Article

Investigation of GOx Stability in Chitosan Matrix: Application for Enzymatic Electrodes

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Abstract: In this study, we designed a new biosensing membrane for the development of an electrochemical glucose biosensor. To proceed, we used a chitosan-based hydrogel that entraps glucose oxidase enzyme (GOx) and we crosslinked the whole matrix using glutaraldehyde, which is known for its quick and reactive crosslinking behavior. Then, the stability of the designed biosensors was investigated over time according to different storage conditions (in PBS solution at temperatures of 4 °C and 37 °C and in the presence or absence of glucose). In some specific conditions, we found that our biosensor is capable to keep its stability for more than six months of storage. We also included catalase to protect the biosensing membranes from the enzymatic reaction by-products (e.g., hydrogen peroxide). This design protects the biocatalytic activity of GOx and enhances the biosensor lifetime.

Keywords: glucose biosensor; chitosan matrix; screen-printed electrode; Glucose Oxidase (GOx); glutaraldehyde crosslinker

1. Introduction

Electrochemical enzymatic glucose biosensors are the most commonly used devices to monitor blood glucose level in diabetic patients. [1–3]. Some of these devices can be implanted subcutaneously in diabetic patients to monitor glucose continuously for up to several weeks, and are called continuous glucose monitoring systems (CGMs). They often contain an electrode, a porous polymer, or hydrogel, encapsulating an enzyme, such as glucose oxidase (GOx), that catalyze glucose oxidation.

However, commercially available biosensors suffer from low stability for long-time operation and a gradual decrease in sensitivity with storage. Due to the enzyme's fragility, enzyme-based biosensors typically show a loss of sensitivity overtime and consequently express short lifetime. The loss of sensitivity implies either a replacement or a regular calibration of the sensors [4,5]. Biosensor lifetime is also limited by inflammatory reactions, or foreign body reactions that isolate the device from healthy interstitial fluid. However, providing sensors with enhanced lifetime would improve patient's quality of life [6,7]. Therefore, it is interesting to develop new methods for stabilizing the enzyme and enhance glucose biosensors lifetime.

Several strategies have been proposed to enhance enzymatic biosensor lifetime such as enzyme immobilization and enzyme engineering [8,9]. Indeed, the first strategy remains the most popular one due to its simplicity. There are different enzyme immobilization techniques including physical adsorption, surface binding by covalent and enzyme encapsulation and/or entrapment in polymeric matrix [10,11]. In recent years, enzymes encapsulation in hydrogel or polymers has attracted great interest because the adsorb water and provide microenvironment favorable for enzyme stability overtime. However, physical and chemical proprieties of these matrixes (i.e., surface area and roughness, pores

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size and distribution, membrane thickness and composition, etc.) play a key role in the stability of encapsulated or entrapped enzymes [12]. Depending on those properties, diffusion of glucose through membranes and then the enzyme accessibility may be affected, which influences the biocatalytic activity of the enzymes. Therefore, the choice of polymers or hydrogel-based matrixes and their nature and composition are of great importance.

Many materials were reported as potential candidates for the development of (bio)membranes such as cellulose [13,14], polycarbonate [15], hyaluronic acid [16], collagen [17,18] and chitosan [19,20]. However, chitosan-based materials are of particular interest due to their particular structural and functional characteristics, i.e., biocompatibility, high permeability, antibacterial and antimicrobial properties, adsorptive characteristics and low cost [21]. Minteer *et al*. suggested that enzymes' encapsulation within the pores/pockets of the hydrophobically modified micellar polymers such as Nafion® and chitosan improves drastically enzymes lifetime [22]. S El ICHI *et al*. reported that laccase encapsulation in chitosan- multi wall carbon nanotubes composite allows designing a biocathode with several months' stability [23,24]. Similarly, in previous work, we investigated the stability of glucose dehydrogenase (GDH) in Chitosan-MWCNTs pellet and showed enhanced GDH stability to more than year [25]. In these studies, the introduction of crosslinkers has been proposed to enhance chitosan stability. Indeed, chitosan is a biodegradable polymer that suffers from loss of stability in aqueous solution over time which requires the use of a crosslinker such as genipin or glutaraldehyde. Conversely to genipin, crosslinking process of chitosan by glutaraldehyde does not demand heating and can be achieved at room temperature, which prevents enzyme denaturation [26,27]. This makes glutaraldehyde an option of choice for the development of a chitosan-based biomembrane.

In the case of first-generation enzymatic glucose biosensor where the enzymatic oxidation of glucose by GOx leads to the production hydrogen peroxide (H_2O_2) and gluconic acid, the chitosan matrix should be capable to keep its stability in the presence of H_2O_2 and of an acidic microenvironment resulting from the accumulation of gluconic acid. The aim of the present work is the design of chitosan based enzymatic glucose biosensors and the study of its stability overtime. Our strategy consisted of crosslinking and encapsulating the enzyme GOx into/with the chitosan matrix. Here, we investigated the adhesion of chitosan on the surface of different electrode materials (gold, platinum and carbon). The stability of the biosensors over time according to different storage conditions (in PBS at 4 °C/37 °C and in the presence or absence of glucose) was also investigated. We also investigated the stability of chitosan-based membranes under dry storage conditions at various temperatures of 4 °C, 37 °C and at room temperature.

2. Materials and Methods

2.1. Chemicals

Glutaraldehyde solution - grade 1, 70 % in water ($C_5H_8O_2$, CAS No: 111-30-8, ref. G7776), Thiol-NH_2 powder (HS-PEG2K-NH_2, ref. JKA5143), Collagen from bovine achilles tendon (CAS No: 9007-34-5, ref. C9879), Chitosan high molecular weight (310 - 375 kDa, > 75 % deacetylated, CAS No: 9007-34-5, ref. 419419), D-Glucose powder (purity > 99.5 %, CAS No: 50-99-7, ref. G8270), GOx from Aspergillus Niger (type X-S lyophized powder, CAS No: 9001-37-0, ref. G7141-50KU), GOx from Aspergillus Niger recombinant (CAS No: 9001-37-0, ref. 345386-10KU) and Catalase from bovine liver (CAS No: 9001-05-2, ref. C1345) were purchased from Sigma-Aldrich. Stock solution of phosphate-buffered saline (PBS 10x) was also purchased from Sigma-Aldrich and utilized for the preparation of working solutions (PBS 1x pH 7.4). All reagents were analytical grade and milli-Q water was used to prepare solutions.

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2.2. *Preparation of hydrogels*

Collagen solution (1 wt %) was prepared by dissolving collagen powder in acetic acid solution (0.1 v/v %). The obtained emulsion was stirred at room temperature for 24 hours until collagen powder was fully dissolved. The obtained gel was stored in the fridge at 4 °C when it is not used.

Chitosan solution (1 wt %) was prepared by dissolving chitosan powder (high molecular weight) in acetic acid solution (0.5 v/v %). The mixture was left to stir at 70 °C for 1 hour to fully dissolve chitosan. The obtained gel was then placed at room temperature to cool down and stored in the fridge at 4 °C when it was not used. The 1 % concentration of chitosan was chosen to limit acetic acid addition as much as possible. A higher concentration of acetic acid is required to dissolve chitosan powder when chitosan concentration increases.

2.3. *Electrochemical apparatus and electrodes*

An SP-300 potentiostat (Biologic Instruments, Grenoble – France) was used for all electrochemical experiments. Screen-printed gold electrodes (auxiliary electrode: Pt and reference electrode: Ag, ref. DRP-250AT-U75) were purchased from METROHM (Villebon-sur-Yvette, France). The working electrodes have a 4 mm diameter characteristic which represent a surface area of 12.56 mm^2.

2.4. *Electrode design*

For chitosan-based materials, it was necessary to crosslink the chitosan-hydrogel by adding glutaraldehyde in order to ensure its stability. Our strategy consisted of combining crosslinking and encapsulating the GOx into/within the chitosan matrix. This allows for large amounts of enzymes to be immobilized and stabilizes the GOx enzyme being used [28]. Indeed, some trials conducted in our lab showed that the chitosan matrixes require the use of an adhesion layer especially dedicated for the commercial gold-based electrodes (METROHM, DRP-250AT). For this purpose, a collagen solution (1 wt %) was used in presence of glutaraldehyde (added at 2 wt % *vs* collagen) and mixed. Five minutes later, 10 µL of the mixture was pipetted and deposed by drop-casting on the working Au-based electrodes and kept at room temperature for complete drying (approx. 1 hour) to provide an intermediate layer that avoided shrinkage of the chitosan matrix on the working Au-based electrodes [29].

For the preparation of chitosan matrix: 10 mg of GOx was added to 1 mL chitosan solution (1 wt %) and the mixture was gently shook for a few seconds (IKA® Vortex). Glutaraldehyde solution (2 wt % related to chitosan) was then added after 5 minutes, 10 µL from the prepared mixture was deposed by drop-casting as a second layer on the collagen layer. Electrodes were kept at room temperature until complete drying (approx. 1 hour). Finally, the sensors were rinsed with distilled water (Figure 1).

2.5. *Stability test of biosensing membranes*

We investigated the stability of the GOx-chitosan-based glucose biosensor at different conditions of storage. The biosensors were first tested in PBS (1X) medium by chronoamperometry. Thereby, the sensors were polarized at + 0.7 V *vs* Ag/AgCl reference electrode. The electrochemical response is then recorded in presence of an increasing concentration of glucose in the bulk solution under a dynamic condition of 200 rpm. This allows to establish the calibration curve ($i_{glucose}$=f ([Glucose]) and to determine the sensitivity of the biosensors at the first day. Herein, the sensitivity corresponds to the slope value of the calibration curve (expressed in nA/ mM).

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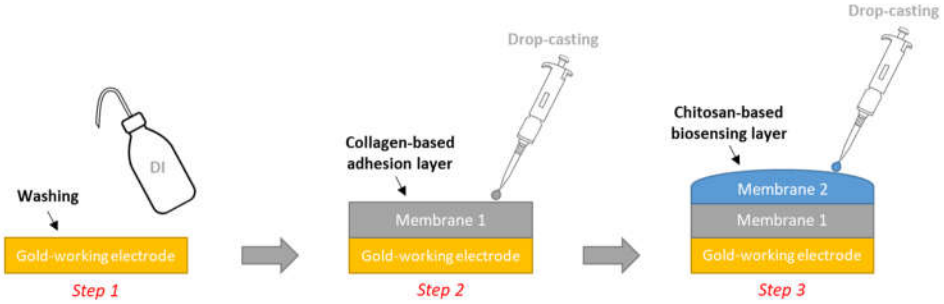

Figure 1. Illustration of the modification process of the working gold-based screen-printed electrode for the fabrication of glucose biosensors.

3. Results and discussion

In order to highlight the immobilization process, it was necessary to understand the reaction that could occur during the fabrication of the bio-based membranes. Indeed, the GOx as well as the chitosan polymer are well-known to have a lot of amine groups in their structures. This feature facilitates the derivatization process. So, in the presence of glutaraldehyde crosslinker (added at 2 wt %), a non-selective reaction leads to obtain three reaction byproducts as represented in Figure 2. The best scenario is reached when the chitosan is crosslinked and bonded to the GOx enzyme through glutaraldehyde chain. This configuration especially prevents from enzyme's release from the chitosan matrix into solution. However, a chitosan polymer could be crosslinked to another chitosan polymer chain as well as the GOx could be also crosslinked and bonded to another GOx enzyme through a glutaraldehyde chain. This reaction randomly happens; thus, the homogeneity of the GOx/chitosan mixture is of great importance in order to obtain reproducible results.

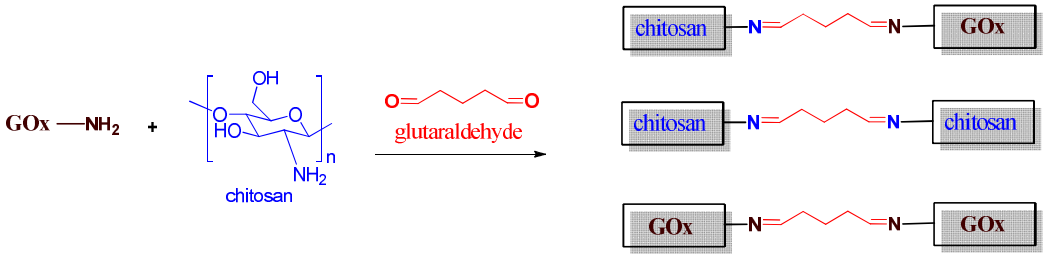

Figure 2. Immobilization process of the enzyme GOx into the chitosan polymer using the glutaraldehyde crosslinker (added at 2 wt %).

Figure 3 shows the amperometric response of GOx-chitosan biosensor to successive additions of glucose. We can see that we have a typical amperometric response as a function of glucose concentration showed a linear relationship (r^2>0.996) in a concentration range of glucose of 0.25 – 1.5 mM (Fig. 3 inset). The apparent Michaelis–Menten constant (K_m^{app}) of the GOx was determined and is near to 1 mM. So far, the biosensors were kept and stored in a phosphate buffer solution (PBS 1X) in the absence or presence of glucose (2 mM) in the solution. After that, the biosensors are retested to investigate the variation of their sensitivities at different times. The results are always normalized according to the value of the sensors' sensitivities obtained at day 1 for each biosensor.

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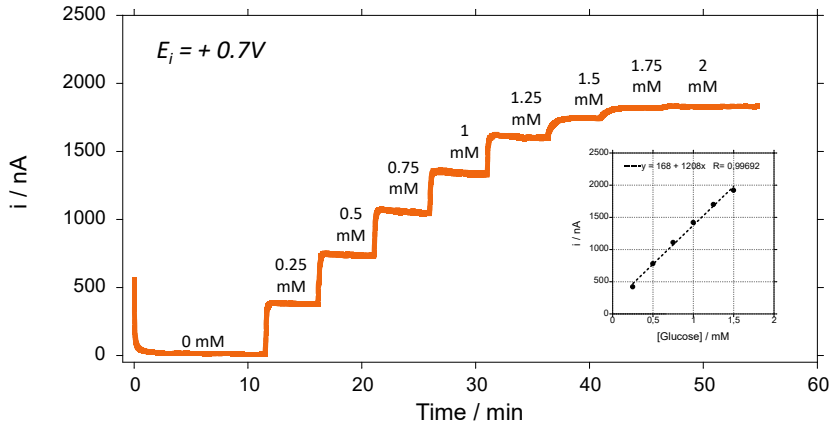

Figure 3. Amperometric responses at + 0.7 V *vs* Ag/AgCl of glucose biosensing) in PBS(1X) medium under hydrodynamic conditions of 200 rpm. Inset: calibration curve of glucose during electrochemical oxidation.

The changes in biosensors sensitivity as a function of the storage day in PBS (1X) in presence of glucose at 2 mM at 37°C (Fig. 4, grey plot) show a stable sensitivity in the first 7 days of storage, with only 10 % decrease in sensitivity after 1 week of storage. The sensitivity drastically decreased after 2 weeks of storage with a 90 % loss of sensitivity (Fig 4), probably due to the degradation of the chitosan-based membrane. Indeed, in the presence of glucose in the storage solution, GOx continuous catalyzes the oxidation of glucose into hydrogen peroxide (H_2O_2) and gluconic acid according to the following reaction [2,30]:

$$Glucose + O_2 \xrightarrow{GOx} H_2O_2 + \ gluconic\ acid$$

However, it is well-known that accumulation of the enzymatic reaction by-products, in particular, H_2O_2 at high concentration levels could contribute to the change in the electrode material properties [31–33]. Herein, the pH of the storage solution was investigated and followed over time. A slight acidification of the medium was detected ($\Delta pH \approx 0.2$) that despite the presence of phosphate buffer, confirming the accumulation of acidic by-products of the enzymatic reaction. Thus, this observation could help to confirm our hypothesis. It is important that we note that the pH value can be lower inside the matrix due to a local acidification of the chitosan nearby GOxs sites. For the biosensors that were stored in PBS without any glucose at 4°C (orange plot), functional stability was observed for more than 6 months of storage. The changes in the biosensors sensitivities over time may be due to the conditioning of chitosan-based matrix. For instance, chitosan material is well known for its high swelling capacity, which could affect the enzyme stability over time [34]. Indeed, it is noteworthy to highlight that the apparent Michaelis–Menten constant (K_m^{app}) of the GOx was not affected during the long storage period and expressed the same value over time of ~ 1 mM.

Until now, and for all these samples GOx and chitosan crosslinking was performed in a single step and that leads to a chitosan matrix where single GOx may be crosslinked to chitosan. As mentioned above one step crosslinking leads to random distribution and crosslinking of GOx. It was interesting to study the effect of the crosslinking order on the stability of the biosensing membranes. To do so, we first crosslinked GOx molecules and subsequently added them to chitosan. Contrary to the first membrane design where the crosslinking was realized non-selectively between the GOx and the chitosan, herein, only one reaction product is expected and considering the GOx that are crosslinked and bonded together. In the latter design, the GOx are entrapped without any covalent bonds within the chitosan polymer. Here, we assume that all glutaraldehyde was consumed because of its high reactivity and thus, we suppose that no more glutaraldehyde was left to react and crosslink the chitosan polymer.

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This new enzyme immobilization design displayed higher stability (Fig. 5, grey plot). The biosensors showed an increased sensitivity of ~ 1.18 for the first week of storage in PBS in the presence of glucose at 2 mM (normalized with respect to first day of operation). This is probably due to the swelling capacity of the chitosan matrixes that increased glucose diffusion to GOx active sites. Afterwards, the biosensors sensitivity decreased progressively over time, probably due to a decrease in the biocatalytic activity of the GOx. After one month of storage, the sensors expressed a normalized sensitivity near 0.25. During this period, the sensors were stored in presence of glucose with continuous enzymatic activity. Despite this decrease in the biocatalytic activity of the GOx, this immobilization method stabilized the redox enzyme that was active for more than one month.

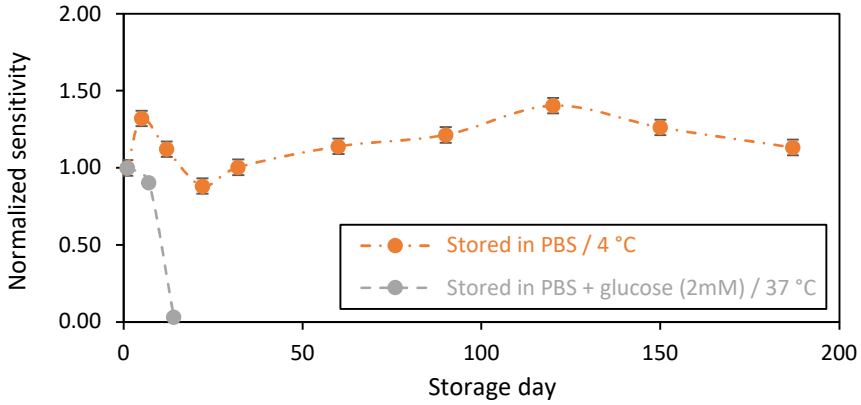

Figure 4. Stability in function of time of the chitosan-based biosensing membranes deposed on gold screen-printed electrodes. Orange plot: biosensors stored in PBS at 4 °C and grey plot: PBS + glucose (2 mM) at 37 °C.

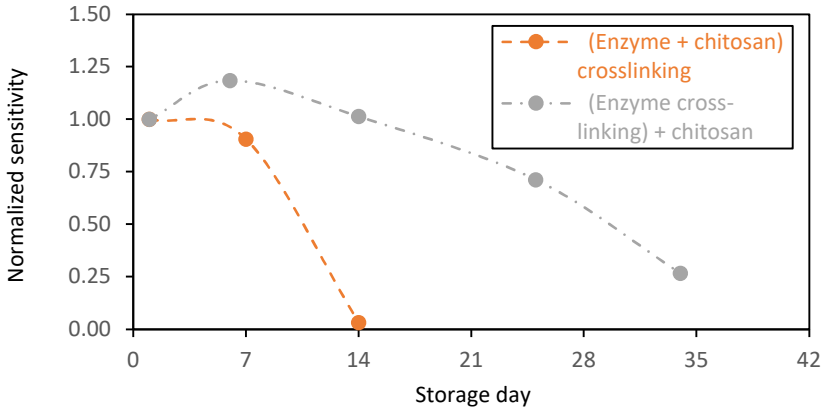

Figure 5. Stability in function of time of the chitosan-based biosensing membranes deposed on gold screen-printed electrodes and stored in PBS + glucose (2 mM) at 37 °C. Orange plot: GOx entrapped and crosslinked and grey plot: GOx entrapped.

We then compared the performance of our GOx-chitosan based glucose biosensors to recombinant GOx (rGOx)-Chitosan based glucose biosensors. Indeed, the rGOx is a structurally modified enzyme on the nanoscopic scale. The recombinant enzyme displays similar properties to the native one but exhibits more advantageous properties for glucose oxidation including the specificity to the substrate (glucose) and an enhanced stability over time [35,36]. Additionally, rGOx might contains more impurities including enzymatic impurities. In particular, rGOx contains catalase as an enzymatic impurity at a higher percentage comparing to the native GOx from Aspergillus niger. Here, it should

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be noted that catalase is known, as oxidoreductase, that catalyzes the decomposition of H_2O_2 into O_2 and water as follows [37]:

$$H_2O_2 \xrightarrow{Catalase} H_2O + O_2$$

According to this reaction, H_2O_2 produced by the GOx or rGOx should be consumed/decomposed locally by the catalase into O_2 and H_2O. It is noteworthy to highlight that this decomposition should be partial to allows to detect and oxidize H_2O_2 on the surface of the electrode. Contrary, complete decomposition of H_2O_2 leads to the elimination of the electrochemical response related to H_2O_2 oxidation.

We tested the stability of the biosensing membranes designed with the GOx or rGOx, and found increased rGOx stability over time (Fig. 6, grey plot). Nevertheless, after 2 weeks of storage in PBS in the presence of glucose (at 2 mM), 30 % decrease in sensitivity was observed, and a 50 % decrease after 3 weeks. This relatively decreases the level of the catalytic current but it probably protects the chitosan-based membrane from intoxication by the local and continuous accumulation of H_2O_2 and prevents the biocatalytic activity of GOx from drastically decrease.

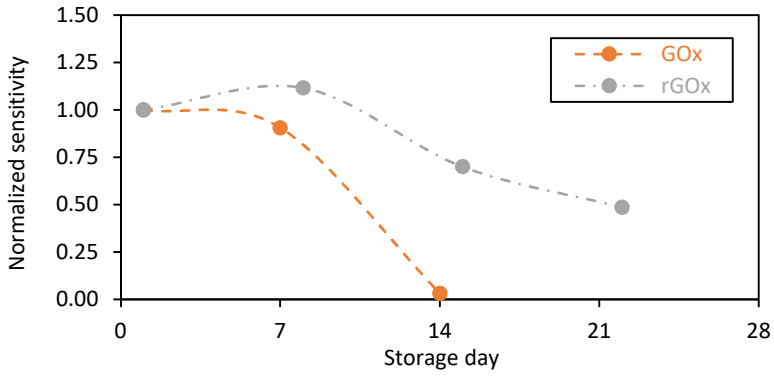

Figure 6. Stability in function of time of the chitosan-based biosensing membranes deposed on gold screen-printed electrodes and stored in PBS + glucose (2 mM) at 37 °C. Orange plot: normal GOx and grey plot: recombinant rGOx.

To verify this hypothesis, we prepared a GOx biosensor in which we added catalase to the native GOx in 2/10 ratio (2 catalase units per 10 GOx units). Each GOx unit oxidizes 1 µmole of glucose while each unit of catalase and theoretically decomposes 1 µmole of H_2O_2 per minute. Therefore, mixing catalase with GOx in a 2/10 ratio was expected to decrease the local H_2O_2 concentration within the membrane, while preserving glucose detection through H_2O_2 reoxidation at the electrode surface.

Unsurprisingly, the catalytic current observed at day 1 in the presence of catalase was lower than in the absence of catalase (Fig. 7a). This could be attributed to the partial decomposition of H_2O_2 by catalase which automatically prevents H_2O_2 diffusion to the electrode surface and reduce by cascade the catalytic current. However, the biosensing membranes containing catalase were also more stable over time (Fig. 7b, grey plot). Indeed, even in the presence of glucose (2 mM), there was a remarkable increase in the sensors' sensitivity that peaked at ~ 3 times than its initial sensitivity after two weeks of storage. This can be attributed to the swelling capacity of chitosan as well as to the change in the physicochemical properties of the biomembranes due to the presence of catalase. Indeed, the K_m^{app} of the GOx was not affected by the presence of catalase. This aspect is of high interest that endorses that the GOx preserved similar properties in the latter conditions.

These results confirm that the catalase added in the vicinity of GOx firstly protects the chitosan-based membrane from the intoxication by the locally accumulated H_2O_2 and secondly improves / protects the biocatalytic activity of the GOx.

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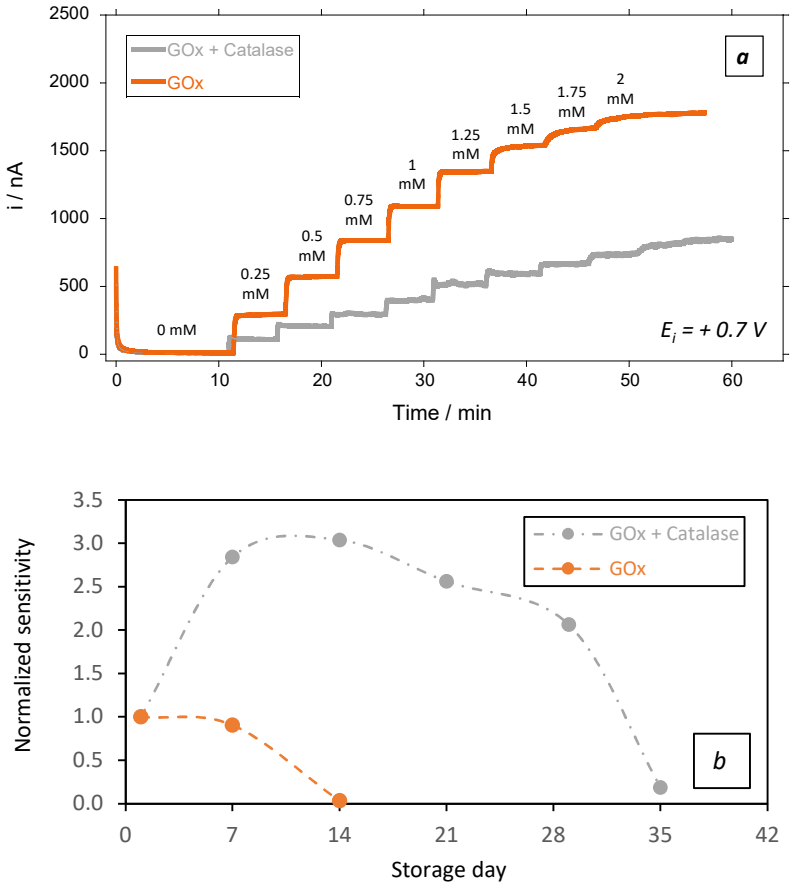

Figure 7. (a) Amperometric responses at + 0.7 V *vs* Ag/AgCl in PBS(1X) medium under hydrodynamic conditions of 200 rpm for testing of biosensors at day 1 in presence of an increased concentration of glucose and (b) Stability in function of time of the chitosan-based biosensing membranes deposed on gold screen-printed electrodes and stored in PBS + glucose (2 mM) at 37 °C: Orange plots: GOx and grey plots: GOx + added catalase.

On the other hand, in addition to the wet storage in PBS(1X) (with or without glucose), it is considerably interesting to investigate also the enzymatic biosensor's stability under dry conditions of storage. In fact, for logistical reasons, there is a need to understand the impact of temperature variation on the enzymatic membrane's stability. For this purpose, the stability of the biosensing membranes was investigated under dry storage condition at various temperatures of 4 °C (realized in the fridge), 37 °C (realized in the oven) and at room temperature. In the cases where the biosensors were stored at 4 °C and at 37 °C, they were kept stable temperatures. However, for the storage at room temperature, the biosensors suffer from humidity and the change in temperature between the day and the night. The storage at 4 °C ensures high humidity level contrary to the storage in the oven (at 37 °C) where the humidity is relatively low. For all the sensors, the enzymatic reaction is stopped during the dry storage while sensors' sensitivity was evaluated at room temperature by establishing a calibration curve for each sensor and comparing their sensitivities on a normalized scale at different times.

The results comparing the stability of the biosensing membranes that were stored dry at the various temperatures for up to 5 months are presented in Figure 8. It is clearly shown that the stability of sensors stored at room temperature (blue plot) was maximum for all the measurements. They expressed stable responses during the first three weeks. After, the biosensing membranes sensitivity decreases of 15 and 60 % at 2 and 5 months, respectively. However, for the sensors stored at 37 °C (orange plot), the sensitivity increases by 20 % after 2 weeks of storage and then falls to 25 % and 55 % after 3 and 5 months, respectively. The sensors stored at 4 °C (grey plot) showed stable responses in

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the first week of dry storage following by a decrease of 23 %, 48 % and 63 % after 20, 90 and 150 days, respectively. These results confirm that temperature of storage (under dry condition) impacts the catalytic activity of the GOx and affects the chitosan biomembranes microenvironment. This should be considered in the future for designing long-term *in vivo* sensor systems.

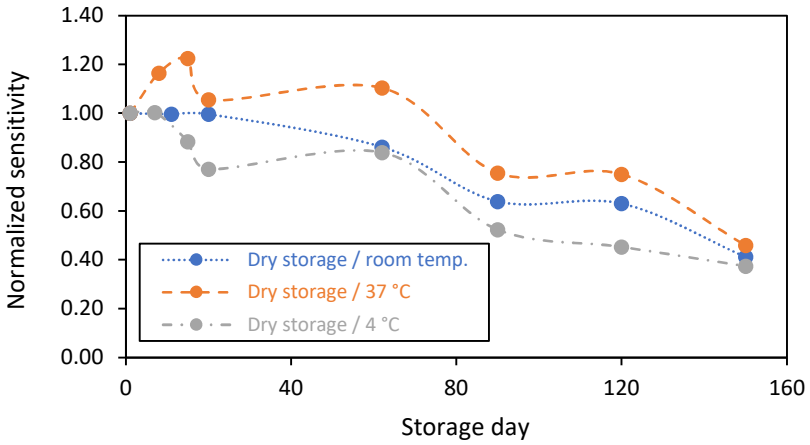

Figure 8. Stability in function of time of the chitosan-based biosensing membranes deposed on gold screen-printed electrodes and stored dry at various temperatures of: (blue plot) at room temperature, (orange plot) at 37 °C and (grey plot) at 4 °C.

5. Conclusions

GOx-based first generation glucose biosensor has been successfully designed and their stability over time under storage and operation conditions has been investigated. Our results show that GOx encapsulation in chitosan matrix is capable of providing high catalytic activity for at least six months.

The stability of the biosensors decreased dramatically when they were stored in glucose 2 mM but prior crosslinking of GOx before its encapsulation into chitosan improved significantly the biosensors stability. In addition, we demonstrated that the addition of catalase enhanced and protected the chitosan-based membrane from the intoxication by the local accumulation of H_2O_2. Overall, our GOx-chitosan based glucose biosensors could stay functional for up to one month, thereby showing high potential for industrial developments in continuous glucose monitors.

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