Paper-based enzymatic electrode with enhanced potentiometric response for monitoring glucose in biological fluids

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Abstract

A novel paper-based potentiometric sensor with an enhanced response for the detection of glucose in biological fluids is presented. The electrode consists on platinum sputtered on a filter paper and a Nafion membrane to immobilize the enzyme glucose oxidase. The response obtained is proportional to the logarithm of the concentration of glucose, with a sensitivity of -119±8 mV·decade^{-1}, a linear range that spans from 10^{-4}M to $10^{-2.5}$ M and a limit of detection of $10^{-4.5}$ M of glucose. It is shown that Nafion increases the sensitivity of the technique while minimizing interferences. Validation with human serum samples shows an excellent agreement when compared to standard methods. This approach can become an interesting alternative for the development of simple and affordable devices for point of care and home-based diagnostics.

Keywords paper-based sensor, potentiometry, enzymatic sensors, glucose, Nafion

1. Introduction

Enzymatic biosensors show attractive analytical features, such as high specificity, good reproducibility, stability, low limits of detection in complex matrices (such as biological fluids), a broad range of applications and a simple detection scheme (Anzai et al., 1998; Evtugyn et al., 1998). For this reason, they are extensively used in clinical, environmental, forensic and food analysis, etc. (Karube and Nomura, 2000; Khan et al., 2008; Wang, 2006; Wang et al., 2009; Wilson and Hu, 2000). Oxidase-type enzymes are widely used since the generation of hydrogen peroxide as a byproduct (Ansari and Husain, 2012; Wilson and Hu, 2000) can be detected using different approaches (Anh et al., 2003; Xiao et al., 1999; You et al., 2011).

Electrochemical detection –in particular amperometric techniques- is widely used because of the outstanding performance and a simple, compact setup (Li et al., 2015; Wang, 2008). Interferences from matrix components are a major challenge that has been solved by the use of permselective

membranes. Nafion, for example, is a polyelectrolyte with negatively charged sulfonate groups employed to overcome the interferences of negatively charged species. The success of amperometry is reflected on the commercial implementation of home glucometers (Invernale et al., 2013). However, emerging social needs –such as the development of wearable (Bandodkar et al., 2016) and low-cost sensors (Maxwell et al., 2013)- are creating a growing demand for alternative approaches combining good analytical performance, robustness, simplicity and low costs. For this reason, potentiometric techniques are attracting a renewed interest, since they display an unrivalled simplicity (Ismail and Adeloju, 2014; Psychoyios et al., 2013; Yang et al., 2014), robustness and low-cost.

Enzyme-based biosensors based on the potentiometric detection of the reaction byproducts were first proposed several decades ago (Pasto et al., 1969). Later, a glucose sensor (Caras and Janata, 1985; van der Schoot and Bergveld, 1988) and a coated-wire sensor to detect urea and penicillin (Anzai and Osa, 1986) were proposed, but never widely adopted. More recently, the use of enzymes and potentiometric detection was explored by Willander *et al*. for the determination of glucose and cholesterol (Israr et al., 2010; Usman Ali et al., 2010), and by Adeloju *et al*. for glucose and phosphate (Adeloju and Moline, 2001; Ayenimo and Adeloju, 2014; Lawal and Adeloju, 2013). Despite of their advantages (Asif et al., 2010; Shukla et al., 2012), most of them have not been validated with real samples.

Finding alternative approaches for the determination of glucose in blood is still a very relevant topic, considering the growing number of people affected by diabetes, particularly in poor regions of the planet (Shaw et al., 2010). Therefore, in line with the cost-reduction approaches using screen-printed techniques (Renedo et al., 2007), nanomaterials and flexible electronics (Guinovart et al., 2013; Novell et al., 2012, Novell et al., 2014), developing a paper-based potentiometric biosensor for glucose may bring significant benefits.

Low-cost paper-based platforms to make affordable analytical tools have been proposed many years ago (Mabey et al., 2004), especially in combination with new materials (Kim et al., 2014; Liana et al., 2012). Although most of these devices are colorimetric assays (Curto et al., 2013; Yetisen et al., 2013), paper-based enzymatic electrodes (Nie et al., 2010) for amperometric detection have been recently proposed. In this work, a novel, simple, robust and sensitive enzymatic paper-based biosensor for the potentiometric determination of glucose is presented. The method is based on the detection of the H_2O_2 generated as a result of the enzymatic oxidation of glucose. A platinum-sputtered paper sensor is used as a redox-sensitive substrate and membrane of Nafion is employed to eliminate interferences and increase the sensitivity of the technique. The results show that this device can accurately predict levels of glucose in body fluids such as serum. Some limitations and potential future applications of these novel sensors in real life scenarios are discussed.

2. Experimental

2.1. Materials and methods

Whatman® Grade 5 qualitative filter paper, Nafion® 117 solution (ca. 5% in a mixture of lower aliphatic alcohols and water), glucose oxidase (GOx) from *Aspergillus niger* type X-S, lyophilize powder (100,000-250.000 units/g) D-glucose, hydrogen peroxide (30 % wt in water), sodium urate, sodium ascorbate and D-Fructose were purchased from Sigma-Aldrich. In all cases, the Nafion solution was used as received. All reagents used were analytical grade and were purchased from Sigma-Aldrich. Phosphate buffer saline (PBS) was prepared at 0.1 M and used in all the experiments. All solutions were prepared using 18.2 MΩ cm^{-1} double deionized water (Milli-Q water systems, Merck Millipore).

Platinum sputtering was performed using a radiofrequency sputtering process (ATC Orion 8-HV, AJA International) operated at 3 mTorr, for 65s at 200W. Filter paper strips were placed inside the sputtering chamber to generate the electrodes. An adhesive plastic mask (0.3 mm thick) coated with an acrylic adhesive on one side (Arcare 8565, Adhesives Research Inc., Limerick, Ireland) was used to expose a given area of the coated paper and isolate the rest of the conductive surface.

Details of the characterization analysis can be found in the supplementary material.

2.2. Electrochemical measurements

Potentiometric measurements were performed using a standard two-electrode (i.e., working and reference) cell configuration, using the paper sensor as a working electrode and commercial reference electrode, in a 4 ml cell in 0.1M PBS (pH 7.4) at 25ºC. A double junction Ag/AgCl/ 3 M KCl reference electrode (type 6.0726.100, Metrohm AG) containing a 1 M LiAcO bridge was used in all the experiments. Electromotive force (EMF) was measured using a high input impedance (10^{15} Ω) EMF16 multichannel data acquisition device (Lawson Laboratories, Inc. Malvern).

2.3. Fabrication of glucose biosensor

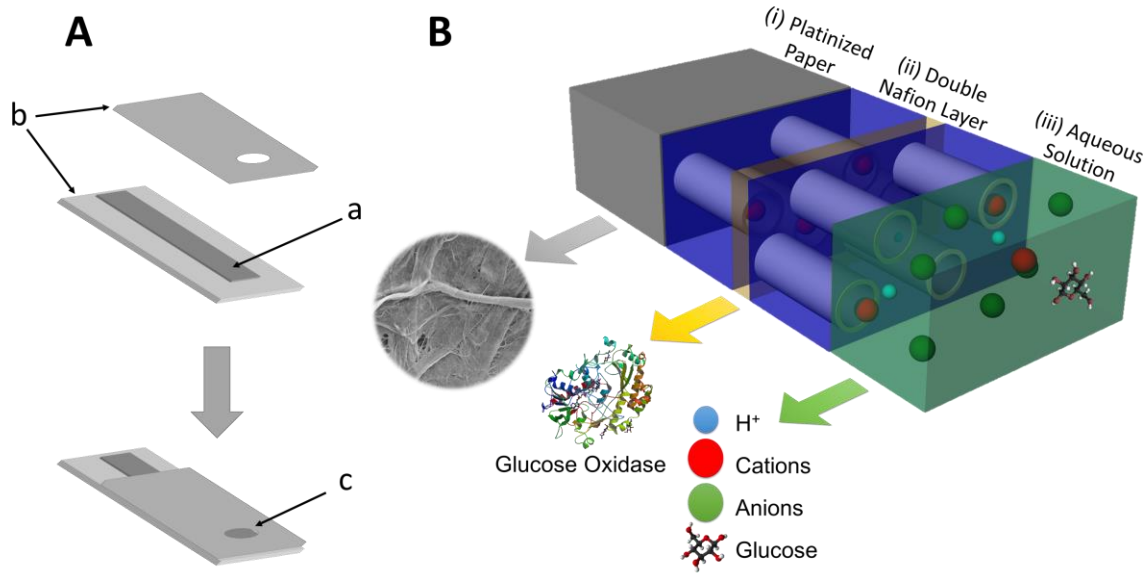

Fig. 1. Illustration of the paper-based biosensor. (A) Fabrication of the electrode, using a strip of platinized paper (a), sandwiched between two plastic masks (b), with a window of electroactive surface(c). (B) Scheme of the enzymatic membrane: (i) Pt-Paper Substrate; (ii) enzyme (GOx) sandwiched between two layers of Nafion, one at Pt-interface and the other at the (iii) solution interface.

Electrodes were made by sputtering Pt on one side of a filter paper, which was then cut into rectangular pieces (20 mm X 5 mm) sandwiched between two rectangular plastic masks. The top mask (15 mm X 10 mm) had a 3 mm diameter circular window and the bottom mask was slightly larger (20 mm X 10 mm), as shown in Fig. 1A. The exposed top of the conductive paper was connected with the reading instrument, and the circular window that will be used as the electrochemically active surface.

These bare Pt electrodes are then functionalized with the biosensing membrane, which is made using Nafion as polymeric coating and glucose oxidase enzyme as the biological receptor. First, the window of each electrode was rinsed with double-distilled water and air-dried. Thereafter, a first layer of Nafion was made by drop casting 4 µl of the Nafion solution air-dried for 60 minutes at room temperature. Thereafter, 20 µL of a solution containing 20 mg mL^{-1} of glucose oxidase (GOx) in distilled water was drop cast on top of the Nafion membrane and the system was left drying overnight at 4ºC. Finally, 2.5 µl of the same solution of Nafion was applied and let dry overnight at 4ºC (See Fig. 1B) This electrode was kept at 4ºC when not in use.

2.4. Analysis of real samples

Serum samples of patients were obtained at a local hospital (Hospital de Sant Pau i Santa Tecla). Values from serum samples were provided by the hospital using hexokinase/glucose-6-phosphate

dehydrogenase colorimetric test as a standard method for further validation of the paper-based potentiometric system.

3. Results and discussion

3.1. Characterization of the platinized paper-based electrodes

Environmental scanning electron microscopy (ESEM) images of the electrodes shows the cross-linked cellulose fibers completely covered by a thin layer of sputtered platinum (Fig. S1A). While it is difficult to assess the thickness of the metal layer on paper, on a flat surface the thickness of the platinum layer is of approximately 100 nm. When a drop of water is added on top of the Pt layer, no percolation through the metallic layer (i.e. no wetting of the paper) is observed. Additionally, this thin layer of Pt shows an electrical resistance of a few ohms, corresponding to a metallic conductor. When the Nafion is added, the surface of the metalized paper is completely covered by the membrane (Fig. S1B), which shows a thickness of approximately 40 μm.

3.2. Electrode response and principle of detection

Preliminary experiments show that the electrode potential decreases linearly with the logarithm of the concentration of glucose as shown in Fig. 2A and 2B. To explore the nature of this response, "blank" measurements were performed using a bare paper Pt electrode and a Nafion coated Pt electrode without enzyme. None of these electrodes respond to the addition of glucose (see inset of Fig. 2A and 2B). Thus, the response must be due to some byproduct of the enzymatic reaction. The reaction catalyzed by the GOx can expressed as:

$$H_2O + O_2 + Glucose \rightarrow Gluconolactone + H_2O_2 \qquad \text{(Equation 1)}$$

Gluconolactone quickly hydrolyses, so the reaction generates gluconic acid and H_2O_2. Similarly, none of the systems respond to the addition of gluconate (Fig. 2C). However, the addition of peroxide produces a response that, in the case of the electrode coated with Nafion, matches the response obtained when adding glucose in the enzymatic sensor (Fig. 2D). Thus, it can be concluded that the response observed is due to the change on the redox potential produced by the enzymatic generation of H_2O_2. Also, the Nafion-coated electrode shows an enhanced response to H_2O_2.

Pt shows a limited and unspecific potentiometric response to H_2O_2. However, we have recently shown (Parrilla et al., 2016) that when Pt electrodes are coated with a layer of Nafion the sensitivity for H_2O_2 increases significantly, while the effect of negatively charged species -such as ascorbate- is reduced. The permselective behavior of Nafion has been already exploited to reduce intereferences in amperometric sensors. The enhanced potentiometric response, though, seems to be the result of the

Donnan potential generated by the Nafion coating. Local variations of pH at the interface (Schasfoort et al., 1990), as well as the complex coupling between the redox and acid-base equilibria have been proposed as the major factor behind this effect. To illustrate this point, experiments with bare platinized papers and Nafion-coated platinized papers were performed (Fig. 2D and 2E), showing the significant increase in the sensitivity obtained when electrodes were coated with Nafion (-140.4±7.4 mV decade^{-1}, N=2) in comparison to bare platinized paper electrode (-53.6±6.2 mV decade^{-1}, N=2) in a linear range of 10^{-5} M to $10^{-3.5}$ M of H_2O_2. Also, unlike the bare Pt electrodes, the Nafion-coated electrodes show a sensitivity for H_2O_2 that is dependent on both, the total electrolyte concentration (Parrilla et al., 2016) and the solution pH (Fig. 2F). Interestingly, optimum conditions for detection of H_2O_2, such as high electrolyte concentrations and pHs slightly about 7, match the conditions usually found in many biological fluids.

Regarding the nature of the response, Nafion is a complex system and the models to describe its structure (Mauritz and Moore, 2004) and interactions with substrates such as carbon or platinum interfaces (Wood et al., 2009) are still a matter of study. Nafion membranes are formed by randomly packed water channels surrounded by partially hydrophilic side branches, forming inverted-micelle cylinders (Schmidt-Rohr and Chen, 2008). The negatively charged sulfonate groups ($-SO_3^-$) provide the permselectivity and strong ion-exchange capabilities that generate a Donnan potential (Naegeli et al., 1986).

All in all, these Nafion-coated enzymatic sensors respond to changes in the redox potential produced by the generation of H_2O_2 in a highly sensitive and selective way. To the best of our knowledge, this is the first time that this type of detection scheme is reported. Thus, this work is significantly different from the potentiometric sensors recently reported by Willander et *al*., which are claimed to be based on the local changes of pH resulting from the enzymatic reaction (Usman Ali et al., 2011).

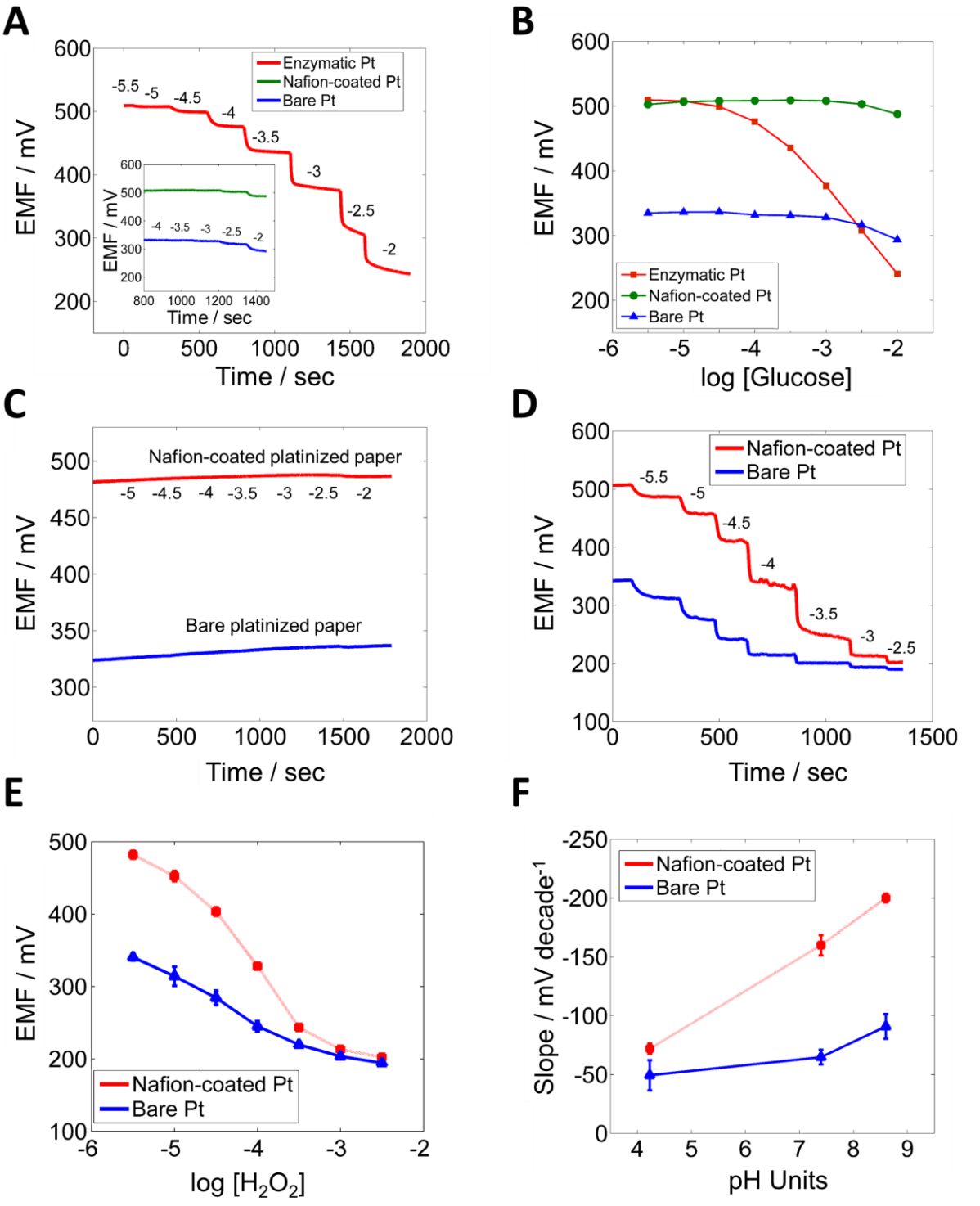

Fig. 2. Response of the electrodes: (A) Potentiometric time-traces and (B) corresponding calibration curves of enzymatic, Nafion-coated and bare platinized paper electrodes upon additions of glucose; (C) potentiometric time-trace for the addition of gluconic acid to bare and Nafion-coated paper electrodes; (D) potentiometric time-trace for the addition of H_2O_2 and (E) corresponding calibration curve for H_2O_2 for bare and Nafion-coated platinized paper electrodes. (F) pH dependence of the sensitivity for H_2O_2 in the range from 10^{-5} M to $10^{-3.5}$ M. All the measurements were performed in 0.1 M buffers, acetic buffer (pH 4.2), PBS (pH 7.4), borate buffer (pH 8.6) at 25ºC. The numbers on the time-traces indicate the logarithm of the concentration of the substance added.

3.3. Optimization of the detection of glucose

Preliminary experiments show that optimum results are obtained when the biosensor is built by sandwiching the enzyme between two layers of Nafion. The first layer of Nafion on top of the Pt electrode plays 3 important roles. First, as it happens in other sensors, it minimizes the interference of negatively charged redox-active substances (Wynne and Finnerty, 2015). Second, as already discussed, it produces an enhancement of the potentiometric response. Third, the Nafion offers a better substrate for direct immobilization of the enzyme. Indeed, when the enzyme is deposited directly onto the Pt surface (results not shown), lower of enzymatic activity is observed, as it has been already described in the literature (Klotzbach et al., 2006). This immobilization is performed simply by direct drop casting of the enzyme solution on top of this first layer of Nafion. After the solution of the enzyme has dried, a second layer of Nafion drop cast on top improves the immobilization –avoiding any leaching of the enzyme on the solution- and it helps to isolate the generation of H_2O_2 from the rest of the solution, avoiding reactions with sample component that could be the potential interferences.

The influence of the amount of enzyme was evaluated by adding a fix volume (15 µL) of solution with different concentrations of enzyme (0.2, 2, 20 mg/mL). Experimental evidence shows that none of these concentrations yield a difference on the final sensitivity obtained. Nevertheless, kinetic factors are improved at the highest concentration of enzyme. Under these conditions, faster production of H_2O_2 allows reaching steady state signal in less than 90 seconds. Alternatively, the amount of enzyme added was modified by adding different volumes (10, 15 and 20 µL) of the highest concentration (20 mg mL^{-1}) of the enzyme solution. In this case, an improvement in the sensitivity for glucose is observed (Fig. 3A), suggesting a better distribution of the enzyme on the membrane during the drying process. Thus, optimum conditions for building the sensor were set at 20 µL of 20 mg/mL of enzyme.

Selectivity of the biosensor was assessed by monitoring the response in presence of redox interferences. Experiments were carried out to evaluate the permselectivity of the first layer of Nafion, which separates the electrode from the solution. Different volumes of the Nafion solution (2, 3.5 and 4 µL) were cast on the electroactive area, verifying that in all cases the whole Pt surface was covered. Therefore, the increase of the volume of solution cast should produce an increase on the thickness of the membrane. To evaluate the performance of this membrane, the potentiometric response to a 100 µM ascorbate solution (upper concentration level typically found in body fluids) was tested. The difference in potential -before and after the addition of ascorbic acid- was used to characterize the selectivity of the system. Ideally this difference should be close to zero. The results are shown in Fig. 3B. Clearly, the degree of interference is significantly reduced as the volume of solution cast (therefore, the membrane thickness) is increased. Nevertheless, with volumes of Nafion solution

higher than 4 µL the sensitivity for glucose is decreased. Therefore, in a compromise between selectivity and sensitivity, a volume of 4 µl of Nafion solution was chosen as optimum drop-cast volume. This volume does not fully eliminate the interference, but it reduces significantly.

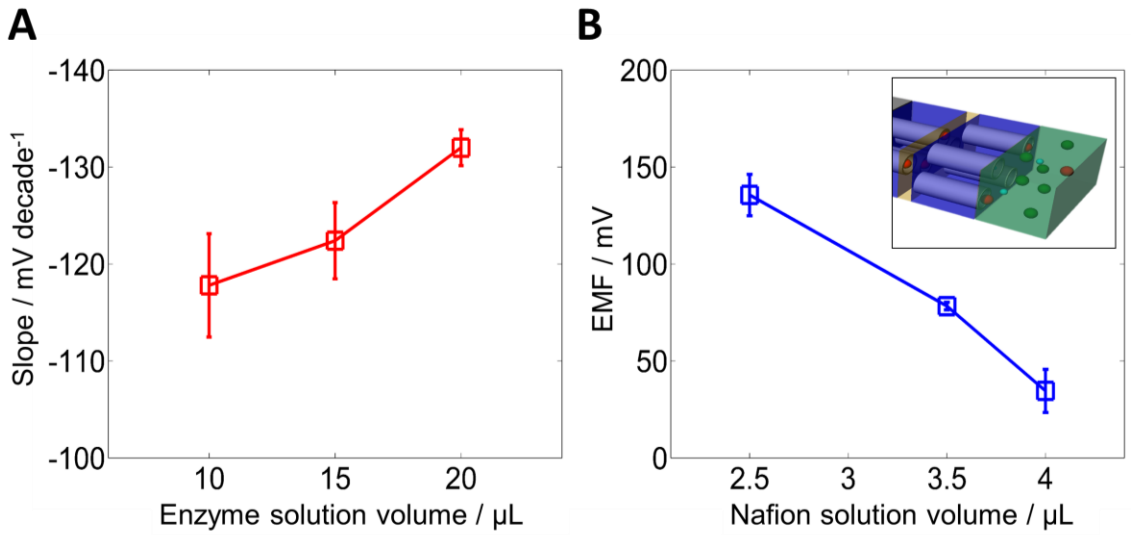

Fig. 3. Optimization of the analytical conditions for the biosensor. (A) Sensitivity for glucose vs volume of solution of enzyme concentration (20 mg/mL) cast. (B) Change on the potentiometric signal after the addition of 10^{-4} M ascorbic acid as function of the increasing volume of Nafion membrane cast. In all cases, error bars correspond to the standard deviation of 3 different electrodes.

3.4. Analytical performance

The enzymatic paper-based electrode was studied by monitoring change in electrochemical potential for increasing concentrations of glucose. Fig. 4A shows the time-response curve of three different electrodes at different potentiometric cells in the concentration range from $10^{-5.5}$ M up to $10^{-1.5}$ M. A decrease in the potential was observed after addition of each glucose standard. Fig. 4B presents the calibration curve on potential and glucose concentration with a linear response from 10^{-4} M to $10^{-2.5}$ M of glucose. The corresponding linear regression equation for the three calibration curves was: EMF (mV) = -118.6±7.6 log[Glucose] - 532.08±34.8, R^2 = 0.99, N=3. The limit of detection was found to be $10^{-4.5\pm0.08}$ M. This is the first time that such high sensitivity is obtained for a potentiometric paper-based enzymatic electrode.

Repeatability test was carried out by performing several consecutive calibration curves for three different electrodes from 10^{-5} M to 10^{-2} M. The comparison between calibration curves (Table S1) yields an outstanding reproducibility among slopes (3.4% RSD, N=3). The only difference found is that, in the third calibration curve, the linear range was shifted towards lower concentrations. In this last case, a decrease on the initial potential of the system was observed, possibly due to some

rearrangement of the Nafion membrane. Considering that the system is intended as a disposable sensor, it can be assumed that the reproducibility of the paper electrodes was highly satisfactory.

The selectivity of the enzymatic sensors is usually associated to the specificity of the enzymatic catalysis. However, some redox sensitive interfering molecules found in real samples can induce to an error in the prediction. It is well-known that some molecules interfere in amperometric glucometer measurements such as uric acid and ascorbic acid. In this work, fructose, uric acid and ascorbic acid were tested at the high level of concentration found in blood (Fig. 4D). For fructose and uric acid addition, the potentiometric response was almost negligible. However, for ascorbic acid addition (10^{-4} M) some small drop of the potential was observed. Thus, despite of the permselectivity of Nafion, some residual electrochemical effect of the ascorbic was observed, possibly due to a slight diffusion of the ascorbic through the hydrophilic membrane. This effect, however, is observed at the highest concentration usually found in blood. Some further work is being currently performed to eliminate this residual effect.

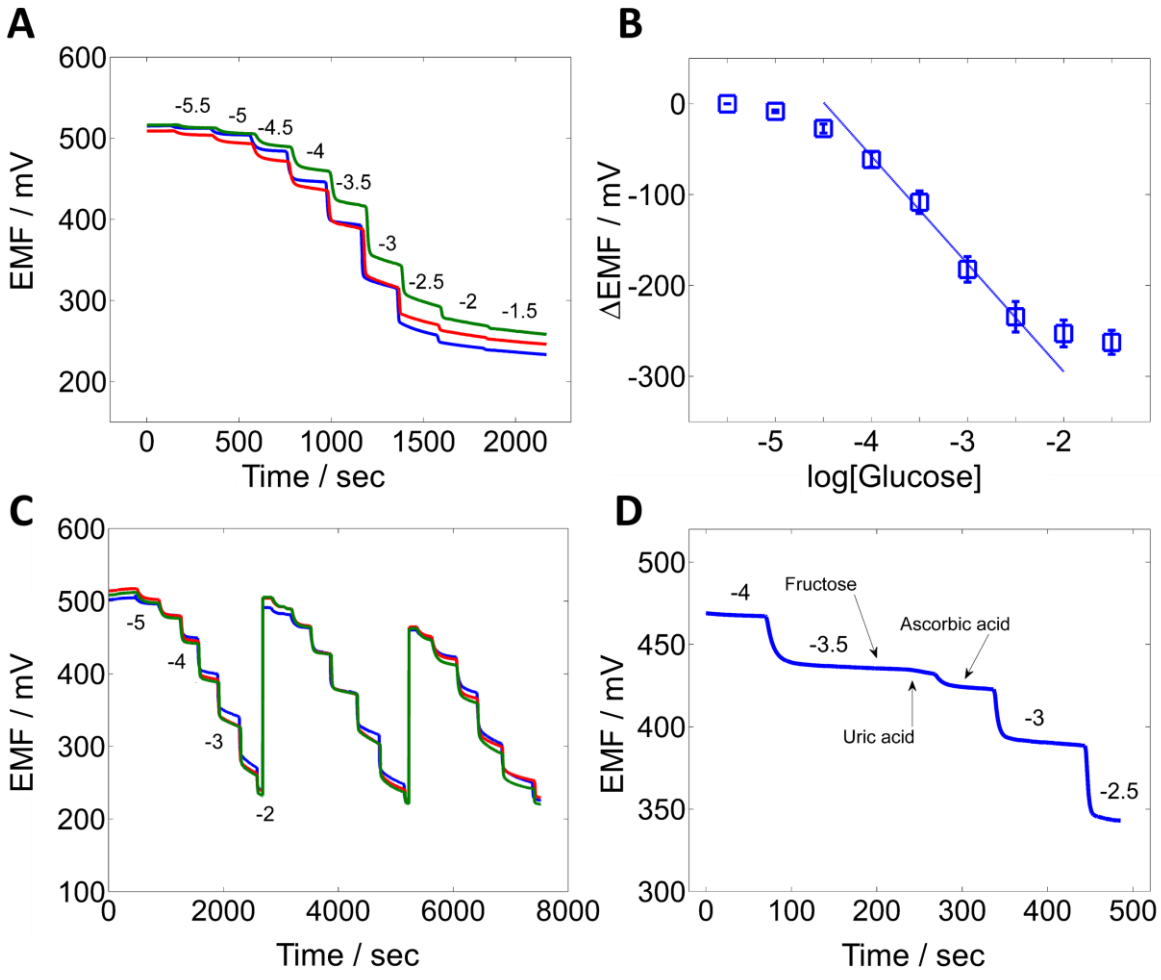

Fig. 4. Analytical performance of the paper-based enzymatic electrode: (A) Time-trace plot of three different electrodes in three separated potentiometric cells, (B) the corresponding calibration curve of enzymatic

electrodes showing the linear range (mean± S.D., N=3). (C) Repeatability test for three different electrodes during consecutive calibration curves for glucose. (D) Time-trace for glucose with a selectivity test to main interferences, such as ascorbate urate and fructose, all added at 0.1 mM level. All the measurements were performed in 0.1 M PBS (pH 7.4) at 25ºC.

3.5 Analysis of real samples

Serum samples from patients provided from a local hospital were used to test the performance of the proposed system. Samples were diluted 1:10 with PBS (0.1 M) to match physiological and the linear range of the sensor. Three different electrodes were calibrated before the analysis and used for each sample. The measurement was performed after a steady-state value was reached, normally two minutes after the addition of the sample. The results (Table S2) show an excellent agreement with the reference method provided by the hospital (hexokinase/glucose-6-phosphate dehydrogenase colorimetric test).

Fig. 5 presents a good linear correlation between both methods. The relationship between values obtained from both methods were between 90.6-111.1 %, indicating that serum matrix had no significant effect on glucose determination. It should be mentioned that the same electrodes were used repeatedly for at least 6 predictions without losing their performance indicating high resistance to biofouling. Nevertheless, ideally the system is conceived to be used as a disposable sensor to make easy to handle and avoid contamination between biological samples.

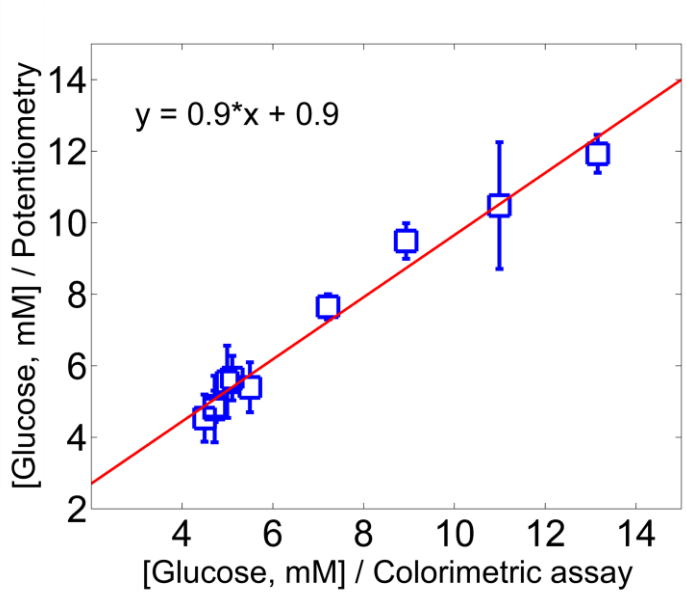

Fig. 5. Comparison of glucose determination (mM) in real samples obtained by the potentiometric paper-based electrode (mean±S.D., N=3) and colorimetric assay (data provided by the local hospital) at 25ºC.

Table 1 provides a comparison of the analytical performances of the enzymatic paper-based electrode described in this study with those of other reported glucose potentiometric biosensors. The system

described in this work reports the highest potentiometric sensitivity and the cheapest method of fabrication of the electrode for the measurement of glucose in real samples.

Table 1. Comparison of analytical performances of potentiometric biosensors for glucose detection.

Sensor[a]	Sensitivity (mV decade^{-1})	Linear range (M)	Limit of detection (M)	Time of response (seconds)	Analysis in real samples	Reference
GOx/Gy/BSA platinum foil	-52	$10^{-3.4}$ to $10^{-2.4}$	---	---	No	Wingard et al., 1984
GOx/ZnONPs/CHIT/PVAL indium tin oxide	---	$10^{-5.7}$ to $10^{-2.9}$	$10^{-6.7}$	---	No	Shukla et al., 2012
GOx/Fe_3O_4NPs/CHIT gold coated glass	27.3±0.8	10^{-6} to $10^{-1.5}$	---	7	No	Khun et al., 2012
GOx/ZnONWs silver coated glass	35	$10^{-6.3}$ to 10^{-3}	---	4	No	Usman Ali et al., 2010
GOx/Fe_3O_4NPs/Ppy MGCE	19.4	$10^{-6.3}$ to $10^{-1.5}$	$10^{-6.5}$	6	Human serum	Yang et al., 2014
GOx/AgNPs over a polymeric membrane Ag-ISE (glass electrode)	---	10^{-4} to $10^{-2.5}$	10^{-5}	---	Beverages	Ngeontae et al., 2009
AuNs/PtNPs/GOx Aluminum foil	33,4	10^{-4} to $10^{-2.1}$	---	---	No	Xu et al., 2013
GOx Iodide electrode	65.2±0.2	10^{-4} to 10^{-2}	---	60-120	Human serum	Karakus et al.,2013
PPy-GOx film PtE	38.3	10^{-3} to 10^{-2}	---	60-120	No	Trojanowicz et al., 1996
PPy-GOx film Pt disk	76,5	$10^{-5.2}$ to $10^{-1.4}$	$10^{-5.2}$	30	No	Ayenimo et al. 2014
Nafion/GOx/Nafion platinized paper	**-119±8**	**10^{-4} to $10^{-2.5}$**	**$10^{-4.5}$ M**	**50**	**Human serum**	**Current work**

[a] GOx – Glucose Oxidase // Gy – Glutaraldehyde // BSA – Bovine serum albumin // ZnONPs – Zinc oxide nanoparticles // CHIT – Chitosan // PVAL – Polyvinyl alchohol // ZnONWs – Zinc oxide nanowires // Ppy – Polypyrrole // PtNPs – Platinum nanoparticles // MGCE - Magnetic glassy carbon electrode // PtE – Platinum electrode

4. Conclusions

A new paper-based enzymatic electrode that shows high sensitivity for the determination of biomolecules such as glucose has been presented. Under the optimal conditions, this sensor exhibits excellent enzymatic activity and high reproducibility for the detection of glucose in a linear range from 10^{-4} M to $10^{-2.5}$ M with a limit of detection of $10^{-4.5}$ M. The use of the hydrophilic negatively charged Nafion membrane avoids minimizing interferences from negative redox active molecules such as ascorbate as well as the biofouling effect. The analytical parameters shown by the paper-based enzymatic electrode can be attributed to the elevated enzyme loading capacity and high stability

provided by the Nafion membrane, which yield a favourable environment for the enzyme catalysed reaction. Furthermore, the use of this Nafion coating allowed the enhancement of the potentiometric detection. This strategy opens a new avenue towards the development of highly sensitive paper-based enzymatic sensors for diseases monitoring. Indeed, with this approach, any laboratory having a basic pHmeter should be able to measure biological substances in a simple, accurate and cost-effective way. Monitoring of different biomolecules should be possible simply by changing the enzyme, a work that is currently being explored in our labs. Additionally, the use of different polyelectrolyte coatings to enhance the selectivity and sensitivity of the potentiometric response is also an area to be further explored. Last, but not least, future prospects should include the integration of a full paper-based potentiometric cell.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi.XXX.

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