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Analysis and optimization of a hydrogel matrix for the development of a sandwich-type glucose biosensor

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ABSTRACT

The development of a sandwich-type biosensor for glucose quantification is presented. This work is focused on the optimization of the enzymatic matrix of the biosensor. The best performance was found for an enzymatic matrix composed by 30% w/w mucin, 70% w/w albumin, 1.35 U glucose oxidase (GOX) per sensor, and glutaraldehyde diluted to 3%. The crosslinking with glutaraldehyde transforms this mixture into a hydrogel that is entrapped between two membranes of polycarbonate. The selected sandwich-type biosensor showed very good response time, sensitivity, stability, and sensor-to-sensor reproducibility.

According to the results presented in this manuscript, a biosensor prepared with very high amount of enzyme would not necessarily increase the analytical signal. Simulated curves are compared with experimental data to explain the dependence of sensitivity on the concentration of enzyme. In addition, this kind of comparison represents a quite simple way to estimate the value of $v_{max} \approx 0.13\ M\ s^{-1}$ from the amperometric response of a sensor prepared with 1.34 U of GOX.

Considering that sandwich-type biosensors are commonly assembled as part of devices where the sample is diluted with buffer, the more than 3 orders of magnitude of linear behavior of this sensor would ensure the possibility for assessing any sample.

1. Introduction

The technology of biosensors offers the possibility for fast, sensitive and selective assessment of diverse analytes usually distributed in a wide variety of clinical, environmental and food samples [1–8]. The success of an amperometric biosensor depends on its reliability and profitability [1–4]. The first aspect is related to the robustness, reproducibility, selectivity, and sensitivity of the enzymatic transducer, while the second depends on the strategy for immobilizing the enzyme to the electrode, the size, portability and simplicity of the analysis [1,4]. The so-called sandwich-type amperometric biosensors are transducers usually applied to devices of intermittent use [1,9].

The immobilization process has strong impact on the catalytic activity of the enzyme because it can work not only as a barrier that protects the enzyme, but also to produce conformational changes in the recognition element and introduce electrostatic interactions with reagents and/or products of the enzymatic reaction [9–14]. As a result, the enzymatic matrix has great influence on the response of the biosensor [5,12,15,16]. Hydrogels are attractive materials for immobilization since they provide suitable scaffolds for trapping the enzyme and present high concentration of water to emulate the aqueous environment [11–13,16,17].

Recently, we have developed a model that simulates the chronoamperometric response of sandwich-type amperometric biosensors [18,19]. In the model it is assumed that the effect of convection stops at the outer membrane and that within the biosensor the flow of reagents and products is controlled only by diffusion. Regarding the enzymatic reaction, the enzyme catalyzes the oxidation of a substrate (S) and the reduction of the mediator (M) according to a conventional ping-pong mechanism [5,14,18–21]. The reactions occurring in the enzymatic membrane can be summarized as:

$$E_r + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E_rS \xrightarrow{k_2} E_o + R \quad (1)$$

$$E_o + M \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} E_oM \xrightarrow{k_4} E_r + P \quad (2)$$

where E_r and E_o are the reduced and oxidized forms of the enzyme while E_rS and E_oM are the intermediate complexes of the enzyme

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with substrate and mediator, respectively. It is also considered that the species M is regenerated at the electrode surface:

$$P \xrightarrow{-2e^-} M \quad (3)$$

In the case of our biosensor, P and M correspond to H_2O_2 and O_2. Eqs. (1)–(3) have been resolved numerically and the respective solution is now used to explain some experimental outcomes of this new system [18,19].

The aim of this work is to describe the optimization of the enzymatic matrix for a glucose sandwich-type biosensor. The selected enzyme, glucose oxidase (GOX), is mixed with mucin and albumin and then crosslinked with glutaraldehyde. The chemical reaction results in a hydrogel that is trapped between two membranes of polycarbonate that help to control the diffusion of species and to reject potential interferences [5,12,14]. Simulated results are compared with experimental data to determine, in a comprehensive way, the maximum amount of enzyme required to prepare the biosensor.

2. Experimental

2.1. Reagents

Base electrolyte solution (0.1 M) was prepared by mixing 0.05 M HK_2PO_4/0.05 M H_2KPO_4 (Merck, Germany). This solution was fixed at pH 7.0 with small amounts of H_2SO_4 (Baker, USA) or KOH (Merck, Germany) and renewed weekly. All solutions were prepared with ultra pure water (18 MΩ cm) from a Millipore Milli-Q system and stored at 4 °C.

A stock solution of 0.1 M glucose (Sigma, USA) was prepared in the base electrolyte. An amount of 0.01380 g of GOX from *Aspergillus niger* (147,900 U g^{-1} of solid, catalog number G-7141, Sigma, USA) was dissolved in 510 μL of base electrolyte to get a solution with 4.0 U μL^{-1} of GOX. From this solution, 5 aliquots of 20 μL were separated into vials and stored at −20 °C. The remaining solution was further diluted to prepare aliquots of 20 μL with 20 U of GOX. These aliquots were also stored at −20 °C.

Diverse dilutions of glutaraldehyde (Backer, USA) were prepared in base electrolyte. Mucin (Sigma, USA) was mortared and stored as dry powder at 4 °C. Bovine serum albumin (Sigma, USA) was used as received. All other reagents were of analytical grade and used as received. Polycarbonate membranes of 0.05 μm pore size (Millipore, USA) were cut in discs of 6 mm diameter.

2.2. Apparatus

All electrochemical experiments were performed with an Autolab PGSTAT 30 Electrochemical Analyzer (Eco Chemie, The Netherlands). The measurements were carried out using a conventional three-electrode system. The counter electrode was a Pt wire, the reference electrode was Ag|AgCl|KCl (3 M) (CH Instruments, USA), and the working electrode was a 2 mm diameter Pt disk (CH Instruments, USA).

2.3. Preparation of the enzymatic matrix

A total mass of 6.0 mg composed by different amounts of mucin and albumin was dissolved in 40 μL of base electrolyte. The proteins were mixed for 5 min and then transferred to a vial containing 20 μL of GOX. As indicated above, two set of vials were prepared, one with 20 U of GOX and another with 80 U of GOX. The resulting 60 μL GOX-matrix system was mixed for extra 5 min and stored at 4 °C.

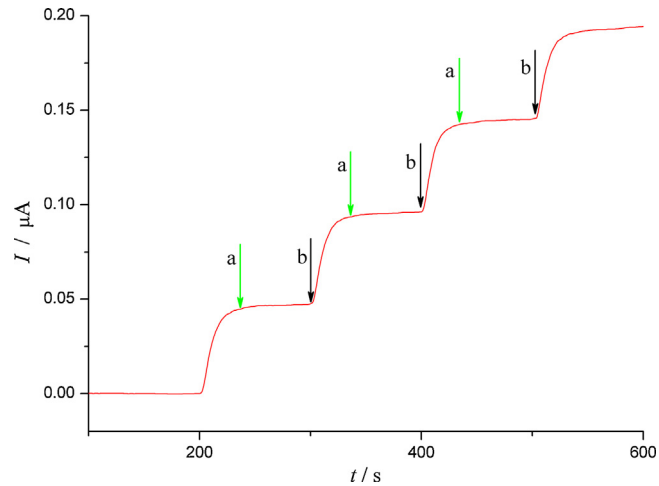

Fig. 1. Chronoamperometric profile resulting from several additions of 0.2 mM glucose. Arrows (a) show when the current has reached 95% of I_{lim}, while arrows (b) indicate new additions and where I_{lim} is measured. Cross-linking time = 5 min, C_{GOX} = 0.27 U per sensor, C_{glut} = 3%, 30% mucin and 70% albumin.

2.4. Construction of the enzymatic electrode

An aliquot of 4 μL GOX-matrix system was mixed with 3 μL of glutaraldehyde and entrapped between two membranes of polycarbonate. The resulting sandwich-type arrangement was placed with precision tweezers at the surface of the Pt working electrode and fixed with a suitable cap [14]. After 5 min, buffer solution was used to rinse the electrode and eliminate the excess of glutaraldehyde and other molecules that did not react with the polymeric matrix.

2.5. Procedure

Once a sandwich biosensor has been assembled and washed with buffer solution it is placed into the electrochemical cell. Electrochemical measurements were performed in base electrolyte solution pH 7.0 at room temperature (23 ± 3) °C. The solution was stirred at 120 rpm during the whole electrochemical experiment. The oxidation of H_2O_2 is measured at 0.65 V and this potential value is applied for 20 min before starting with the additions of samples with glucose. After this equilibration time, the base current of the system decayed practically to zero and it is constant enough to start measuring. The current corresponding to this equilibration period is not recorded.

3. Results and discussion

3.1. Effect of the hydrogel composition

Fig. 1 shows the typical chronoamperometric response corresponding to the calibration curve of one of the several sandwich-type biosensors prepared in this work. The arrows (a) and (b) indicate where the response time ($t_{95\%}$) and the limiting current (I_{lim}) are measured, respectively. The value of response time corresponds to the time where the current reaches 95% of I_{lim}. These two parameters are very important for developing a biosensor with relatively good signal of current and response time. The analysis of these two parameters is presented in Fig. 2.

The values of the slopes corresponding to calibration curves prepared with different concentrations of glutaraldehyde, mucin, and albumin are presented in Fig. 2A. Every data point corresponds

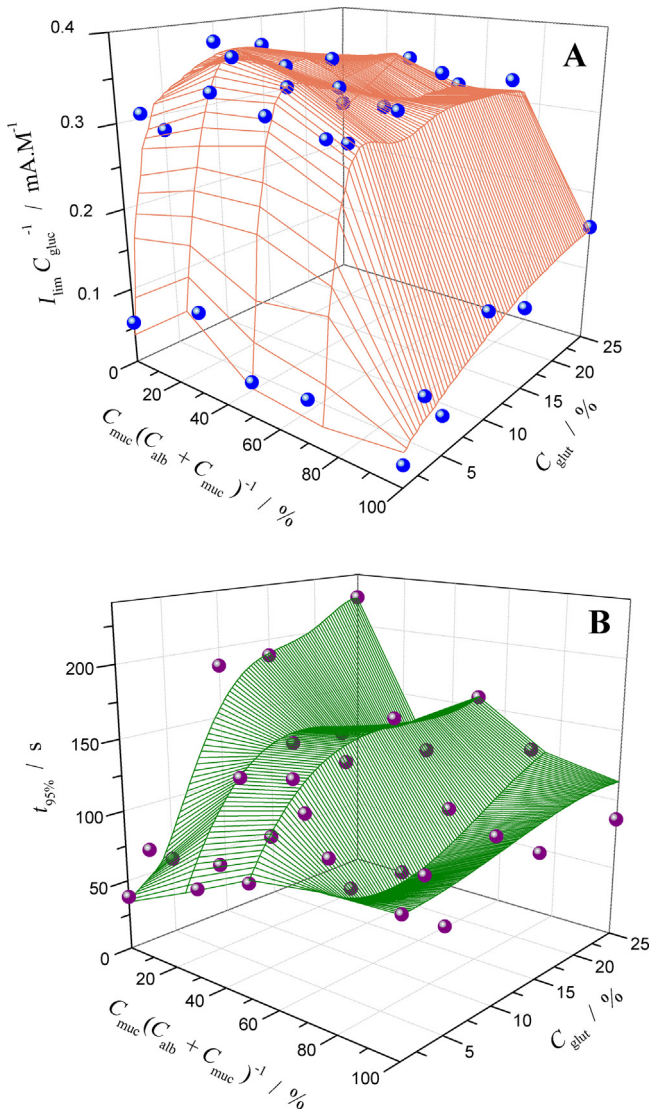

Fig. 2. Dependence of (A) sensitivity ($I_{lim}\,C_{gluc}^{-1}$) and (B) response-time at 95% of I_{lim} ($t_{95\%}$) on the % of glutaraldehyde and on the mucin/albumin mass ratio employed for the construction of the enzymatic matrix of a GOX-biosensor. The time for the cross-linking reaction was 5 min, C_{GOX} = 0.27 U/sensor.

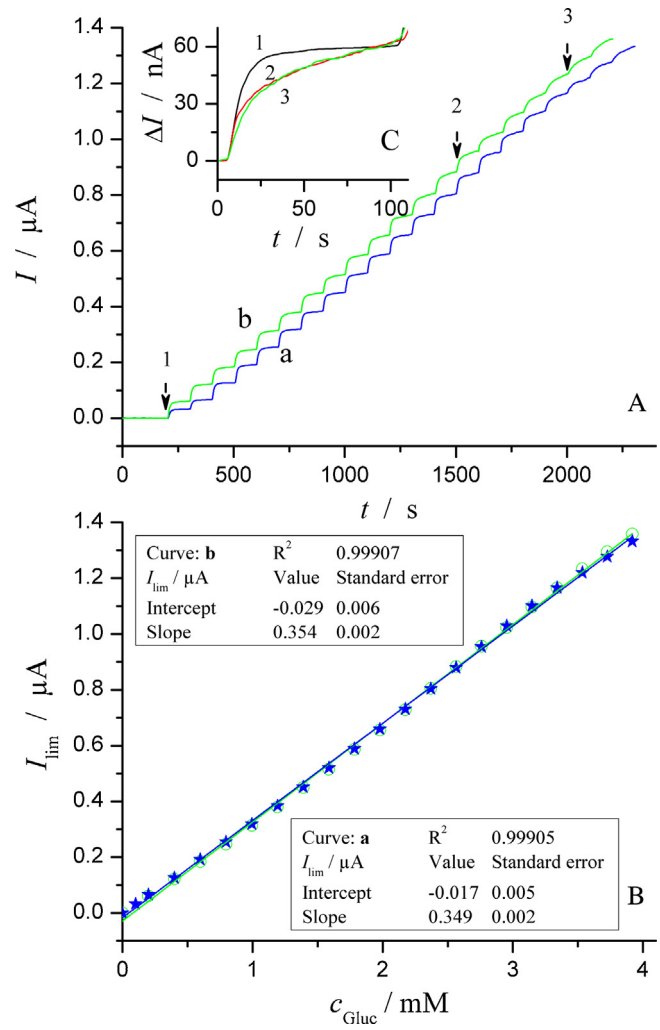

Fig. 3. (A) Chronoamperometric profiles for 2 independent enzymatic matrixes composed by 2.7 U/sensor of GOX. (B) Calibration curves of each biosensor calculated from the values of I_{lim}. (C) Chronoamperometric profiles for the additions 1, 2, and 3. The first two additions of curve (a) correspond to 0.1 mM glucose, all others are of 0.2 mM glucose. Cross-linking time = 5 min, C_{glut} = 3%, and 30% mucin.

to parameters averaged from at least 3 calibration curves. The sensitivity of the GOX-sensor is rather low when the concentration of glutaraldehyde is lower than 3% and when the enzymatic matrixes are prepared only with mucin. On the contrary, the value of I_{lim} increases when higher amount of glutaraldehyde and albumin is used. The response of I_{lim} remains practically constant for those enzymatic matrixes prepared with more than 3% of glutaraldehyde and when at least 30% of the mass of the hydrogel corresponds to albumin. Actually, there is a slight maximum of I_{lim} when the enzymatic matrix is prepared with 5% of glutaraldehyde and 30% of mucin. This behavior can be explained considering that glutaraldehyde is a cross-linker species that reacts with the amino groups of other molecules, while mucin is a glycoprotein that has very few amino groups exposed to the solution [12,22]. As a consequence, those hydrogel matrixes prepared with low amount of glutaraldehyde and/or high amount of mucin will not be significantly crosslinked and the enzyme would escape from the sandwich during the washing and measuring steps.

As stated above, it is also necessary to consider the dependence of response-time on the concentration of glutaraldehyde and albumin to decide what enzymatic matrix provides the best conditions for a biosensor of these characteristics. Fig. 2B shows that those sensors prepared with 100% of albumin and relatively high concentration of glutaraldehyde have the slowest response-time of the set of analyzed sensors. On the contrary, the fastest response is found for the sensors prepared with the lowest concentration of crosslinker. Also the biosensors built with high percentage of mucin exhibit responses faster than those with higher amount of albumin in their composition. Apparently, a high level of cross-linking limits the diffusion of reagents and products through the enzymatic matrix and so, it increases the response-time of the sensor [12].

The selection of the most suitable enzymatic matrix was performed in two steps. First, it was calculated the ratio between the data of Fig. 2A and B, which corresponds to the ratio between sensitivity and response-time for each sensor. Second, it was evaluated the linear range and the sensor-to-sensor reproducibility of the sensors selected from the first step. After this analysis, it was selected a hydrogel composed by 30% mucin and 70% albumin crosslinked with glutaraldehyde diluted to 3%. This enzymatic matrix presented not only the best ratio between sensitivity and response-time of the analyzed sensors, but also had very good sensor-to-sensor reproducibility when compared with other matrix compositions.

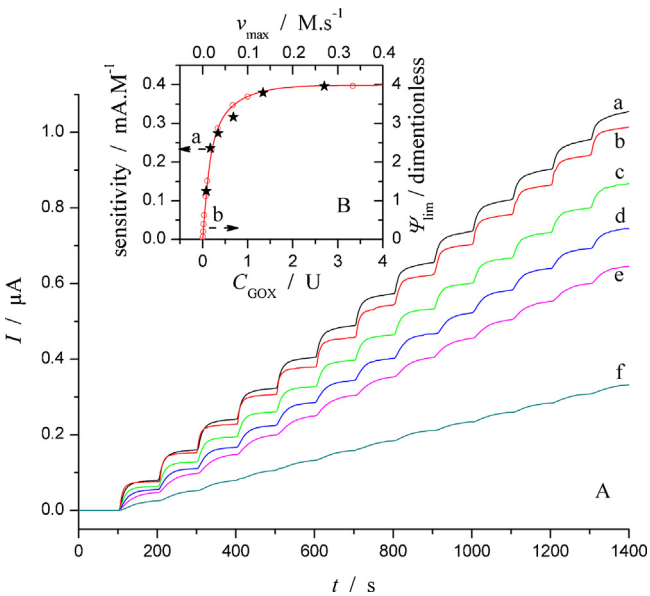

Fig. 4. (A) Chronoamperometric profiles for enzymatic matrixes composed by diverse concentrations of enzyme. Responses correspond to several additions of 0.2 mM glucose. Cross-linking time = 5 min, C_{glut} = 3%, 70% albumin and 30% mucin, C_{GOX}/U per sensor = (a) 2.7; (b) 1.34; (c) 0.67; (d) 0.34; (e) 0.17 and (f) 0.08. (B) Dependence of (a) the experimental I_{lim} on C_{GOX} and of (b) the theoretical Ψ_{lim} on v_{max}. In the model $D_{glucose} = 1\times10^{-6}$ cm^{2}s^{-1}, $D_{H2O2} = 5\times10^{-6}$ cm^{2}s^{-1}, $D_{O2} = 1\times10^{-5}$ cm^{2}s^{-1}, C_{O2} = 0.274 mM, $K_{O2} = K_{glucose}$ = 0.1 M, v_{max} = 0.10 mM s^{-1}, $C_{glucose}$ = 0.1 mM, sensor thickness 100 μm.

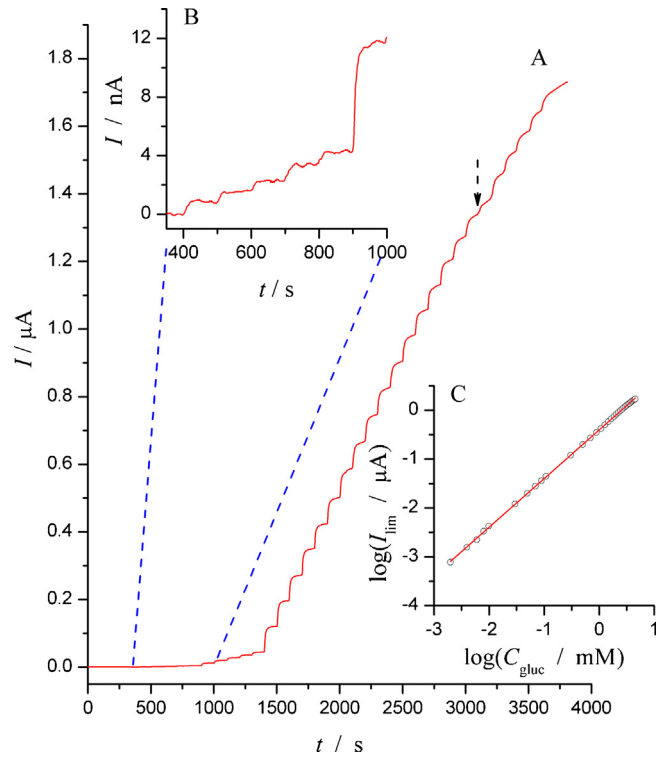

Fig. 5. (A) Chronoamperometric profile of a biosensor prepared with C_{glut} = 3%, 30/70 mucin/albumin, C_{GOX} = 1.34 U. Additions are of 2 μM (Inset B), 20 μM, and 0.2 mM glucose. (C) Logarithmic dependence of I_{lim} on C_{GOX}.

3.2. Sensor-to-sensor reproducibility

Fig. 3 shows chronoamperometric responses of two glucose biosensors prepared with the same amount of enzyme. As it can be observed both curves are very similar. In this regard, the first two additions of curve (a) were made with 0.1 mM glucose instead of 0.2 mM, in order to simplify the distinction of both curves. From the analysis of a set of six calibration curves corresponding to independent sandwich-type biosensors, prepared from the same vial of enzymatic matrix, it was found that the slope may vary up to 10% around an average value equal to 0.36 mA M^{-1} (not shown). This result points out the very good sensor-to-sensor reproducibility of this system. However, as it was indicated above, it depends on the selected enzymatic matrix as well as on the control of the crosslinking reaction time [12,14].

The inset 3C compares the shape of 3 steps of curve (b) that have been indicated with arrows in Fig. 3A. It can be noticed that the response-time of the sensor increases for solutions with high concentration of analyte. This fact particularly occurs for solutions with more than 2.4 mM glucose. At first, it was considered that this issue could be associated with the lost of the enzyme that was not properly crosslinked to the matrix and that was slowly released to the bulk. However, the comparison of diverse chronoamperometric profiles showed that several sensors prepared with different concentration of glutaraldehyde increase their response-time when C_{gluc} > 2.4 mM, not shown. As a result, we concluded that the lost of linearity depends on the diffusion of reactants, particularly oxygen, through the enzymatic matrix and that this problem can be minimized by reducing the concentration of enzyme.

3.3. Effect of the amount of GOX

Fig. 4 shows chronoamperometric responses of sensors prepared with different concentration of GOX. Every addition corresponds to 0.2 mM glucose. In all cases, the hydrogel is prepared with a mixture 30/70 of mucin/albumin. After the addition of the respective amount of enzyme, the mixture is crosslinked with 3 μL glutaraldehyde previously diluted to 3%. All sensors present linear response in the analyzed range of glucose concentration.

Recently, we did theoretical work evaluating diverse aspects of sandwich-type biosensors [18,19]. Particularly, it was pointed out that the response-time of those sensors should decrease and the steady-state current (Ψ_{lim}) should rise by increasing the value of v_{max}, which is proportional to the concentration of enzyme [18]. Both outcomes are in agreement with the shape of the chronoamperometric curves exhibit in Fig. 4A. However, in our previous manuscript it was predicted that the steady-state current (Ψ_{lim}) should increase linearly with the value of v_{max} [18]. Instead of it, Fig. 4B shows that Ψ_{lim} increases linearly only for v_{max} < 0.01 M s^{-1}, curve (b). Above this value, the dependence of Ψ_{lim} on v_{max} deviates from the expected linear behavior and Ψ_{lim} reaches a plateau for v_{max} > 0.3 M s^{-1}. The equations used to calculate theoretical results are included in Appendix A, while further description of the model can be consulted elsewhere [18].

The comparison of theoretical and experimental results provides a quite simple way to estimate that a sensor prepared with 1.34 U of GOX would have a value of $v_{max} \approx 0.13$ M s^{-1}, curves (a and b). This fast approximation can be performed simply by comparing the curved region of theoretical and experimental profiles of Fig. 4B. Moreover, these data indicate that it does not worth to use higher concentration of GOX, since the response of the sensor is limited by the diffusion of glucose and O_2, the natural mediator of this enzymatic reaction.

3.4. Detection limit and linear range of the selected matrix

From the above results it is possible to consider that a hydrogel composed by 3% of glutaraldehyde, a mass ratio 30/70 of mucin/albumin, and C_{GOX} = 1.34 U would be the most suitable enzymatic matrix for developing a biosensor with relatively

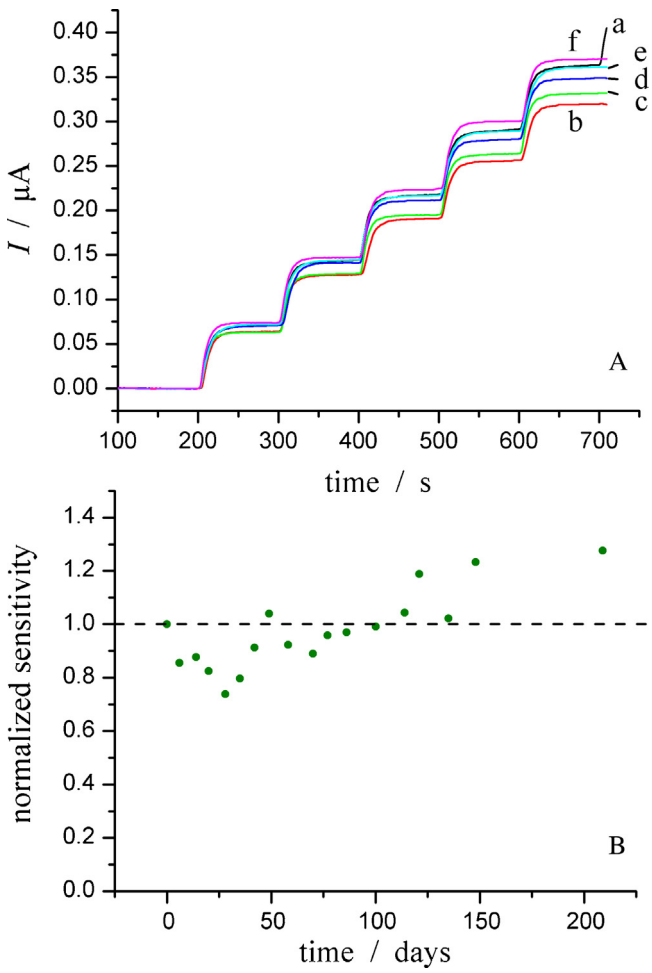

Fig. 6. (A) Chronoamperometric profiles recorded: (a) 0; (b) 14; (c) 42; (d) 77; (e) 100 and (f) 135 days after the assembling of the biosensor. The sensor was prepared with 1.34 U GOX, 30/70 mucin/albumin, 3% glutaraldehyde and stored in buffer pH 7 at 4 °C after every calibration curve. Additions correspond to 0.1 mM glucose. (B) Evolution of the sensitivity of the sensor as a function of the days of its assembling.

good sensitivity, linear range, and response time. Fig. 5 shows a chronoamperometric profile of a sandwich-type biosensor prepared with this selected enzymatic matrix. As it can be observed, several additions of 8 μL have been made to the electrochemical cell. The cell was initially filled with 4 mL of buffer pH 7 and standard solutions of 1, 10 and 100 mM glucose were used to determine the detection limit (LOD) and linear range of the sensor. Fig. 5B shows the chronoamperometric response of the sensor after additions of 2 μM of glucose, while the last addition of glucose in Fig. 5A gives a final concentration of 4.7 mM.

According to IUPAC, the value of LOD can be calculated as 3 times the ratio between the standard deviation of the blank signal (σ_b) and the slope of the calibration curve [23]. The value of σ_b was calculated from a set of data recorded for 400 s before the first standard addition of glucose, providing a value of LOD = 0.9 μM. Although this value would be consistent with the data presented in Fig. 5B, this definition of LOD does not consider the quality of the linear regression analysis. In this regard, the linear regression of data for $C_{gluc} \leq 3.5$ mM (arrow) has a squared correlation coefficient (R^2) equal to 0.9999, while the inclusion of data up to $C_{gluc} = 4.7$ mM decreases the value of R^2 to 0.9990. Both regression analyses have practically the same value of slope, but their errors are notably different [24]. Thus, it has been calculated a value of LOD = 3 μM by using 3 times the standard deviation of the y-intercept (σ_y) divided by the slope of the calibration curve calculated for data with $C_{gluc} \leq 3.5$ mM. The last equation was suggested by the International Conference on Harmonisation (ICH). This way for calculating the value of LOD is consistent with the data of Fig. 5, considers the instrumental noise of the signal, and also the dispersion of data around the linear regression curve [24].

Fig. 5C is a logarithmic plot where it can be clearly observed that the sensor has excellent range of linear behavior since the linear dependence of I_{lim} involves more than 3 orders of magnitude.

3.5. Stability of the proposed sandwich-type biosensor

Fig. 6 shows chronoamperometric profiles of a sandwich-type biosensor prepared with an enzymatic matrix composed by 30/70 mucin/albumin, 1.34 U GOX, and 3% glutaraldehyde. Every addition of glucose increases the concentration in 0.2 mM. After measuring a calibration curve, the biosensor is rinsed and then stored in buffer pH 7 at 4 °C. As it can be observed, the shape of chronoamperometric curves remains practically unchanged after 7 months of use. Actually the sensitivity of the sensor decreased during the first month and then started to increase. After the 4th month the electrochemical response became even higher than the initial signal. The sensor response remained almost constant until the end of the time considered for this analysis, Fig. 6B. After this evaluation period, the biosensor still did not significantly change its LOD and kept its linear behavior within the range of 3.5 mM (not shown).

4. Conclusion

The analysis and optimization of an enzymatic matrix suitable for the development of a sandwich-type glucose biosensor has been presented. The enzymatic matrix is a hydrogel composed by GOX, mucin and albumin crosslinked with glutaraldehyde. The selected enzymatic matrix showed excellent performance for the detection of glucose. The proposed biosensor has an average response time of (35 ± 5)s, high sensitivity, remarkable stability, high sensor-to-sensor reproducibility, and very low production cost.

The linear response of the sensor involves solutions with glucose concentrations that go from micro to milimolar. Although, the value of LOD calculated from σ_b would indicate that the sensor can detect additions of glucose =0.9 μM, the ICH suggests the use of σ_y for estimating LOD. In this way, the instrumental noise of the signal and the dispersion of data around the linear regression curve are considered in the value of LOD [24]. Accordingly, a value of LOD = 3 μM was calculated as 3 times σ_y divided by the slope of the calibration curve calculated for data of $C_{gluc} \leq 3.5$ mM. This value of LOD is consistent with the data presented in Fig. 5 and it would be more reliable than that obtained from σ_b.

The results presented in this manuscript point out that a biosensor prepared with the same geometric characteristics and with $C_{GOX} > 1.3$ U would not necessarily increase the analytical signal. Besides, very high amount of enzyme might slightly affect the linear range of this kind of sensors, due to the depletion of oxygen within the enzymatic membrane. Simulated curves are compared with experimental data to explain the dependence of sensitivity on the concentration of enzyme. In addition, this kind of comparison represents a quite simple way to estimate the value of $v_{max} \approx 0.13$ M s^{-1} from the amperometric response of a sensor prepared with 1.34 U of GOX.

Considering that sandwich-type biosensors are commonly assembled as part of devices where the sample is diluted with buffer, the more than 3 orders of magnitude of linear behavior of this sensor would ensure the possibility for assessing any sample. The accuracy of this biosensor might be compromised by the effect

of electroactive interferents that are commonly present in samples such as blood or serum. The response of the biosensor when exposed to real samples will be discussed in a future work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2015.01.063.

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