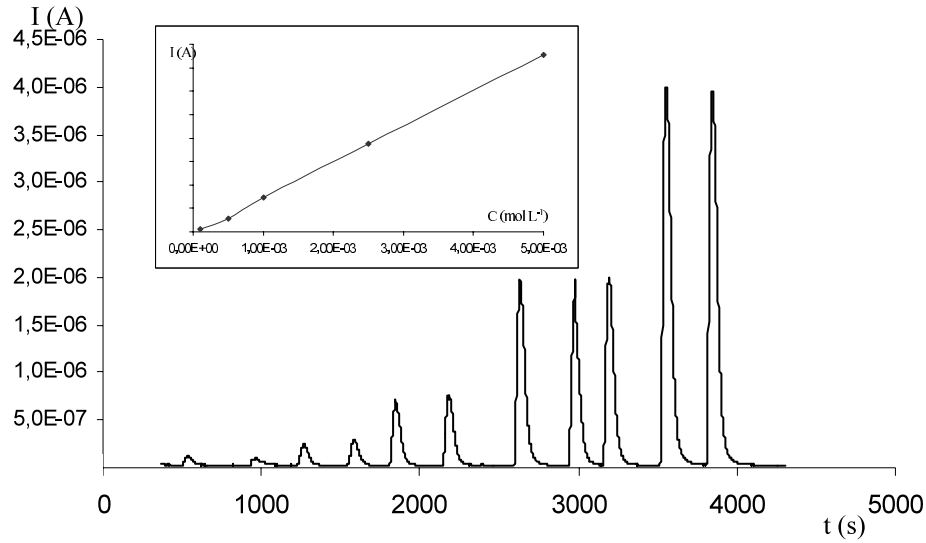

Fig. 2. Typical response curve of the GOX immobilised sol–gel glass sensor and diagram.

large sample consumption, particularly important in clinical analysis.

The effect of flow rate was evaluated by measuring the height and area of analytical signals. Lower flow rates gave rise to a large interaction between the substrate and enzyme, favouring the biocatalytic reaction and consequently leading to a greater amperometric response. For this reason, we proceeded to study this parameter between the 0.25 to 1.0 ml min^{-1} interval. No significant analytical signal variation was observed in the interval studied, although values greater than 0.75 ml min^{-1} yielded less reproducible analytical signals. Therefore, we opted for a value of 0.5 ml min^{-1}, as a compromise situation between the sensitivity and the sampling rate obtained (30–35 samples per h).

The factors that influenced the enzymatic activity such as pH, temperature and concentration and buffer composition were equally optimised, in an effort to obtain maximum sensitivity.

The influence of pH of the phosphate buffer in the transport solution was studied in the 6.0–8.0 interval. The optimum pH was shown to be 7.4, this value giving the greatest amplitude of amperometric signal and greatest analytical sensitivity.

The carrier solution temperature was varied between 25 and 42 °C, over which no significant variations in the analytical signal obtained were registered. As such, we opted to work at room temperature (25 °C).

The ionic strength was evaluated in terms of the carrier solution concentration of phosphoric buffer, having obtained an optimum response for a 0.01 mol l^{-1} concentration. For higher concentration values (0.1 mol l^{-1}) the biosensor response was slightly lower.

The dependency of the substrate concentration on the biosensor was studied, having shown a behaviour typical of Michaelis–Menton. The response increased up to a concentration value of 5×10^{-3} mol l^{-1} and thereafter remained practically constant (Fig. 2). A linear response was obtained within the 1×10^{-4}–5×10^{-3} mol l^{-1} concentration range.

Experiments were conducted to evaluate the biosensor stability over time. The variation in analytical signal obtained from the injection of a 1×10^{-3} mol l^{-1} standard glucose solution was verified during a period >45 days (Fig. 3). This showed that the amperometric signal increased slightly in the initial applications, thereafter maintaining itself stable for a period of about 45 days, before decreasing after approximately day 45 of utilisation. The analytical signal in this period varied between 1.36×10^{-6} and 1.16×10^{-6} A

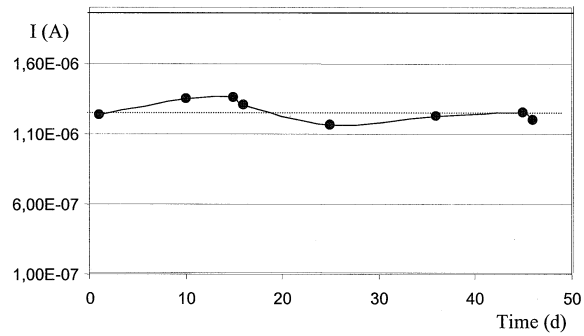

Fig. 3. Evaluation of the biosensor stability over time. Analytical signal corresponding to a 1×10^{-3} mol l^{-1} glucose solution.

for a 1×10^{-3} mol l^{-1} glucose solution. The biosensors constructed permitted more than 500 substrate determinations to be carried out being easily recycled at the end of their useful life.

3.3. Interferences

The effect of some possible interfering substances on the glucose biosensor response was investigated. To study the substrate specificity, interferences were evaluated by intercalation of 1×10^{-3} mol l^{-1} solutions of twelve carbohydrates in the FIA optimised system (Table 2). Glucose gave the highest response (set as 100%). Only mannose yielded a considerable response (14%), while the other carbohydrates gave very low signals. In any case, as these carbohydrates are not generally present in parental solutions and human serum, they would not interfere with the measurement that we tried to carry out.

Table 2
Substrate specificity of the glucose sensor

Carbohydrate	Signal normalised to glucose (%)
Glucose	100
Lactose	0.6
Lactulose	0.1
Sucrose	0.4
Melibiose	0.7
Maltose	0.6
Raffinose	0.1
Xylose	0.1
Mannose	14
Fructose	1.4
Galactose	8.7
Cellobiose	0.4
Ascorbic acid	80

*Glucose signal was set as 100%. **1×10^{-3} mol l^{-1} solutions.

Table 3
Results obtained from glucose determination in electrolytic solutions and human serum by the proposed method (FIA) and the reference procedure (Ref)

Sample	FIA (mol l^{-1})	Ref (mol l^{-1})	DR (%)
1	8.98×10^{-4}	9.08×10^{-4}	−1.10
2	1.77×10^{-3}	1.76×10^{-3}	0.57
3	1.55×10^{-3}	1.49×10^{-3}	4.03
4	2.60×10^{-3}	2.55×10^{-3}	1.96
5	2.72×10^{-3}	2.77×10^{-3}	−1.81
6	2.80×10^{-3}	2.71×10^{-3}	3.32
7	5.15×10^{-3}	4.90×10^{-3}	5.10
8	2.85×10^{-3}	2.76×10^{-3}	3.26
9	1.01×10^{-3}	1.02×10^{-3}	−0.98
10	9.37×10^{-4}	9.44×10^{-4}	−0.74
11	6.93×10^{-4}	7.16×10^{-4}	−3.21

Ascorbic acid strongly interferes because of the high operating potential of 0.70 V, at which value reducing agents can be oxidised. The current increased by 80% in the presence of 1×10^{-3} mol l^{-1} ascorbic acid.

3.4. Determination of glucose in samples

The present system was used for glucose determination in 11 samples. Table 3 shows the analytical data related to the analysis of eight samples of electrolytic solution for iv administration commonly found in the Portuguese market together with three human serum samples. Accuracy of the results given by the FIA method (C_F) was assessed by comparison with the results provided by the reference method (C_R). A linear relationship was obtained and is expressed as follows: $C_F = 7 \times 10^{-5}(\pm 7 \times 10^{-5}) + 1.05(\pm 0.03)C_R$ mol l^{-1} ($R = 0.9993$). A Student two-tail paired t-test was also applied yielding a value of 0.1353, which is less than the theoretical value of 1.624 for a confidence interval of 95%. It may thus be concluded that there are no significant differences between the proposed method and the reference procedure.

The within-run precision of FIA methodology was assessed by calculating the relative standard deviations after ten successive injections of two typical samples with respective concentrations of 1.55×10^{-3} and 9.37×10^{-4} mol l^{-1}. The values obtained showed good precision with a relative standard error $< 1.5\%$.

4. Conclusions

sol–gel technology is both simple and inexpensive. This immobilisation technique is of low-cost creating the possibility of employing it to other enzyme systems for electrochemical sensor development. The proposed FIA system involves both a low reagent and sample consumption together with a good sampling rate (30–35 samples per h). The system developed here allows glucose determination over a wide concentration range, with an enzyme consumption < 0.20 mg per biosensor, permitting up to 500 determinations and with a useful life of ≈ 45 days.

Acknowledgements

The authors gratefully acknowledge the Praxis/P/QUI/10109/1998 program for financial support.

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