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Electrodepositable alginate membranes for enzymatic sensors: An amperometric glucose biosensor for whole blood analysis

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ABSTRACT

Simple and disposable point of care systems are usually the best solution for chronic patients to get a rapid diagnosis in home care context. However, their main drawback relies on the poor reliability derived from the low stability of the bio-recognition elements and low quality of the transducers. In the current work, we study the use of electrodeposited calcium alginate hydrogels as a biocompatible matrix in the development of enzymatic amperometric biosensors for whole blood analysis, to enhance the enzymes stability and to protect the transducer from biofouling. The alginate electrodeposition involves the controlled Ca^{2+} release, so the gel thickness can be modulated. In the biosensor, horseradish peroxidase (HRP) and glucose oxidase (GOD) were electrodeposited within the hydrogel and the activity of the bi-enzymatic system was analyzed chronoamperometrically using 3,3′,5,5′-Tetramethylbenzidine (TMB) as the mediator. Besides enzyme entrapment, the obtained gels protected the transducer from biofouling, enabling the reuse of the transducer after hydrogel removal and re-electrodeposition. The biosensors showed good analytical characteristics to glucose determination in whole blood samples, discriminating among healthy and hyperglycemic samples, with good sensitivity (−0.27 μA cm^{-2} mM^{-1}), low limit of detection (126 μM) and long lineal range (2–12 mM).

1. Introduction

The development of simple and low-cost methods based on biosensors for the detection of biomarkers is nowadays one of the main challenges in analytical science (Vashist et al., 2015; Bahadir and Sezgintürk, 2015) as an alternative to sophisticated benchtop instruments like high performance liquid chromatography or mass-spectrometry, among others. In the field of clinical analysis, as well as environmental monitoring and food industry, amperometric biosensors are a first choice for the simplicity, low-cost and robustness of amperometric transducers, the portability and low energetic requirements of amperometric instrumentation and the sensitivity and selectivity of biological molecules (e.g. enzymes) commonly used as recognition elements (Desmet et al., 2015). A very representative case of success is the glucometer with test strips, where all these advantages are exploited in the best-selling biosensor ever. However, the lack of accuracy of these systems is still a motive of concern (Huysal et al., 2015; Johnson and Baker, 2001; Tonyushkina and Nichols, 2009). First concerns are technological, due to the low accuracy of the fabrication protocols that affect measurements reliability and reproducibility. Even more important is biosensor dysfunction, resulting from the high sensitivity of enzymes to the environmental factors and the immobilization protocol that may affect their activity (Bamberg et al., 2005). Enzyme immobilization is thus a critical process in biosensors development for two reasons: (i) can disrupt the enzyme activity but, at the same time, (ii) can maintain the structural integrity of the enzymes for longer times, once immobilized. Classical immobilization protocols such as physisorption, covalent bonding or cross-linking rely on poor stability of the biomolecule in the sensor surface or loss of activity due to steric hindrance or reaction with amino acids from the active site (Sassolas et al., 2012). Entrapment on biocompatible matrices, emerges as an auspicious alternative for (i) enabling the immobilization of high amount of biocomponent in a 3D architecture, (ii) avoiding orientation problems or inactivation of the biomolecule for reaction with the recognition or active centres, (iii) providing a wet environment (hydrogels) to improve biomolecules stability and (iv) acting as a diffusion barrier that minimizes the poisoning of the electrode and allows working with complex real samples without pre-treatments (waste water, milk, blood, etc.). In this sense, alginate hydrogels have positioned as one of the most promising biocompatible matrices for enabling biomolecules entrapment at very soft experimental conditions, i.e. room temperature and pH 7. The advantage of alginate is that this linear copolymer, combining (1,4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G), spontaneously cross-links in presence

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of divalent cations (e.g. Ca^{2+}) without requiring temperature or irradiation and therefore, without compromising the integrity and activity of the biomolecule (Mørch et al., 2006). Calcium ions may be directly added to alginate solution or in situ generated (Kuo and Ma, 2001), which provide these membranes with high versatility. These excellent gelling conditions have been exploited in the entrapment of enzymes (Abu-rabeah et al., 2005; Liu et al., 2009; An et al., 2014) and cells (Betz et al., 2013) (Cheng et al., 2011b), as well as in tissue engineering (Hunt and Grover, 2010) and drug delivery (Lee and Mooney, 2012). Recently, it has been described the use of alginate membranes in the entrapment of enzymes or cells for biosensors development (An et al., 2014; Perullini et al., 2015; Liu et al., 2009). However, electrodepositable alginate hydrogels have never been applied to biosensors development for glucose analysis in whole blood samples.

In the present work, we present electrodepositable calcium alginate enzymatic membranes here employed in the development of a glucose biosensor for whole blood samples by entrapment of GOD and HRP (GOD/HRP cascade reaction (Rondeau et al., 1999)) using screen printed gold electrodes (SPGEs) as electrochemical transducer. Enzyme electrodeposition is performed at soft reaction conditions (i.e. room temperature, pH 7) and does not compromise enzyme integrity or activity. The electrodeposition of the matrix presents three more advantages: localized membrane deposition, reproducibility and reusability of the transducer by elimination and in situ re-electrodeposition (refreshing) of the enzymatic membrane. Selectivity is achieved because the matrix grows only over the working electrode surface. Furthermore, applying a constant current is enough to growth the matrix, so defining its intensity and duration is enough to obtain a reproducible process. The matrix can be easily redissolved after the detection using Ca^{2+} quelating agents such as phosphate buffered saline (PBS) or citrate buffers (low reactive solutions), and the same electrode can be re-used for a next matrix synthesis and analyte detection only performing a fast electrochemical cleaning between measurements. Electrodeposited alginate matrix characteristics, mediator diffusion through the matrix, transducer protection and amperometric response of the biosensor to glucose from buffer, plasma and whole blood samples are discussed in this work.

2. Materials and methods

2.1. Reagents

Alginic acid sodium salt, $CaCl_2$ (≥ 93%), 2-(N-morpholino)ethanesulfonic acid hydrate (MES hydrate, ≥99%), NaOH (≥ 98%), 3,3′,5,5′-Tetramethylbenzidine (TMB, ≥ 98%), H_2O_2 (30% in H_2O), D-(+)-Glucose (≥ 99.5%), NaCl (ACS reagent, ≥ 99%), trisodium citrate dihydrate (USP testing specifications), acetic acid (ACS reagent, ≥ 99.7%), HRP (Type VI-A, essentially salt-free lyophilized powder, 250–330 units mg^{-1}) and GOD from *Aspergillus niger* (Type X-S, lyophilized powder, 100–250 units mg^{-1}) were purchased from Sigma-Aldrich. $CaCO_3$ (≥ 98.5%), $K_3[Fe(CN)_6]$ (ACS reagent, ≥ 99%), dimethyl sulphoxide (DMSO, 99.5%), Na_2HPO_4 (ACS reagent, ≥ 99%), citric acid anhydrous (99.5%), sodium acetate anhydrous (ACS reagent, 99%), imidazole (ACS reagent, 99%), HCl (ACS reagent, 37%), H_2SO_4 (96%) and ethanol absolute (≥ 99.5%) were purchased from Panreac. KCl (≥ 99%) and KH_2PO4 (ACS reagent, ≥ 99.5%) were purchased from Fluka.

All chemicals were used as received and aqueous solutions were prepared using de-ionized water with a resistivity of 2 M Ω cm. Used PBS contains NaCl (8 g L^{-1}), KCl (0.2 g L^{-1}), Na_2HPO_4 (1.42 g L^{-1}) and KH_2PO_4 (0.24 g L^{-1}). TMB 50 mM stock solution was daily prepared using DMSO as solvent. Whole blood ([glucose] = 5.4 mM) was extracted from a volunteer and stored at 4 °C until measurement.

SPGEs (Dropsens, 220BT) with Au working and counter electrode and Ag pseudo-reference electrode were used as transducer for the biosensor.

2.2. Equipment

For all electrochemical measurements, a Dropsens μSTAT8000 potentiostat controlled with Dropview 8400 software was used. For profilometry measurements, an optical profilometer pLμ2300 from Sensofar controlled with PLμ Confocal Imaging Profiler was used. Basal glucose concentration in the blood sample was measured with TRUEresult® test strips and glucometer. The pH of buffer was established using a Crison pH & ION-Meter GLP 22+. The images of alginate hydrogel were taken with a digital microscope camera DigiMicro 2.0 Scale. The plasma was separated from blood cell by centrifugation at 13,000 rpm for 15 min with a Mikro 20 Hettich centrifuge.

2.3. Optimization of hydrogel membrane deposition

Electrodeposition of calcium alginate hydrogels was optimized by considering the magnitude of the applied current and the duration of the electrodeposition step. The proposed mechanism (Cheng et al., 2011a) consists of the rupture of $CaCO_3$ particles due to the protons generated by water electrolysis and the subsequent cross-linking reaction between different alginate molecules by Ca^{2+} ions (Fig. 1).

The precursor composition was fixed at 1% w/v of sodium alginate, 0.5% w/v of $CaCO_3$ and different enzyme concentrations (only HRP in case of H_2O_2 biosensor and HRP-GOD in case of glucose biosensor). According to bibliography (Zhang et al., 2016), the random distribution of the two enzymes within the hydrogel should not affect the performance of the biosensor.

2.4. Amperometric detection

The selected enzymes for the biosensor, GOD (1) and HRP (2), catalyse the following reactions:

$$\beta\text{-}D\text{-}Glucose + O_2 \xrightarrow[GlucoseOxidase]{} D\text{-}Glucono\text{-}1,5\text{-}lactone + H_2O_2 \quad (1)$$

$$H_2O_2 + TMB_{red} \xrightarrow[HorseradishPeroxidase]{} H_2O + TMB_{ox} \quad (2)$$

The oxidized form of TMB (TMB_{ox}) is reduced electrochemically on the working electrode of the biosensor, recording a negative current. The intensity of this negative current is therefore proportional to the initial quantity of H_2O_2 or glucose present in the sample, depending on the biosensor. The detection potential *versus* Ag pseudo-reference electrode is selected from a cyclic voltammetry of TMB and set at 0.1 V (below the reduction potential).

For measurements, 50 μL droplets (including sample, buffer and mediator) were deposited on the biosensors ensuring the complete coverage of the three electrodes. In the case of real samples (plasma and whole blood), 40 μL of sample were mixed with 2 μL of 50 mM TMB and 8 μL of glucose solution in water at different concentrations yielding to different final glucose concentrations. Due to diffusion limitations, 2 min were stablished as incubation time prior to measurement to ensure the stability of the recorded signal. In case of direct H_2O_2 measurement, the reaction rate was so fast that incubation was not necessary.

3. Results and discussion

3.1. Optimization of electrodeposition process

The physico-chemical properties of the hydrogel depended on the electrodeposition conditions. The characteristics of the obtained hydrogels were analyzed under several conditions: duration of the electrodeposition step (from 30 to 180 s) and current applied (from 50 to 150 μA). Fig. S1 shows the aspect of the alginate hydrogel under these conditions. The electrodeposition current affected the consistency of

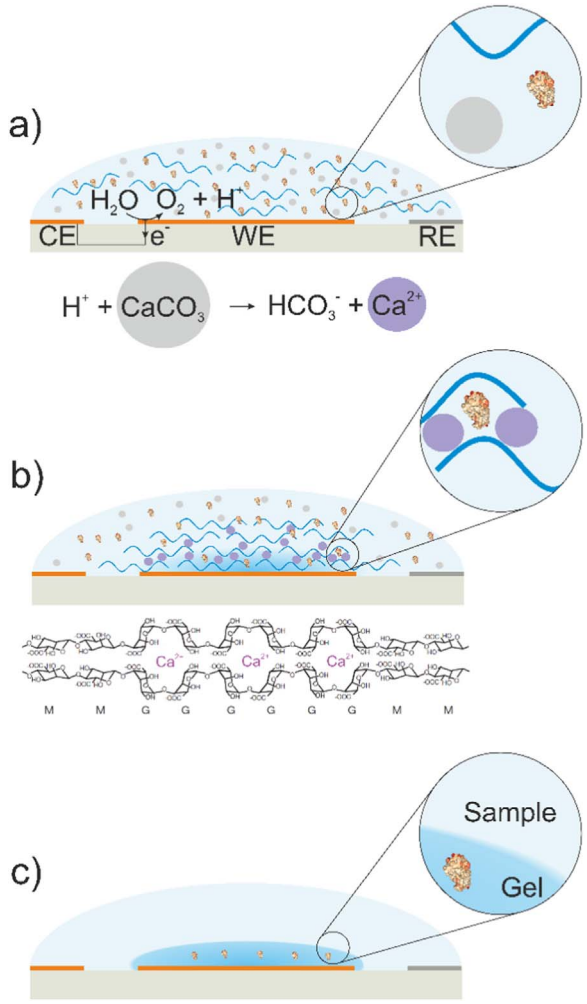

Fig. 1. Cross section scheme of the biosensor for different steps of the alginate hydrogel electrodeposition: a) A solution containing sodium alginate (blue curly lines), $CaCO_3$ and enzymes of interest is dropped over the transducer and an anodizing current of 100 μA is applied to the working electrode to liberate protons that dissolve the $CaCO_3$ particles. b) The released Ca^{2+} cations react with the alginate monomers in a cross-link reaction among the G-blocks of this copolymer. c) When the hydrogel is formed, the transducer is rinsed with distilled water to discard the precursor. Finally, only the hydrogel remains attached to the working electrode, retaining the enzymes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the hydrogel. More consistent and rigid gels were obtained when applying higher currents due to the higher number of cross-linking events taking place. Electrodeposition currents around above 100 μA provided stable and consistent hydrogels suitable for enzymatic membranes development.

The duration of the electrodeposition step, on the other hand, showed high correlation with the presence of bubbles in the final gel. The bubbles formation was associated to the co-generation of O_2 during electrodeposition (Fig. 1). Electrodeposition times above 90 s were discarded for producing high amounts of bubbles, even when applying low currents. The presence of bubbles made heterogeneous membranes that detached from the electrode surface. Below 90 s of electrodeposition homogenous, transparent and stable hydrogels with restricted area of electrodeposition were obtained on the working electrode. Alginate hydrogels electrodeposited at optimal conditions (90 s and 100 μA) are illustrated in Fig. S2.

Apart from bubbles formation, the electrodeposition time also influenced the thickness of the hydrogel. Thicker hydrogels were obtained after longer electrodeposition times (Fig. 2a) in a very localized electrodeposition process where hydrogels were only produced in the working electrode area (see the pronounced border in Fig. 2b). The lineal interval (electrodeposition time *versus* gel thickness) was broad so the quantity of hydrogel generated, as well as the amount of entrapped enzyme, may be precisely modulated with modifying the electrodeposition time.

The thickness of the hydrogel, and consequently the electrodeposition time, also affected the diffusion of analytes (and mediators) through the membrane. The diffusion of ferricyanide molecules (1 mM solution) for hydrogels electrodeposited during 30, 60, 90 and 120 s was evaluated by cyclic voltammetry. Results are illustrated in Fig. 3a–d. Diffusion rate was determined by monitoring the increment of the intensity of the redox peaks of ferricyanide in consecutive cyclic voltammetries with electrodes covered with hydrogels of different electrodeposition times. Under the same experimental conditions, diffusion rate showed correlation with the electrodeposition time (and thickness) of the hydrogel, being smaller for hydrogels electrodeposited during longer times (Fig. S3 and Fig. 3e–f). This diffusion restriction associated to the thickness of the membrane should be taken into consideration when incubation times of the samples in the biosensor were calculated.

3.2. Hydrogel stability

Alginate hydrogels are sensitive to cation chelating agents which disaggregate them with facility. The stability of alginate hydrogels to contact with buffers of different compositions was evaluated. PBS and citrate buffers, containing Ca chelating agents, disaggregated the gel

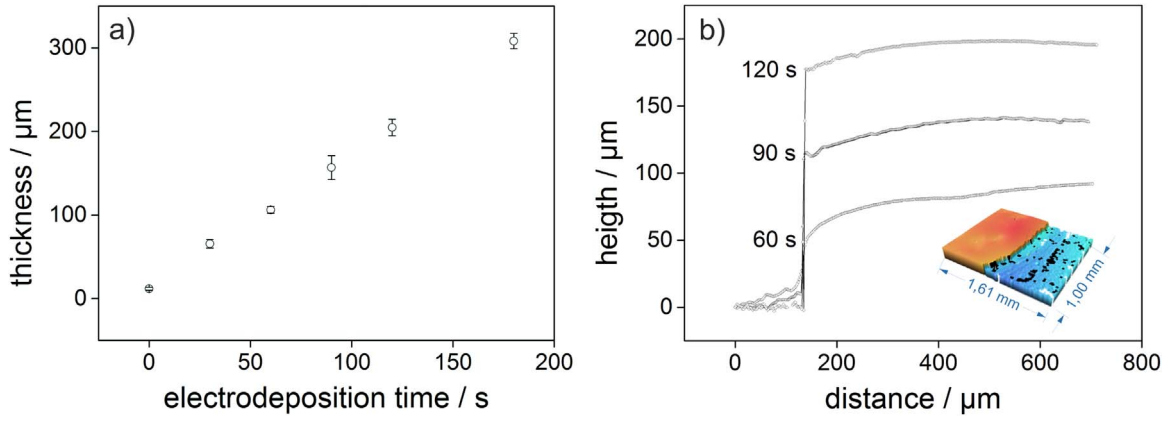

Fig. 2. Optimization of alginate thickness a) Hydrogel thickness dependence on the electrodeposition time, measured by profilometry. b) Profiles of three alginate gels, deposited with different electrodeposition times. Profilometry image of 90 s electrodeposited hydrogel profile represented in the inset. Gel precursor: Sodium alginate 1% w/v and $CaCO_3$ 0.5% w/v. Applied current between WE and CE: 100 μA.

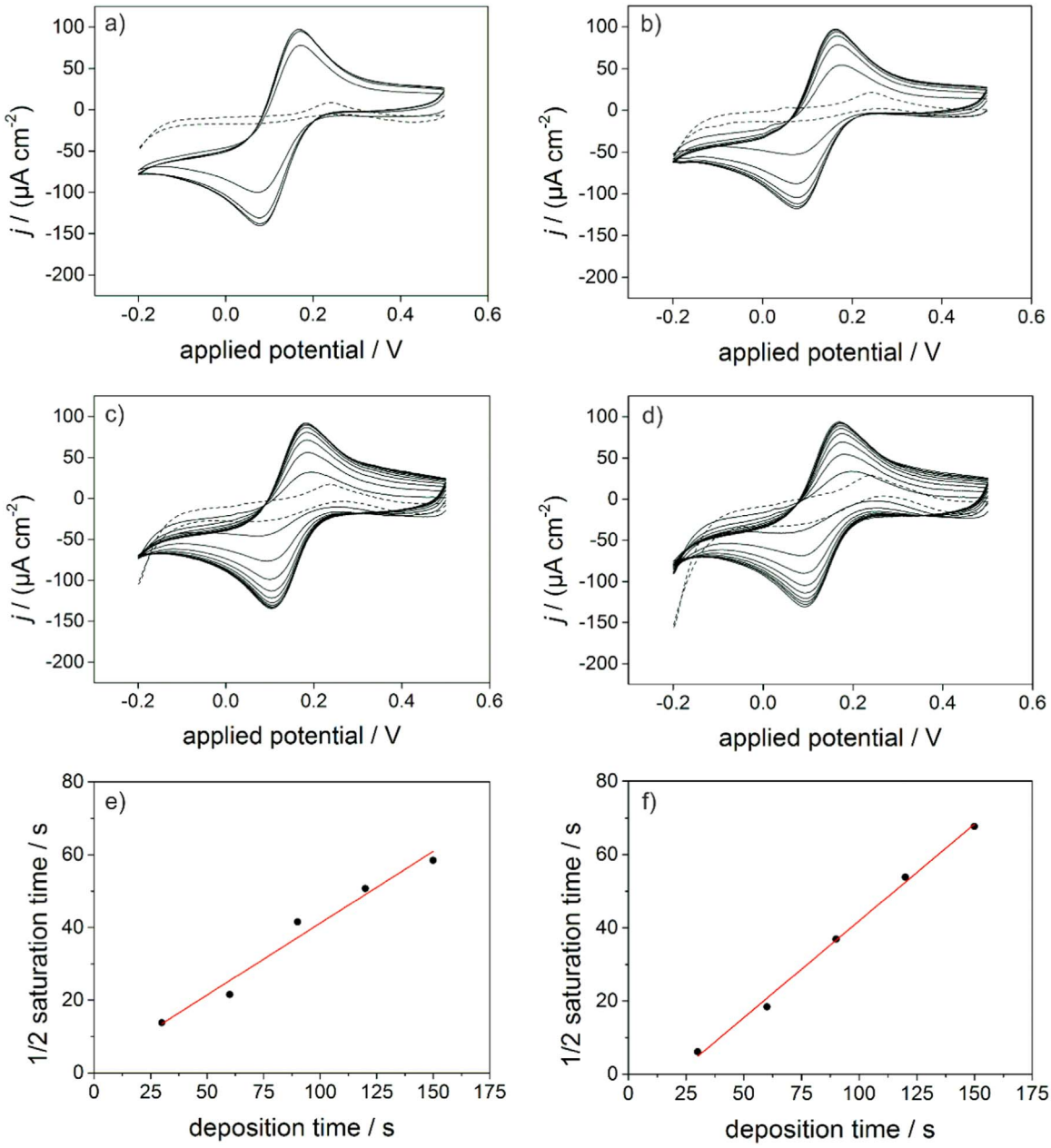

Fig. 3. Diffusion limitation by the hydrogel. Hydrogels electrodeposited applying 100 μA during a) 30 s, b) 60 s, c) 90 s and d) 120 were immersed in a buffer solution containing 1 mM $K_3[Fe(CN)_6]$ and successive cyclic voltammetries were recorded obtaining different saturation responses of the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox peaks. Dashed line represent a cyclic voltammetry with no $K_3[Fe(CN)_6]$. e) Anodic and f) Cathodic redox peaks current saturation time dependence on the electrodeposition time of the hydrogel (thickness). Peak values extracted from cyclic voltammetries. Medium: MES-NaOH (0.2 M, pH 7), 0.145 M KCl, 0.01 M $CaCl_2$ and 1 mM $K_3[Fe(CN)_6]$. Scan rate: 50 mV s^{-1}.

after less than 10 min, whereas alginate hydrogels remained stables in Imidazol-HCl and MES-NaOH buffers (Fig. S4). MES-NaOH (0.2 M, pH 7), in combination with 0.145 M KCl (to emulate physiological samples saline concentration) and 0.01 M $CaCl_2$ (to maintain the gel cohesion), was chosen for electrochemical measurements. PBS, on the other hand, was used to clean the electrodes between successive electrodepositions and for biosensor refreshing.

3.3. Protection of the transducer from biofouling

In the analysis of biological samples, molecules and cells susceptible to be adsorbed on the transducer surface are widely present therefore biofouling becomes an issue (Wisniewski and Reichert, 2000). The use of membranes has been the ordinary strategy to avoid transducer dysfunction. In the case of alginate hydrogels, the enzymatic membrane may have the dual function as biocompatible support for the enzyme and as size-exclusion barrier avoiding cell and molecule diffusion to the transducer surface. To evaluate this second function, cyclic voltammetries of the bare electrode (uncovered) and the electrode protected with an alginate membrane (covered) were compared when measuring whole blood samples containing 1 mM $K_3[Fe(CN)_6]$. It is clear from Fig. S5 that redox peaks (Fig. S5a and S5b) current density were much smaller when the electrode was not covered, probably due to a dramatic reduction of the active area of the electrode associated to biofouling. The filtering capacity of alginate hydrogels could be observed with the bare eye. When a drop of blood is added to a gel matrix and washed several times with water, it was appreciated how the liquid pass through the gel but the red cells remain in the edge of the membrane (Fig. S5).

3.4. Optimization of enzyme and mediator content

In biosensor's development, the composition of the enzymatic membrane and the concentration of enzymes and mediator should be adapted to the requirements of the final application. Since this biosensor is based on the coupled activity of HRP and GOD, the relative concentration of these enzymes should also be considered. To avoid HRP inactivation for exposure to high H_2O_2 concentrations (Krainer and Glieder, 2015), a HRP/GOD molar ratio around 8 was established, according to bibliography (Ferri, 2001). Maintaining this ratio, enzyme concentrations between 5 to 100 μg mL^{-1} GOD were evaluated (Fig. S6). Optimal conditions providing with a clear Michaelis-Menten behaviour, good sensitivity and linear range were obtained for concentrations of HRP and GOD of 11 μg mL^{-1} and 5 μg mL^{-1} respectively. Under these conditions, we could assume that the biosensor response decayed for enzyme-substrate saturation and not because of an electron transfer or diffusion limitation at the transducer surface (Silverstein and Goodney, 2010).

In the case of the mediator, TMB was chosen for presenting a low detection potential (0.1 V vs. Ag pseudo-reference), far from potential interfering molecules (e.g. ascorbic acid). As shown in Eqs. (1) and (2), TMB is oxidized in presence of glucose using the bi-enzymatic system of GOD and HRP. The oxidized TMB is then reduced at the WE and the registered current is proportional to the amount of glucose present. In Fig. S7 it is evident how the anodic current decreases while the cathodic current increases when this reaction takes place.

The concentration of mediator influenced the lineal range of the biosensor and should be optimized. Two TMB concentrations were compared, 0.5 and 2 mM, by enzymatic membranes containing optimal concentrations and ratio of enzymes (Fig. 4). Biosensors with 0.5 mM TMB presented good sensitivity (0.36 ± 0.00-μA cm^{-2} mM^{-1}) and LOD (11.31 μM) but a short linear range between 0 and 4 mM (R^2 = 0.999). The increase of TMB to 2 mM, nevertheless, expanded the linear range from 2 to 12 mM (R^2 = 0.993), enabling to distinguish between healthy (3.8 and 6.5 mM) and hyperglycemic subjects (> 7 mM) with sensitivity (0.32 ± 0.02-μA cm^{-2} mM^{-1}) and without compromising the detection capacity of the system (LOD = 270 μM). A TMB concentration of 2 mM was thus selected for biosensor development.

There is no response of the biosensor when one of the two enzymes is not present in the alginate matrix neither with both enzymes present but in absence of glucose (inset Fig. 4a).

The biosensor response was compared to a reference biosensor (same transducer) with the enzymes free in solution and without alginate electrodeposition (Fig. 4c). It is remarkably a higher sensitivity (0.74 ± 0.03-μA cm^{-2} mM^{-1}) without hydrogel for glucose calibrations in buffer.

3.5. Enzymatic stability within alginate hydrogels

The conditions nearby the transducer when the anodization is occurring may affect the activity of the enzymes, not only because of direct charge transference but also because of regional pH changes to acidic values (Dumitraşcu et al., 2016). However, previous studies report that the hypothetical pH shift is restrained by the buffering effect of $CaCO_3$ (Cheng et al., 2011a), remaining above pH 6 even close to the transducer when $CaCO_3$ is present at 0.5 w/v % and a current of 6 A m^{-2} is applied (7.95 A m^{-2} in this work).

The stability of the enzymes in the alginate matrix over time was evaluated and compared with enzymes absorbed on the electrode surface. The enhanced sensitivity commented above remained constant for 15 days, decreasing to 50% of initial activity in one month. Comparative studies with adsorbed enzymes revealed a decrease in a 50% of the sensitivity only one day after the biosensor fabrication (Fig. S8).

3.6. Reusability and repeatability of the biosensor

In closed systems (e.g. LOCs), where the sensing compartment is not accessible, electrodeposition becomes a great advantage, as it allows to introduce a liquid precursor and to immobilize the hydrogel with the recognition element only over the electrochemical transducer, just by a simple anodization step. Furthermore, if this membrane is degradable, several electrodeposition events can be performed, using fresh or different recognition elements each time and expanding the transducer lifetime. In the case of alginate it is even easier since, as already exposed, it may be dissolved using simple calcium chelating solutions such as PBS. The re-usability limitation is then due to the transducer stability and not to the recognition part (this can be regenerated when required). The re-usability of the transducer was thus evaluated by electrodepositing alginate membranes and removal with PBS. Concretely, the removal protocol included addition of PBS and electrochemical activation by cyclic voltammetries in 0.5 M H_2SO_4 (5 cycles; 0 and 1.5 V vs. Ag pseudo-reference electrode). As shown in Fig. S9, eight consecutive calibration curves of glucose were performed using the same transducer and refreshing the alginate matrix. The slope of the linear calibration (sensitivity) decreases only a 10% between the 1st and the 8th calibration. This result demonstrated the reusability of the system and their potential application in closed systems such as Lab-on-a-chip architectures.

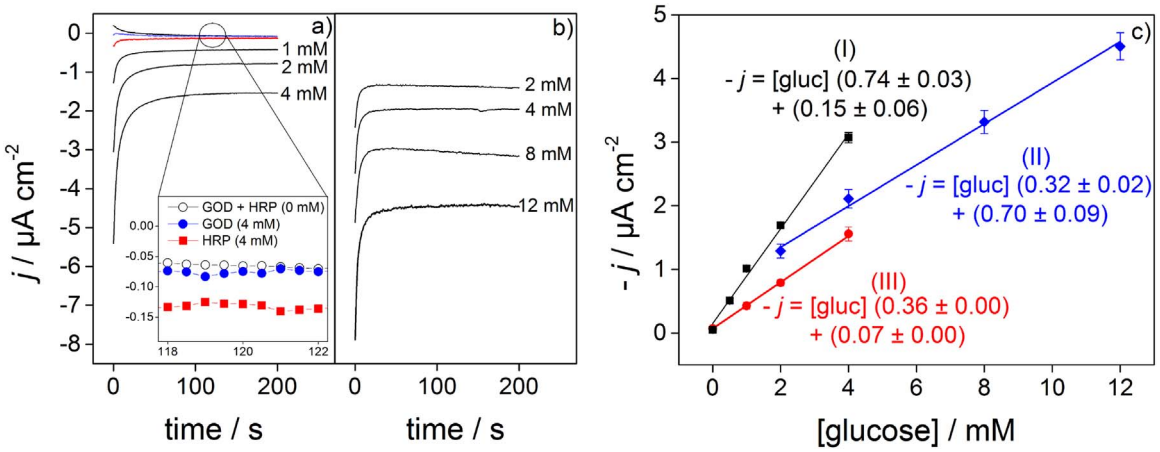

Fig. 4. Amperometric response of biosensors using two concentrations of TMB as mediator a) 0.5 mM (from 0 to 4 mM glucose) and b) 2 mM (from 2 to 12 mM glucose). Negative control responses are included (4 mM glucose), where only one of the enzymes is present (inset in a)). c) Calibration curve for glucose: (I) Biosensor without alginate membrane and 0.5 mM TMB, (II) With alginate membrane and 2 mM of TMB and (III) With alginate membrane and 0.5 mM of TMB (n = 6). Medium: MES-NaOH (0.2 M, pH 7), 0.145 M KCl, 0.01 M $CaCl_2$, TMB (0.5 mM or 2 mM). Detection potential: 0.1 V vs. Ag pseudo-reference electrode.

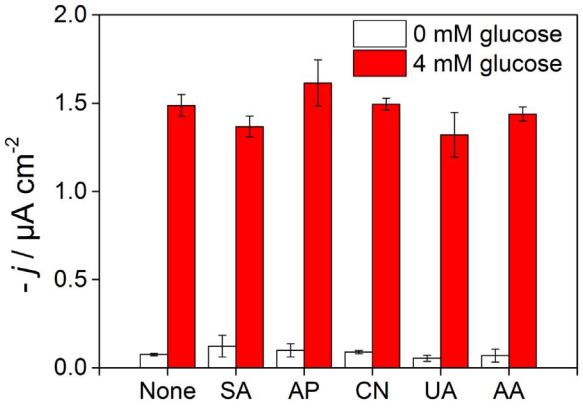

Fig. 5. Amperometric current density responses of the biosensor in presence of interfering species: None (no interfering species), SA (0.75 mM), AP (0.35 mM), CN (0.25 mM), UA (0.5 mM) and AA (0.15 mM). Medium: MES-NaOH (0.2 M, pH 7), 0.145 M KCl, 0.01 M $CaCl_2$, TMB (0.5 mM or 2 mM in case of AA) (n = 3). Detection potential: 0.1 V vs. Ag pseudo-reference electrode.

3.7. Interference test

Common blood interfering substances such as salicylic acid (SA), acetaminophen (AP), creatinine (CN), uric acid (UA) and ascorbic acid (AA) may have positive or negative contribution to the amperometric signal obtained with the presented biosensor. In this context, the maximum common concentration of these molecules in blood (Medscape) is added to the buffer solution and, both in absence (0 mM glucose) and presence (4 mM glucose), the current response at 0.1 V vs. Ag pseudo-reference electrode are measured.

As shown in Fig. 5, there are not significant differences in the biosensors response due to the presence of these interfering species (Kruskal-Wallis and Dunn's multiple comparison tests, $P < 0.05$), except in case of ascorbic acid. In this case, at 0.5 mM TMB, almost no current was registered. This molecule is well known as antioxidant, so the provoked interference is related to the reduction of the previously oxidized TMB in the GOD-HRP cascade reaction. This problem was resolved with an increment of TMB from 0.5 to 2 mM. Then, a normal response to glucose was register due to the molar excess of TMB respect AA (2 mM and 0.15 mM respectively). This result is very important in order to stablish the proper conditions to proceed blood samples where the AA is present.

3.8. Biosensor response with whole blood samples

With the optimized biosensor we proceed to measure human samples of plasma and whole blood, directly (health) and spiked (pre-diabetic and diabetic) to comply with the hyperglycaemic conditions. Real blood samples required a re-optimization of the enzymes concentration to adapt the response of the biosensor, lower in this case due to matrix effects, to the glucose concentration. A proper calibration with real samples required HRP and GOD concentrations of 55 µg mL^{-1} and 25 µg mL^{-1} respectively, maintaining the TMB concentration at 2 mM. As shown in Fig. 6, good linear responses were then obtained both in plasma (R^2 = 0.995) and whole blood (R^2 = 0.996). It is remarkable that the sensitivity and the LOD is similar in both cases (whole blood and plasma), concluding that the presence of cells did not interfere the obtained results and confirming the good performance of the alginate matrix as size-exclusion barrier, in contrast to the response to spiked samples of a reference biosensor (enzymes in solution and no hydrogel membrane protecting the transducer) where the sensitivity is very low in case of plasma and negligible in case of whole blood (dashed lines in Fig. 6).

With this design of membrane, it was possible to distinguish between healthy, pre-diabetic and diabetic patients with high precision in a single, fast and sensitive assay.

4. Conclusions

A simple, reusable and easily-fabricated biosensor for glucose detection in whole blood samples was developed using electrodeposited calcium alginate as enzymatic membrane. The geometrical selective electrodeposition of the hydrogel, besides the possibility of immobilize different enzyme types or concentrations, makes the fabrication very reproducible and adaptable to different samples. Furthermore, the hydrogel is easily re-dissolved with common buffers (PBS, citrate, among others) so it is possible to reuse the same electrochemical transducer for successive enzyme immobilization steps and analytes detection. This easy removing and deposition of new membranes lead to costs and time saves, since no extra calibration is needed. The alginate also demonstrates to be a good barrier protecting the electrodes from biofouling, which permits a reliable measure of whole blood glucose concentration, and also capable of maintaining the enzyme activity longer times than other simple fabrication method such as adsorption. In fact, it is critical to use this protective membrane in case of real samples analysis (plasma and whole blood) as almost no

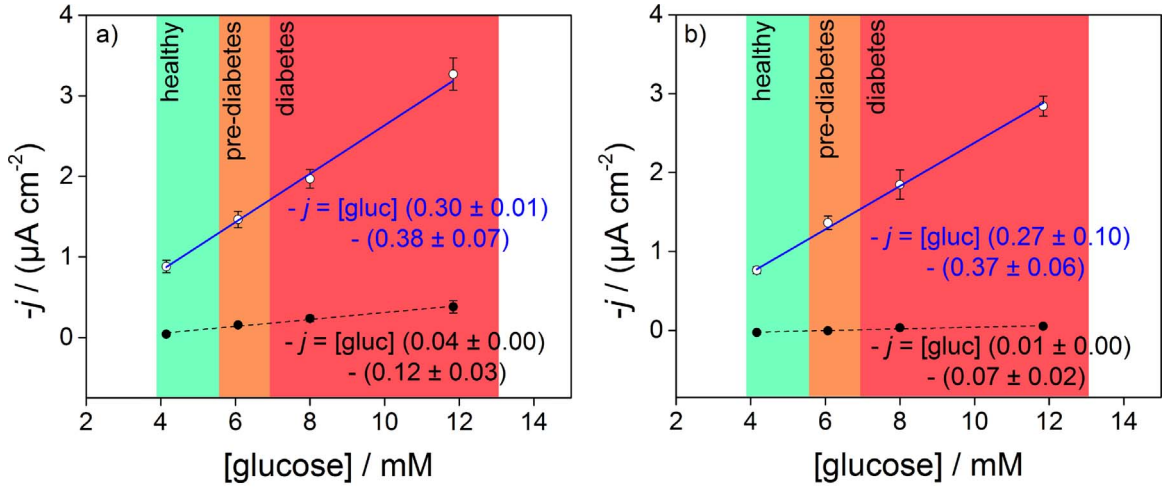

Fig. 6. Calibration curves of biosensor to different glucose concentrations in a) human plasma and b) human whole blood (n = 6). Dashed line represent the response of a biosensors with same amount of enzymes in suspension but no alginate membrane on the WE. Mediator: 2 mM 3,3′,5,5′-tetramethylbenzidine. Detection potential: 0.1 V vs. Ag pseudo-reference electrode. Stabilization time of the chronoamperometry: 3 min.

response is obtained with bare transducers. Good sensitivity of the biosensor was achieved, for buffer, plasma and whole blood measurements, modulating the enzymatic content of the membrane and the mediator concentration, making it possible to distinguish normal from hyperglycemic glucose concentrations.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2017.05.051.

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