Sensors and Actuators B xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Research Paper

A robust and practically free of charge intermittent use glucose biosensor

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ARTICLE INFO

Article history:
Received 25 July 2017
Received in revised form
15 September 2017
Accepted 2 October 2017
Available online xxx

Keywords:
Biosensor
Glucose
Chronoamperometry
Long-term stability
Intermittent use
Diabetes

ABSTRACT

In the past decades, great effort has been put in finding new electrode surface modifiers and enzyme immobilizer agents that prevent the enzyme leakage, minimize the effect of interfering species, retain the enzyme bioactivity, and enhance the sensor sensitivity. In this work, a sandwich-type glucose biosensor that keeps its sensitivity and operational linear range for more than a year is presented. After 5 months of intermittent use, where the biosensor was exposed to more than 500 standard additions, it presented a limit of detection of 5 μM, and the linear behavior was from 5 μM to 3 mM with a value of $r^2 = 0.999$. Besides, after 7 months of its assembling, the biosensor was employed for assessing the glucose concentration of real serum samples and its performance was compared with the response of a commercial autoanalyzer. A year later, the biosensor still exhibited very good performance of its analytical parameters.

The performance of identical sandwich-type biosensors is analyzed when they are exposed to three different storage conditions. Simulated curves are compared with experimental data to explain the dependence of sensitivity and response-time on the aging and storage conditions of the biosensors.

1. Introduction

Biosensors can be defined according to the way in which they are utilized. On the one hand, they can be used for qualitative, semi-quantitative, or quantitative analysis, but on the other hand, they can be used for a single, intermittent or continuous measurement process [1]. Within this last classification, single use biosensors are the most common type of sensor when the analysis of glucose in blood is required [1]. These sensors are user friendly because they contain the selective and the transducer elements in a cell, which is the single use test strip. Since they cannot be calibrated while they are in use, they are mechanically created to provide sufficient sensor-to-sensor reproducibility. This kind of sensors is the most used for periodical testing of diabetes, a disease that has been declared as a global epidemic by World Health Organisation [2,3].

Unfortunately, the possibility of a cure for diabetes seems to be unrealistic in the short term [3]. There is a highly lucrative market though, which is the main driving force in the area of commercial devices for blood glucose monitoring [3]. In this regard, it is important to consider that there are wide gaps between academic achievements, commercial developments, and social needs related to sensor research [1]. Academic researchers and large diagnostic companies are both focused on the improvement of easy-to-use strips and continuous monitoring devices. To achieve their goals, they are using different strategies commonly related to nanotechnology. However, while academic researchers are focused on the sensitivity and detection limit of their sensors, they do not put too much attention on the cost per strip or the cost per analysis, which is one of the main concerns of diagnostic companies [3]. Typically it is considered that the price of a sensing strip is low enough if it is around or below U$ 1. This asseveration is true when it is compared with the price of a strip developed in an academic research lab. However, the cost per analysis is still quite expensive and not too accurate when it is compared to other laboratory methods [1].

The market of strips for glucose analysis is segmented with Roche, Minimed, LifeScan, Dexcom, Bayer, and Abbott as the key players. Their commercial devices fulfill the accuracy, precision, and reliability required by the International Organization for Standardization (ISO). In this regard, the ISO 15197:2003 specifies that 95% of the individual glucose results must be within $\pm 15\ mg\ dL^{-1}$ for samples with glucose concentration $<75\ mg\ dL^{-1}$ and that the error must be within 20% if the glucose concentration is $\geq 75\ mg\ dL^{-1}$ [3]. After reading those requirements, a simple question should come up in the mind of most researchers: Why are not those parameters more rigorous? Perhaps the physicians do not require more accuracy or perhaps this is the actual state of art for

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https://doi.org/10.1016/j.snb.2017.10.010
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Table 1
Comparison of the analytical performance of different GOx electrodes.

Electrode matrix	Immobilization method	Detection range/mM	Effect of interfering species	Storage Stability	Electroactive species	References
Glucose oxidase (GOx)/polyaninline (PANI)	Electrochemical doping	0.005–10	Low	95% activity after 30 days	H_2O_2	[9]
Chitosan(Chit) Grafted Pd nanoparticles (NPs)		0.002–1	Low	91% activity after 60 injections or 94% 3 weeks	H_2O_2	[10]
GOx/PANI/ /polyisoprene	Entrapment	0.01–12	Negligible	5 months	H_2O_2	[11]
GOx/Pt NPs/mesoporous silica NPs	Crosslinking	0.001–26	Negligible	90% activity after 1 month (used > 100 times)	H_2O_2	[12]
GOx/PANI/polyacrylonitrile	Entrapment	0.002–12	Negligible	100 days	H_2O_2	[13]
Polymethyl methacrylate/bovine serum albumin (BSA) core-shell NPs	Crosslinking	0.2–9.1	Very low	1 month	H_2O_2	[14]
multi-walled carbon nanotubes (MWNTs)/Chit/BSA/Ferrocene/GOx	crosslinking	0.01–30	Negligible	95% activity after 350 injections or 99% 30 days	Ferrocene	[15]
GOx/cytochrome C/Au NPs/PANI nanospheres	Immobilization of GOx via Nafion	0.01–3.2	Very low	92% activity after 30 days	H_2O_2	[16]
GOx/Chit hydrogel/Au NPs	Physical entrapment	0.012–3	Negligible	91% activity after 4 weeks	Ferrocene	[17]
Au NPs on eggshell membrane	crosslinking	0.008–1	Very low	87% activity after 10 weeks	Oxygen	[18]
Nafion/GOx/(MWNTs)/PANI/Prussian blue	Physical entrapment	1–11	Very low	90% after 30 days (used 15 times)	H_2O_2	[19]
Polycarbonate/Mucine/BSA/GOx	crosslinking	0.005–3	Low	Close to 100% after 1 year and > 500 injections	H_2O_2	This manuscript

most single use biosensors. Table 1 summarizes some biosensors listed in recent published reviews [3–8].

As it can be observed most of these biosensors offer excellent limit of detection (LOD) and linear range. Also, they have quite good stability and present low or negligible effect of interfering species. However, it is not clear if they can assure the same LOD or linear range after a week or a month of assembling. Furthermore, nor the sensor-to-sensor reproducibility neither the linear range are typically analyzed after a month of assembling. Eventually, some of those reasons would be associated with the definition of ISO 15197:2003 [3].

Intermittent use biosensors correspond to another commercially available methodology of analysis in which a flow stream is commonly used to refill the cell and reuse the sensor. This other kind of biosensors typically exhibits much better precision, accuracy, and sensitivity than single use biosensors. Moreover, the cost per data point of intermittent use biosensors is very modest, since they are commonly used for weeks or months instead of for few minutes [1]. Even though this kind of sensors is very promising, they have at least two important disadvantages. First, they are not as easy-to-use as the well-known glucose test strips and second, they do not provide the same economical profits [1]. Sandwich-type biosensors are a kind of intermittent use biosensor where the enzyme is stored into a hydrogel placed between two diffusion membranes. The diffusion membranes are hydrophilic and they can be modified to stop the passage of interfering species [20,21]. The performance of enzymatic biosensors is dramatically affected by the physicochemical characteristics of the microenvironment that surrounds the enzyme [22–24]. The activity and stability of the enzyme, as well as the response-time of the sensor are the outcome of the immobilization process, the sensor geometry, and of the electrode material that was selected for building up the sensor. Usually, the stability of these biomolecules is increased when they are in contact with molecules presenting glycosidic groups [23,25].

In this opportunity, it is analyzed the performance of a sandwich-type glucose biosensor that has been intermittently used for more than a year. The cost of each sensing membrane would be around a quarter (U$ 0.26) and it can be stored in buffer or into an empty vial. Chronoamperometric experimental curves have been simulated to explain how different storage conditions affect the behavior and performance of the biosensor. Finally, the glucose concentrations of real serum samples are compared with the

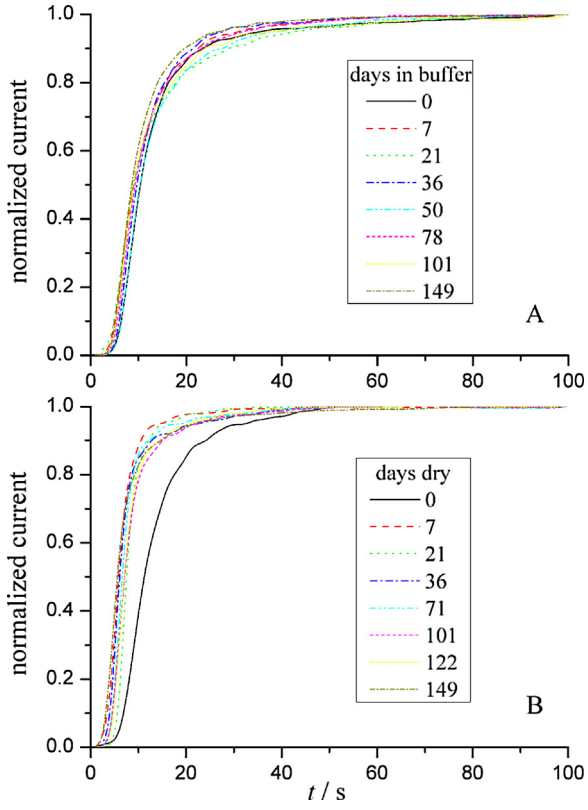

Fig. 1. Normalized chronoamperometric profiles of two glucose biosensors after the addition of 0.2 mM glucose. The curves correspond to the response of each biosensor after different days of assembling. The biosensors were stored in buffer (A) and in an empty vial at 4 °C (B).

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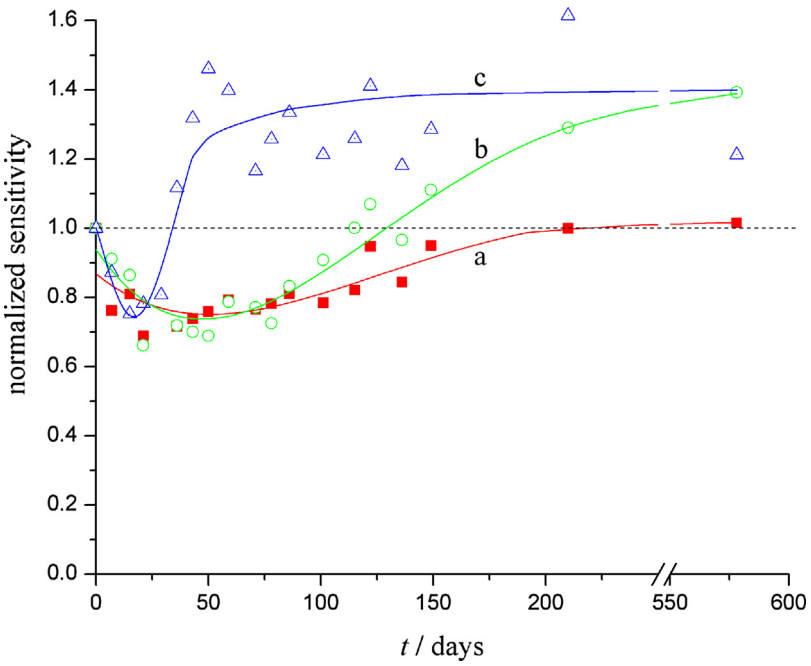

Fig. 2. Normalized sensitivity of three glucose biosensors after different days of assembling. The biosensors were stored: in buffer at 4 °C (a), in an empty vial at 4 °C (b), and in an empty vial at room temperature (c).

standard hexokinase-spectrophotometric method employed in a hospital of Cordoba City. For the comparison, it is used a 222 days old sandwich-type glucose biosensor that was intermittently used and stored in an empty vial at room temperature. Accuracy and precision are very good when compared with the requirements of ISO 15197:2003 [3].

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 M Ω 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.

A solution of glutaraldehyde 25% v/v (Backer, USA) was prepared in base electrolyte. Mucin type III (catalog number M1778, Sigma, USA) was mortared and stored as dry powder at 4 °C. Bovine serum albumin (catalog number A4503, 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 (Whatman Nuclepore, catalog number WHA111103, Sigma, 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 disc (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. Proteins were mixed for 5 min and then transferred to a vial containing 20 μL with 40 U of GOX. The resulting 60 μL GOX-matrix system was mixed for extra 5 min and stored at 4 °C until the construction of each enzymatic electrode.

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 polycarbonate membranes. The resulting sandwich-type arrangement was placed at the surface of the Pt working electrode and fixed with a suitable cap [20]. After waiting for 5 min, the sandwich-type biosensor is placed in a beaker with 4 mL of buffer solution to 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 at pH 7.0 and 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 the equilibration time, the base current of the system decayed practically to zero and it is constant

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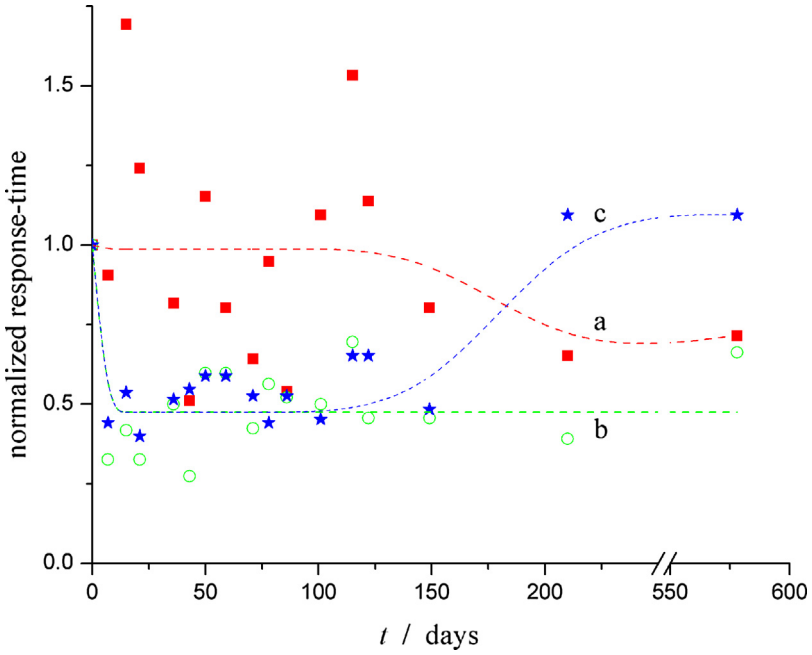

Fig. 3. Normalized response-time of three glucose biosensors after different days of assembling. The biosensors were stored: in buffer at 4 °C (a), in an empty vial at 4 °C (b), and in an empty vial at room temperature (c).

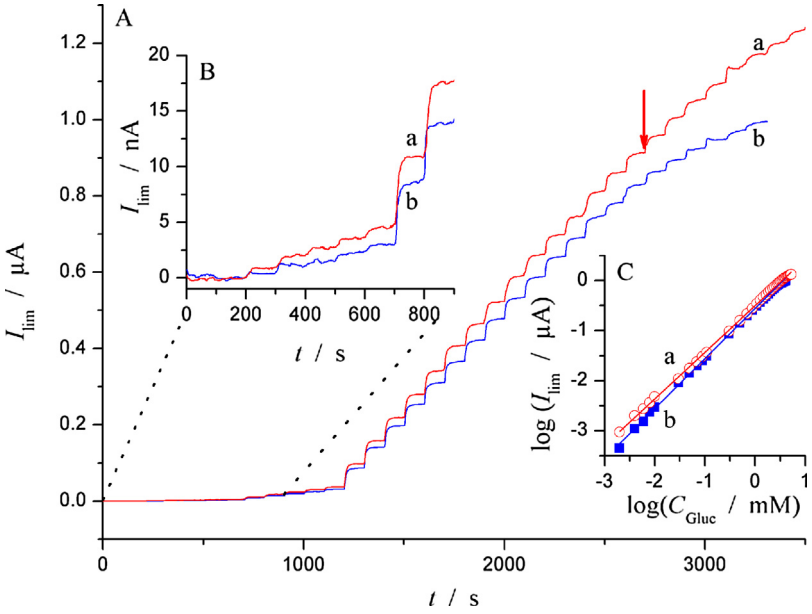

Fig. 4. (A) Chronoamperometric profiles of two sandwich-type biosensors. The additions of glucose correspond to: 2 μM (Inset B), 20 μM, and 0.2 mM. (C) Logarithmic dependence of I_{lim} on C_{Gluc}. Both curves correspond to biosensors exposed to intermittent use for 222 days. The biosensors were stored at 4 °C, dry (a) and in buffer (b).

enough to start measuring. The current of this equilibration period is not recorded.

Three identical sandwich-type biosensors were constructed for the analysis of long-term stability. One sensor was stored at 4 °C in phosphate buffer pH 7 (sensor A), another biosensor was stored in an empty vial at 4 °C (sensor B), and the third biosensor was stored in an empty vial at room temperature (sensor C).

2.6. Comparison with a reference method

Serum samples were provided by Hospital Privado SRL in vials with walls protected with heparin. The glucose concentration of those samples was previously quantified at the clinical laboratory of the hospital where the standard hexokinase-spectrophotometric method was employed. The samples were analyzed within 24 hs of their reception. The analyses of samples were performed with a 7 months old biosensor stored dry at room temperature, sensor C. After the equilibration time, 40 μL of serum was added to a vial

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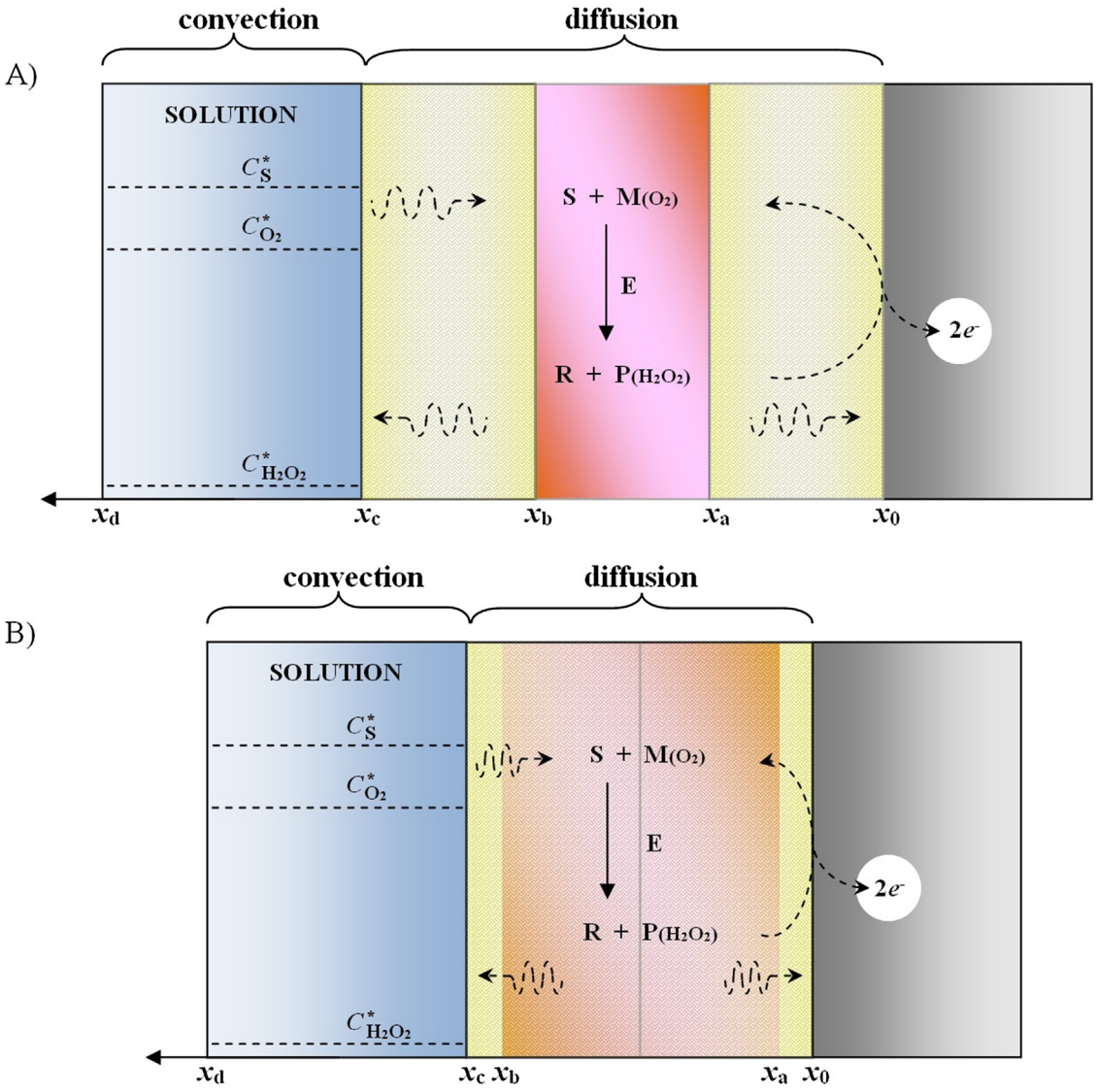

Fig. 5. Schemes of a sandwich-type amperometric biosensor when the enzymatic matrix does not permeate into the membranes (A) and when the sensor is successively dehydrated and rehydrated (B).

containing 4 mL of buffer. The signal was recorded 100 s after the addition.

3. Results and discussion

3.1. Long-term stability of the sensor

Fig. 1 shows the responses of two sandwich-type biosensors. Both sensors were assembled in the same way and with the same composition, but stored under different conditions. The biosensor of Fig. 1A was used and then stored in a vial with buffer at 4 °C, while the biosensor of Fig. 1B was used, rinsed with buffer, and subsequently stored in an empty vial at 4 °C. The responses have been normalized to simplify the comparison of their chronoamperometric profiles. The biosensor of Fig. 1A exhibits very reproducible profiles. The shape of these chronoamperograms is practically the same irrespectively of the day of analysis. As a result, all curves show well-defined steady-state currents to calculate the analytical sensitivity of the sensors. Besides, the physicochemical, geometrical, and biocatalytic characteristics have not apparently changed from one week to another. These data indicate that the composition of the enzymatic matrix is not only suitable for providing a good signal to the sensor, but also for ensuring good stability to the enzyme [26].

A different situation is observed in Fig. 1B, since the signal changes from the first day of analysis. In this experiment it was intended to study how robust was our recently developed glucose biosensor under critical storage conditions. Curiously, after a week of assembling, the chronoamperometric profiles of the sensor achieved the steady-state in a period shorter than that of a freshly prepared sensor. This biosensor, stored under practically dry conditions, presented its fastest response after a week of assembling, while the slowest corresponded to its day zero.

When the sensitivity of the biosensors is analyzed as a function of the assembling time, the current signal of each sensor decays after the first week of assembling. More precisely, the response of current becomes around 80% of the one observed for a freshly prepared sandwich-type biosensor, Fig. 2. This figure summarizes the information of the set of biosensors that were tested for more than a year. During this time, at least 20 calibration curves involving more than 10 standard additions of glucose were performed to each biosensor.

After the first week, the sensors stored into the fridge kept the sensitivity relatively constant for approximately 3 months, curves

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(a and b). Then, gradual increase of their sensitivity was observed during the following 5 months. In the case of curve (a), the increment on sensitivity was from 80% to 100% while in the case of the biosensor associated with curve (b), the increment of sensitivity was to around 130% of the signal measured when it was just prepared. As it can be observed, both biosensors kept the final values of sensitivity for around a year.

The change on sensitivity was more abrupt in the case of the biosensor stored at room temperature, curve (c). The sensitivity of this last biosensor decayed to around 80% during the first days. However, after a month of use, its analytical signal increased close to 130% of its initial value. Although biosensor C kept its chronoamperometric response relatively constant for more than a year, its sensitivity evidenced variations from one week to another. Those variations of sensitivity should not be important for a sandwich-type biosensor that is employed for intermittent use. However, the variations observed for sensor C were clearly more important than those of the biosensors stored in the fridge.

Fig. 3 shows the dependence of the response-time on the assembling time of each biosensor. The data have been also normalized to provide a simpler comparison of curves. The biosensor stored in buffer is the one that shows the highest dispersion on the response-time. However, all the values associated with its response-time were around an average value of $(5 \pm 2) \times 10^1$ s. The error was calculated from the standard deviation of data.

The sensors stored under dry conditions showed different dependence of the response-time on the storage-time. After the first week, the biosensors stored under dry conditions significantly decreased their response-time. The average value of their response times were (23 ± 6) s for the sensor stored inside the fridge and (25 ± 4) s for the sensor kept at room temperature. In both cases the response-time was practically half that of a freshly prepared sandwich-type glucose biosensor. After the fifth month, however, the biosensor stored dry at room temperature increased its response-time to around 55 s, while the one that was stored in the fridge kept its response-time unvarying, curves (b and c).

Parameters such as sensitivity and response-time are very important for a biosensor [27]. In this regard, depending on the storage conditions, the developed sandwich-type glucose biosensors have shown good performance on both parameters for more than 1.5 years. Fig. 4 shows calibration curves measured on the 222nd day after assembling the sandwich-type biosensors. Both biosensors were stored into the fridge at 4 °C, one in buffer and the other one dry. Besides, both sensors were utilized for the construction of several calibration curves before being employed for obtaining those plots, see Figs. 1–3. The arrow of Fig. 4A corresponds to a glucose concentration (C_{Gluc} = 3 mM). The coefficient of determination up to this concentration value was ($r^2 \geq 0.999$) and we consider that it is the highest concentration where the relationship between the analyte concentration and current is actually linear for a sensor.

The values of standard deviation, calculated from the last 200 s of the baseline of each calibration curve, were 2×10^{-5} μA for curve (a) and 1×10^{-5} μA for curve (b). The ratio between those values and the slope of respective calibration curves would provide the limit of detection (LOD) of each sensor. However, the resulting values, 0.2 μM for curve (a) and 0.1 μM for curve (b), cannot be detected with any of these sensors, see inset 4B. In this regard, the use of 3 times the standard deviation of the intercept obtained from the linear regression analysis provides more realistic values for the value of LOD [26–28]. The resulting LOD values are 5 μM and 8 μM for the biosensors stored dry and in buffer, respectively. Both values are in agreement with the data shown in inset 4B, in which current signals resulting from the successive addition of 2 μM glucose are shown.

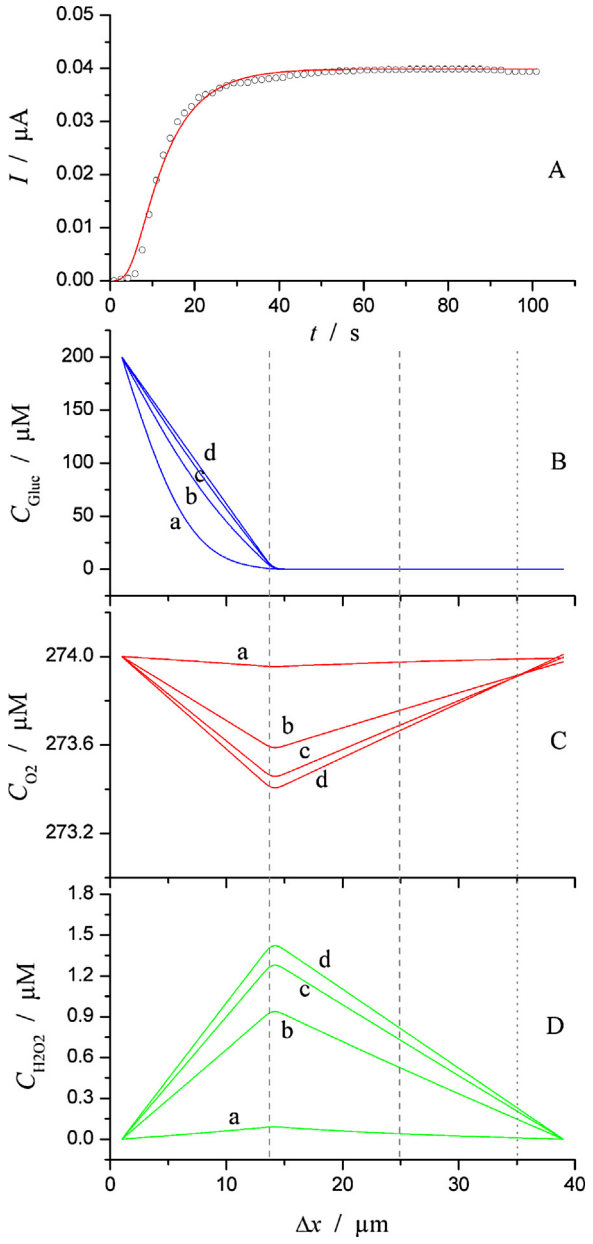

Fig. 6. (A) Experimental and simulated chronoamperometric profiles of a recently prepared sandwich-type glucose biosensor after the addition of 0.2 mM glucose. Concentration profiles of substrate (B), oxygen (C) and hydrogen peroxide (D) into a 38 μm thick sandwich-type glucose biosensor. The profiles were calculated: (a) 4 s, (b) 14 s, (c) 24 s, and (d) 60 s after the addition of 0.2 mM glucose. Dashed lines indicate the limits of polycarbonate membranes. Dotted lines indicate the limits of the enzymatic matrix. Other parameters are in the experimental section.

3.2. Why do old sensors respond faster than new sensors?

The developed sandwich-type biosensor has shown very good characteristics of sensitivity, linear-range, stability, and response-time. However, it is rather curious to find out that a biosensor has faster response-time when it is stored under dry conditions than in a buffer solution. To answer this question it is necessary to consider the model proposed for this kind of biosensors [24,29]. Fig. 5A shows a scheme of a freshly prepared biosensor with two diffusion membranes of polycarbonate. The thickness of this kind of membranes has been estimated to be close to 13 μm [29]. These membranes allow the diffusion of analytes and contain the enzymatic matrix. The enzymatic matrix is a hydrogel with a thickness close to 20 μm that provides a suitable environment for contain-

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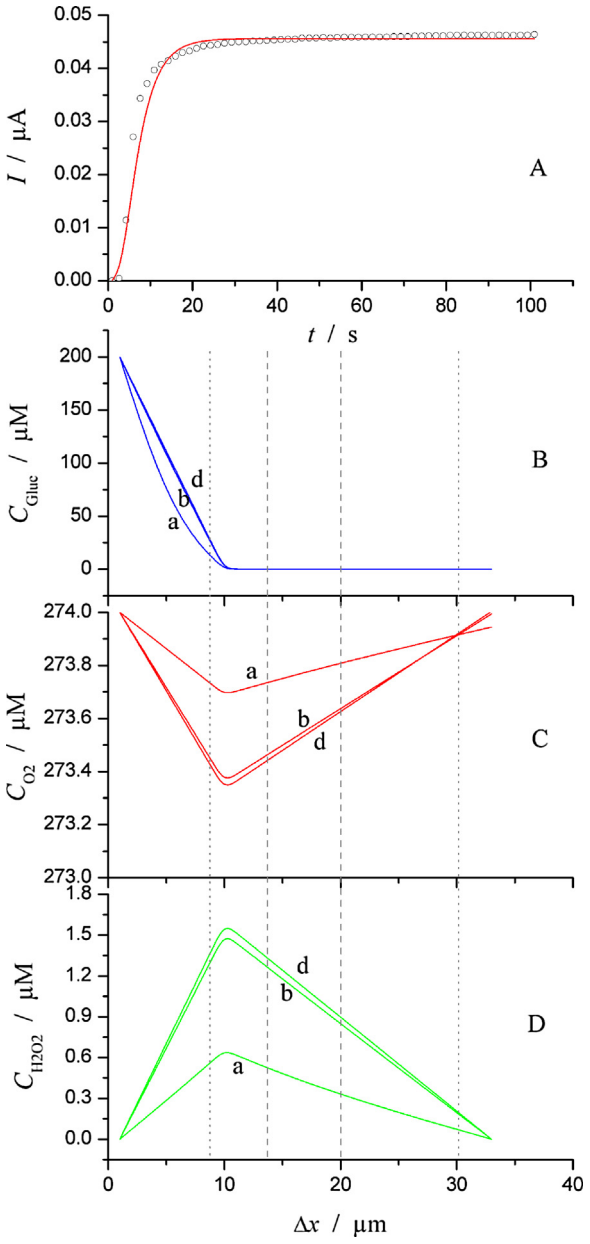

Fig. 7. (A) Experimental and simulated chronoamperometric profiles of a sandwich-type glucose biosensor, stored in an empty vial at 4 °C for 149 days, after the addition of 0.2 mM glucose. Concentration profiles of substrate (B), oxygen (C) and hydrogen peroxide (D) into a 33 μm thick sandwich-type glucose biosensor. The profiles were calculated: (a) 4 s, (b) 14 s, and (d) 60 s after the addition of 0.2 mM glucose. Dashed lines indicate the limits of polycarbonate membranes. Dotted lines indicate the limits of the enzymatic matrix. Other parameters are in the experimental section.

ing the enzyme [26,29]. The schemes presented in Fig. 5B would correspond to a biosensor that was dried and rehydrated several times. As a consequence of the dehydrating-rehydrating sequence the enzymatic matrix shrinks and swells back diffusing into the polycarbonate membranes. Since the enzymatic matrix is not dehydrated when the sensor is stored in buffer, no change is expected on the thickness of sensors stored in an aqueous solution. This hypothesis was included into our model to evaluate experimental chronoamperometric responses and to describe the concentration profiles of involved species.

Fig. 6A shows experimental and simulated chronoamperometric responses of a freshly prepared sandwich-type biosensor. Since the diffusion coefficient of glucose is much slower than those of O_2 and H_2O_2, the diffusion of substrate through the outer membrane limits the response-time of the sensor. Also, it has to be considered that the amount of active GOX within the sensor is relatively high [26]. As a result, it is expected high rate of the enzymatic reaction as well as fast consumption of substrate at the enzymatic matrix. From the simulation of the chronoamperometric curve, it is possible to get an insight about how the concentrations of involved species are changing within the biosensor. In a previous manuscript, the thickness of polycarbonate membranes was estimated to be 13 μm, which is a value consistent with that informed by the provider [29]. Fig. 6B–D show the concentration profiles of glucose, O_2, and H_2O_2. As stated above, those data are obtained from the simulation of the chronoamperometric curve of Fig. 6A.

Dashed lines indicate the limits of polycarbonate membranes, while dotted lines point out the limits of the enzymatic matrix. At 13 μm there is a dashed line that indicates the outer limit of both, the membrane and the enzymatic matrix. According to the model, the enzymatic matrix would extend from the edge of the outer polycarbonate membrane to 9 μm within the inner membrane. This is because the enzymatic matrix can diffuse into the inner membrane during the assembling of the biosensor [29]. Therefore, glucose molecules have to diffuse through 13 μm to react with the enzyme. At that point it is rapidly consumed by a highly concentrated and active enzyme [26]. It is interesting to note that, the concentration profile of glucose is practically linear within the outer membrane after 24 s of the analyte addition. This fact would indicate that the sensor is close to the steady-state condition, Fig. 6B. Another fact that points out the proximity of the steady-state is that the concentration gradient of glucose does not change significantly during the following seconds.

Typically, the diffusion coefficients of O_2 and H_2O_2 are higher than that of glucose. As a result, the concentration profiles of those species are usually linear and their maximum or minimum concentration values also need at least 24 s to achieve the steady-state condition, Fig. 6C and D. This is because the concentration gradients of those species depend on the concentration profile of glucose and on the thickness of the sensor. The maximum and minimum concentrations of those species develop at the edge of the enzymatic matrix that is next to the outer membrane due to the high concentration of enzyme and relatively low concentration of substrate used in the experiment [26,27].

Fig. 7A shows experimental and simulated chronoamperometric profiles corresponding to a sandwich-type biosensor after 149 days of being assembled. This biosensor was not only stored in an empty vial at 4 °C, but also it was used for the construction of several calibration curves since its assembling. The data associated with its sensitivity and response-time are summarized in Figs. 2 and 3. After 5 months, the response-time of the biosensor became practically the half of its initial value and its sensitivity was even 20% higher than that of a freshly prepared biosensor. To understand the improvement on these analytical parameters it is necessary to take a look to the concentration profiles of involved species. Fig. 7B–D show the concentration profiles of glucose, O_2, and H_2O_2 obtained from the simulation of the chronoamperometric curve of Fig. 7A. Again, dashed lines indicate the limits of polycarbonate membranes and dotted lines the limits of the enzymatic matrix. As it can be observed, the enzymatic matrix would have moved some micrometers into the outer membrane. As a result, the biosensor has become thinner than a freshly prepared sandwich-type biosensor and the analyte has to diffuse less distance than within a new sensor. This is because the biosensor losses water during the storage period. Accordingly, the enzymatic matrix shrinks and permeates into the polycarbonate membranes. Permeation of the enzymatic matrix within the external membrane is more important, because it was crosslinked into the inner membrane. Thus, the biosensor appears to be thinner than a freshly prepared biosensor and the

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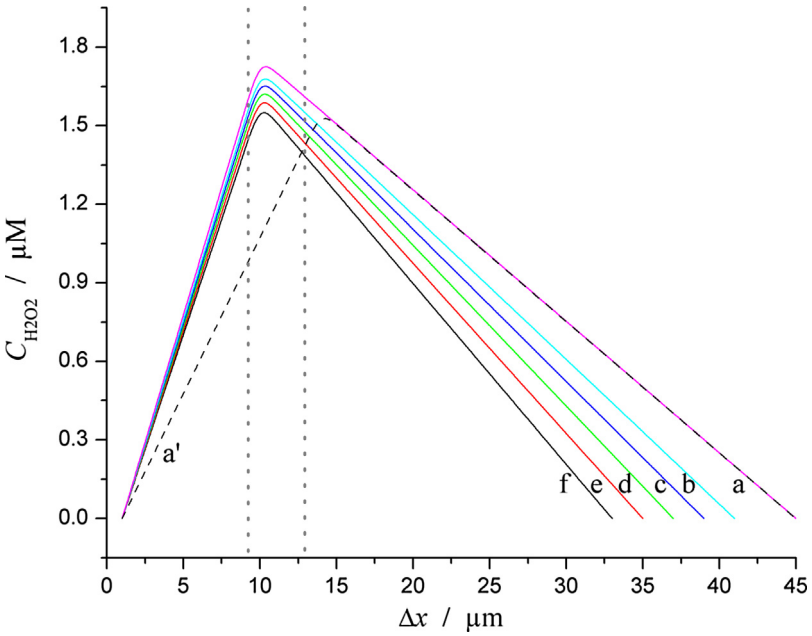

Fig. 8. Concentration profiles of hydrogen peroxide within sandwich-type glucose biosensors of different thickness. Profiles calculated at 60 s after the addition of 0.2 mM glucose and for C_{O2} = 0.274 mM. Dotted lines indicate the limits of the enzymatic matrix.

analyte has to diffuse shorter distance for reaching the enzyme. As a result, the permeation of the enzymatic matrix within the external membrane would diminish the response-time of the sensor.

Fig. 7B–D shows that not only the concentration profile of glucose, but also those of O_2 and H_2O_2 reach steady-state conditions after around 20 s. Although this result is consistent with the response-time of the experimental chronoamperometric curve, it does not explain the 20% of increment on the sensitivity of the biosensor. Actually, the sensitivity of the biosensor depends on the distance that H_2O_2 has to diffuse from the region of its maximum concentration to the electrode surface [27]. The magnitude of this distance defines the concentration gradient of H_2O_2 and so, the signal of current. As it was indicated above, when the enzymatic matrix permeates into the membranes, it diminishes the thickness of the external membrane as well as the cross-section of the sensor. The former process decreases the response-time of the sensor, while the latter improves its sensitivity.

Fig. 8 shows theoretical concentration profiles of H_2O_2 calculated for a set of hypothetical sandwich-type biosensors in which the thickness is gradually changed. All curves correspond to concentration profiles of H_2O_2 where the sensors have achieved the steady-state condition. To ensure this, all curves were calculated after 60 s of the analyte addition. The thickness of the sensor can be inferred from the points where the concentration of H_2O_2 becomes zero. Curves (a) and (a') exhibit concentration profiles corresponding to biosensors of the same thickness. From their analysis, it is possible to realize that the size and position of the maximum of H_2O_2 change with the position of the enzymatic matrix. However, both curves would have the same concentration gradient for H_2O_2 at the electrode surface. The position of the maximum can be associated with the response-time of the sensor while the concentration gradient determines the sensitivity of a given biosensor. Therefore, curves (a–f) are concentration profiles for a set of biosensors that should have identical response-time, but different values of sensitivity. In this regard, the sensor of curve (f) should be the one with the highest sensitivity, because it has the largest concentration gradient of H_2O_2 at the electrode surface.

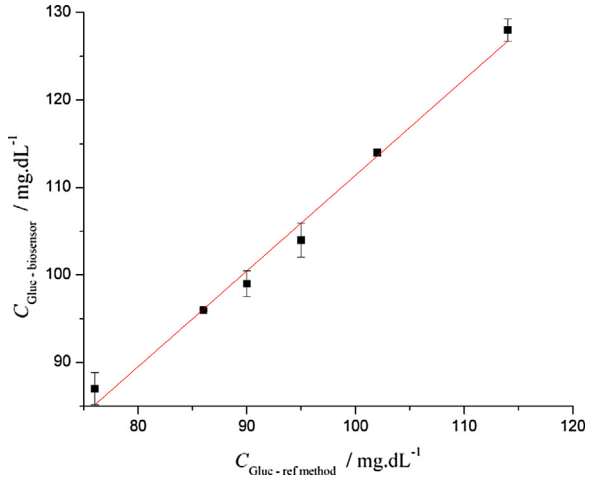

Fig. 9. Comparison of the glucose content in serum samples assessed by a sandwich-type glucose biosensor stored in an empty vial for 222 days at room temperature with the data supplied by a hospital.

3.3. Analysis of real samples

Once the performance and robustness of the developed sandwich-type biosensors have been described, it is interesting to evaluate its performance against real serum samples. The samples were provided by the Hospital Privado SRL in vials with walls protected with heparin. Fig. 9 shows a plot where the response of the developed sandwich biosensor is compared with the standard hexokinase-spectrophotometric method used in the hospital. The analyses of those samples were performed with a 7 months old biosensor stored in an empty vial at room temperature, sensor C. As it can be observed, the correlation between both set of data is very good. The slope obtained from the linear regression is 1.09 ± 0.05 and the dispersion of data is below 5%. In this regard, this is a biosensor that was exposed to around 500 standard additions associated with diverse calibration curves through 222 days, and whose data still fulfill the requirements of ISO 15197:2003 [3].

Please cite this article in press as: L. Colombo, F. Garay, A robust and practically free of charge intermittent use glucose biosensor, Sens. Actuators B: Chem. (2017), https://doi.org/10.1016/j.snb.2017.10.010

4. Conclusions

The behavior of a robust sandwich-type glucose biosensor that has been intermittently used for more than a year has been analyzed and discussed in this manuscript. The performance of the biosensor has been evaluated under three different storage conditions. In all cases the sensitivity of the sensors decreased almost 30% of the former signal during the first month of use. However, after a stabilization period, the sensitivity of all sensors raised back reaching even more than 100% of their former values. The biosensor stored in buffer at 4 °C showed the most stable behavior since it kept its response-time constant and slowly recovered the 100% of sensitivity after 5 months of use. The glucose biosensors stored in empty vials showed a more curious behavior since, after a year of use, they presented faster response-time and higher sensitivity than a freshly prepared sensor. This behavior was explained by simulating their chronoamperometric responses with a previously developed model [24,29]. Also, the glucose concentrations of real serum samples were compared with the standard hexokinase-spectrophotometric method of a hospital. This study was performed employing a 222 days old sandwich-type glucose biosensor that was intermittently used and stored at room temperature in an empty vial. Although the membranes of this sensor were not treated to minimize the effect of interfering species, the accuracy and precision of this sensor showed to be very good when compared with the requirements of ISO 15197:2003. Actually, most commercial strips for glucose analysis are also affected by typical interfering species [3].

The cost of each sandwich-type biosensor would be around a quarter U$ 0.26, it can be used for the analysis of several samples (>500), and it can even be stored at room temperature. Moreover, the sensitivity of this kind of biosensors can be determined from a simple calibration curve. In this regard, intermittent use biosensors such as the one presented in this work should not only be considered as a cheaper alternative, but also as a more accurate option with regards to the well-known strips for glucose analysis.

Acknowledgements

Financial support from CONICET, FONCYT and Secyt-UNCis gratefully acknowledged. F. Garay is a permanent research fellow of CONICET.

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Please cite this article in press as: L. Colombo, F. Garay, A robust and practically free of charge intermittent use glucose biosensor, Sens. Actuators B: Chem. (2017), https://doi.org/10.1016/j.snb.2017.10.010