MODULATING THE MIXED POTENTIAL FOR DEVELOPING BIOSENSORS: DIRECT POTENTIOMETRIC DETERMINATION OF GLUCOSE IN WHOLE, UNDILUTED BLOOD.

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ABSTRACT

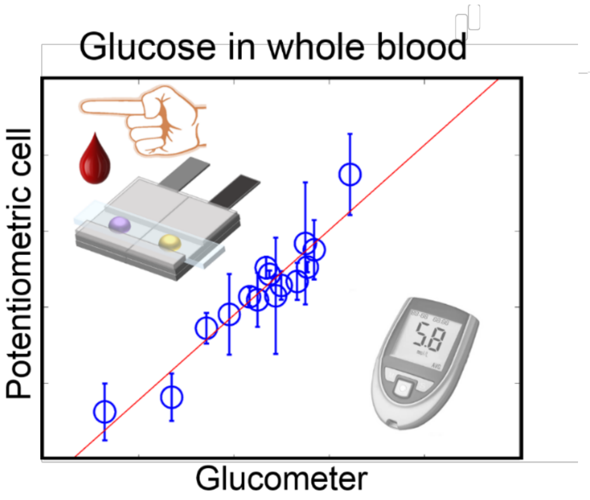
The growing demand for tools to generate chemical information in decentralized settings is creating a vast range of opportunities for potentiometric sensors, since their combination of robustness, simplicity of operation and cost can hardly be rivalled by any other technique. In previous works, we have shown that the mixed potential of a Pt electrode can be controlled with analytical purposes using a coating of Nafion, thus providing a way to develop a potentiometric biosensor for glucose. Unfortunately, the linear range of this device did not match the relevant clinical range for glucose in blood.

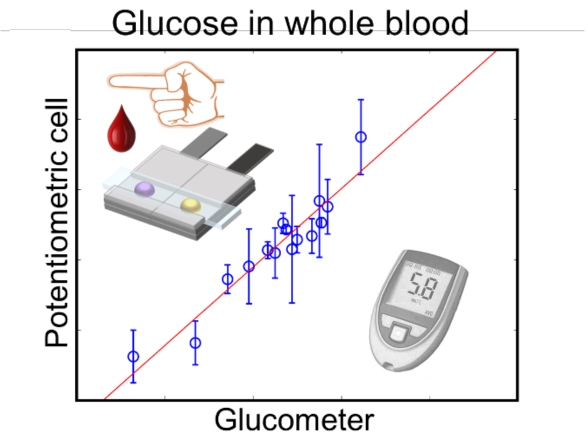
This work presents a novel strategy to control the mixed potential that allows the development of a potentiometric biosensor for the direct detection of glucose in whole, undiluted blood without any sample pretreatment. By changing the ionomer, the analytical response can be tuned, shifting the linear range while keeping the sensitivity. Aquivion, a polyelectrolyte from the same family as Nafion, is used to stabilize the mixed potential of a platinized paper-based electrode, to entrap the enzyme and to reduce the interference from negatively charged species. Factors affecting the generation of the signal and the principle of detection are discussed. Optimization of the biosensor composition was achieved with particular focus on the characterization of the linear range and sensitivity. The accurate measurement of blood sugar levels in a single drop of whole blood with excellent recovery is presented.

KEYWORDS

Potentiometry; paper-based sensor; glucose; mixed potential; whole blood detection

Graphical Abstract





1. INTRODUCTION

Biosensors have traditionally played a major role in areas such as healthcare (Sekretaryova et al., 2014), drug discovery (Wang, 2006), food safety (Gamella et al., 2010), environmental monitoring (Rana et al., 2010), homeland security and defense (Arduini et al., 2007), among many others (Mahato et al., 2017). More recently, with the growing interest in distributed sensing approaches -such as in point of care and telemedicine- biosensing platforms to generate information on-site in real time have become increasingly important. A centerpiece in the successful development of these tools is the compromise between the analytical figures of merit and some practical aspects regarding the robustness, operational simplicity and cost. This combination, the real conundrum of the distributed analytical platforms, is well represented by the home glucometer. This device has been recently explored as a universal point of care tool (Lisi et al., 2020), although still, many challenges regarding affordability and simplicity remain unsolved.

The problem of sensor affordability has been extensively explored during the last decade with the use of low-cost substrates such as paper, textile, rubber or plastics (Novell et al., 2012; Parrilla et al., 2016; Rius-ruiz et al., 2011). While there are still ongoing efforts on this area, the progress achieved so far offers an attractive outlook for the development of affordable chemical sensing devices (López_Marzo and Merkoçi, 2016).

The operational simplicity remains as one of the most challenging aspects of distributed sensors, since any activity involving the user –sampling and sample conditioning such as dilution, pH adjustment, etc- must be minimized. Elimination of the user intervention is being extensively explored using wearable and implantable devices, but there are still limitations regarding the type of information that these approaches can provide. For this reason, determinations in a single drop of whole, undiluted samples –mostly blood- are still the norm. Simple sample conditioning steps can be performed using the capillary-driven flow through cellulosic substrates (Arroyo et al., 2020; Erenas et al., 2019; Ruecha et al., 2017). Ion-exchange membranes (Grygolowicz-Pawlak et al., 2013) and multi-electrode arrays have been used to minimize interferences (Wang et al., 2018) and improve selectivity. All these solutions, however, require a tradeoff between overall complexity and performance. Ideally, distributed tools must provide suitable sensitivity, be impervious to the matrix components and match the linear ranges of interest. This requires detection approaches that show high sensitivity, wide (or adjustable) linear ranges, instrumental simplicity and matrix-insensitive response.

We have recently proposed a novel approach for the detection of hydrogen peroxide, a molecule ubiquitously found in a plethora of biosensing devices (Parrilla et al., 2017a). Unlike most of current optical and electrochemical methods, which require a redox mediator, we have proposed a simple, highly sensitive and mediator-free approach for the detection of H_2O_2 using the potentiometric response of a Pt electrode coated with a layer of Nafion. The advantages of this approach have been demonstrated through the development of a paper-based potentiometric cell for the determination of glucose in blood (Parrilla et al., 2017b). The low-cost platform is sensitive, robust and simple. However, because of the mismatch between the dynamic range of the sensor and the clinical range of interest, a 1:10 dilution of the blood sample was required to perform the measurement (Cánovas et al., 2017). This seriously impairs the applicability in distributed settings.

Nevertheless, some preliminary evidence has shown that the response of the Pt electrode can be modulated by the choice of polymeric coating used (Baez et al., 2020). This opens the way to developing detection schemes with tunable analytical parameters. This work is devoted to show that by the modification of the ionomer used, the figures of merit for the detection of peroxide can be changed. Thus, a potentiometric sensor for the direct detection of glucose in whole, undiluted blood can be performed. Replacing the Nafion membrane by its analogous, shorter side-chain Aquivion, the linear range can be shifted toward the range of interest, without affecting the sensitivity of the detection. This approach may become a platform to build robust, simple, versatile and low-cost sensors for distributed settings.

2. MATERIALS AND METHODS

2.1 Instrumentation and electrochemical measurements

Electromotive force (EMF) measurements were performed with a high input impedance (10^{15} Ω) EMF16 multichannel data acquisition device (Lawson Laboratories, Inc. Malvern). All the calibration curves and measurements were always carried out by triplicate (N=3).

Prior to real samples detection, the device was calibrated with three standards of glucose 1, 3.16 and 10 mM in artificial serum. The cleaning of the cell (after the measurement of a blood sample) can be evaluated by the recovery of the open circuit potential initial value. This indicates that the system is ready to receive a new sample. This procedure, which was repeated using three different paper-based devices, allows measuring several samples in few minutes, including the calibration curve, washing, etc.

2.2 Fabrication of the glucose biosensor

Enzymatic paper-based electrodes were built using a platinum sputtering approach as was described previously (Parrilla et al., 2017b). The working electrode was built using Glucose oxidase (GOx) entrapped between two layers of a polymeric matrix. The deposition of the polymeric matrices (either Nafion or Aquivion) was carried out by drop casting. For the reference electrode (Guinovart et al., 2014), a total of 9 µL of a reference membrane consisting of 78 mg PVB and 50 mg NaCl in 1 mL of methanol was drop-cast. To build the miniaturized cell, the enzymatic sensor was placed in between the sampling cell and connected to the measuring device through the conductive ends (Figure S1). This cell was kept at 4ºC when not in use. The sampling cell was designed and optimized for a sample volume of 25 µL (Cánovas et al., 2017).

3. RESULTS AND DISCUSSION

3.1 Mixed potential mechanism

When a platinum electrode is immersed in an aqueous solution lacking of a well-defined redox couple, the open circuit potential is established through a mixed potential (MP) mechanism (Baez et al., 2020). In essence, MP are produced when the electrochemical potential gradient is balanced through redox reactions with different solution components. Oxidation and reduction reactions on the surface of the electrode produce anodic (i_a) and cathodic (i_c) exchange currents. While each current is a separate potential-dependent charge-transfer kinetic process, under steady state they must be balanced to avoid charge accumulation. Therefore, the MP is the electrode potential required to make the two exchange currents reaching a similar absolute value ($i_a + i_c = 0$). Theoretical approximations to MP balancing the Tafel equations of each exchange current show that the voltage-concentration relationships do no follow a Nernstian behavior. For this reason –unlike conventional potentiometry- extraordinarily high values of sensitivity can be reached.

We have recently shown that the MP of Pt can be effectively controlled using a Nafion membrane on the surface of the electrode (Baez et al., 2020). As a permselective barrier, Nafion helps to reduce the concentration of anions on the electrode surface. This stabilizes the surface charge, reduces interferences and minimizes the competitive binding with spectator species. The acidic characteristics of Nafion enhance the local proton concentration and minimize the effect of convection. The polarity of the membrane allows reducing the effect of biofouling. Last, but not least, the solubility of O_2 in Nafion is 20 times higher than in water, so the membrane acts as an oxygen reservoir. For this reason, this Nafion-coated electrode shows an enhanced response to hydrogen peroxide that has been exploited for detection of glucose (Cánovas et al., 2017; Parrilla et al., 2017b). Unfortunately, a sample dilution is required. Tuning the analytical parameters to avoid this step is crucial for a distributed analytical tool.

3.2 Polyelectrolyte coating

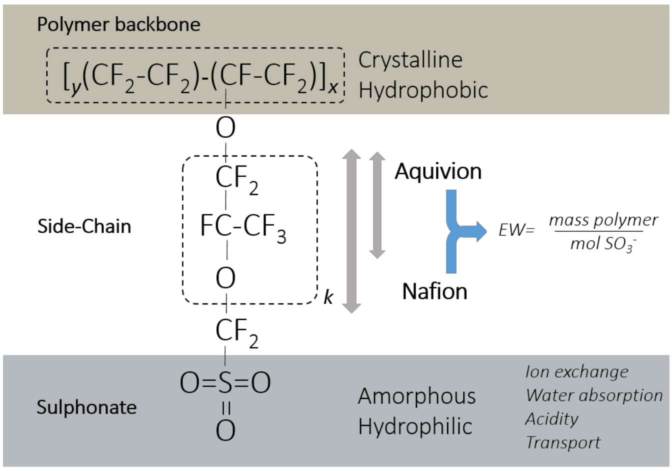


Figure 1. Ionomers structure and main characteristics

It has been shown that the response to peroxide can be modulated by using different type of ionomers (Baez et al., 2020). In particular, changing the ionomer allows shifting the range of response to peroxide. Nafion and Aquivion are members of the perfluorinated sulfonic-acid (PFSA) ionomers, a type of polymers with a crystalline polymeric backbone (polytetrafluoroethylene) and an amorphous side-chain with a pendant negatively charged sulphonate group, which is associated to some counter-ion. These polymers show great chemical and mechanical stability and a remarkable ionic conductivity. The reason for these unique property lies on the strong differences between the polymeric backbone and the sulphonate groups, which produces a phase separation upon the addition of solvents. When hydrated, these materials form nanochannels in a segregated hydrophilic phase that allows the transport of water and facilitates the ionic mobility. Therefore, a key parameter used to characterize these polymers is the equivalent weight (EW) a value that provides an indication of the ion-exchange and water uptake capacity of the material (Figure 1). Typical *EW* values for Nafion are in the order of 1100 g/mol, and for Aquivion slightly below 1000. It is important to note that this difference is enough to modify some of the properties of the polymers, but it does not have a marked impact on the mechanical and morphological properties of the membrane. When comparing Nafion and Aquivion, general macrostructural and morphological features are similar, although microstructural differences in terms of nanochannels formation, water uptake, ionic conductivity and transport can be expected. The *EW* is usually changed by modifying the length of the side-chain. Nafion is a long side-chain, while Aquivion is a short side-chain PFSA. For this reason, Aquivion presents a higher ion-exchange and water uptake capacity, which gives enhanced transport properties.

3.3 Analytical performance

Platinized paper-based electrode was constructed and characterized for the detection of glucose in PBS pH 7.4 (Figure S1). A comparison of the analytical performance can be seen in Figure 2A and B with the corresponding analytical parameters reported in Table S1 (6 µL of polymer coating for Aquivion). Interestingly, similar sensitivity is obtained in both cases, while the linear range is shifted to higher concentrations when using Aquivion. The upper limit of the linear range is 3 mM for Nafion, while is up to 17.8 mM for Aquivion. While it does not seem much in absolute terms, this is of extreme importance since it allows matching the clinical range of interest in blood. For healthy, non-diabetic conditions, ranges between 4 and 7 mM are expected. As a preliminary conclusion, this result would allow for direct measurement of glucose in blood or serum.

In both cases, the electrodes show a comparable standard (initial) potential (200 mV increase respect to a bare Pt electrode). In addition, the similarities in the slopes suggest that the change of the type of ionomeric membrane does not affect the actual mechanism of the reaction. Then, the changes observed can be ascribed to variations in the exchange current density. This is not surprising, since this parameter depends on the nature of the electrode and the physical and chemical characteristics of the environment (Baez et al., 2020). As a minor drawback, the response time for the sensors based on Aquivion suffer a threefold increase (Table S1, from 80 s for Nafion up to 215 s for Aquivion). This is possibly due to a slower diffusion across the ionomeric layer due to a higher charge density of the coating.

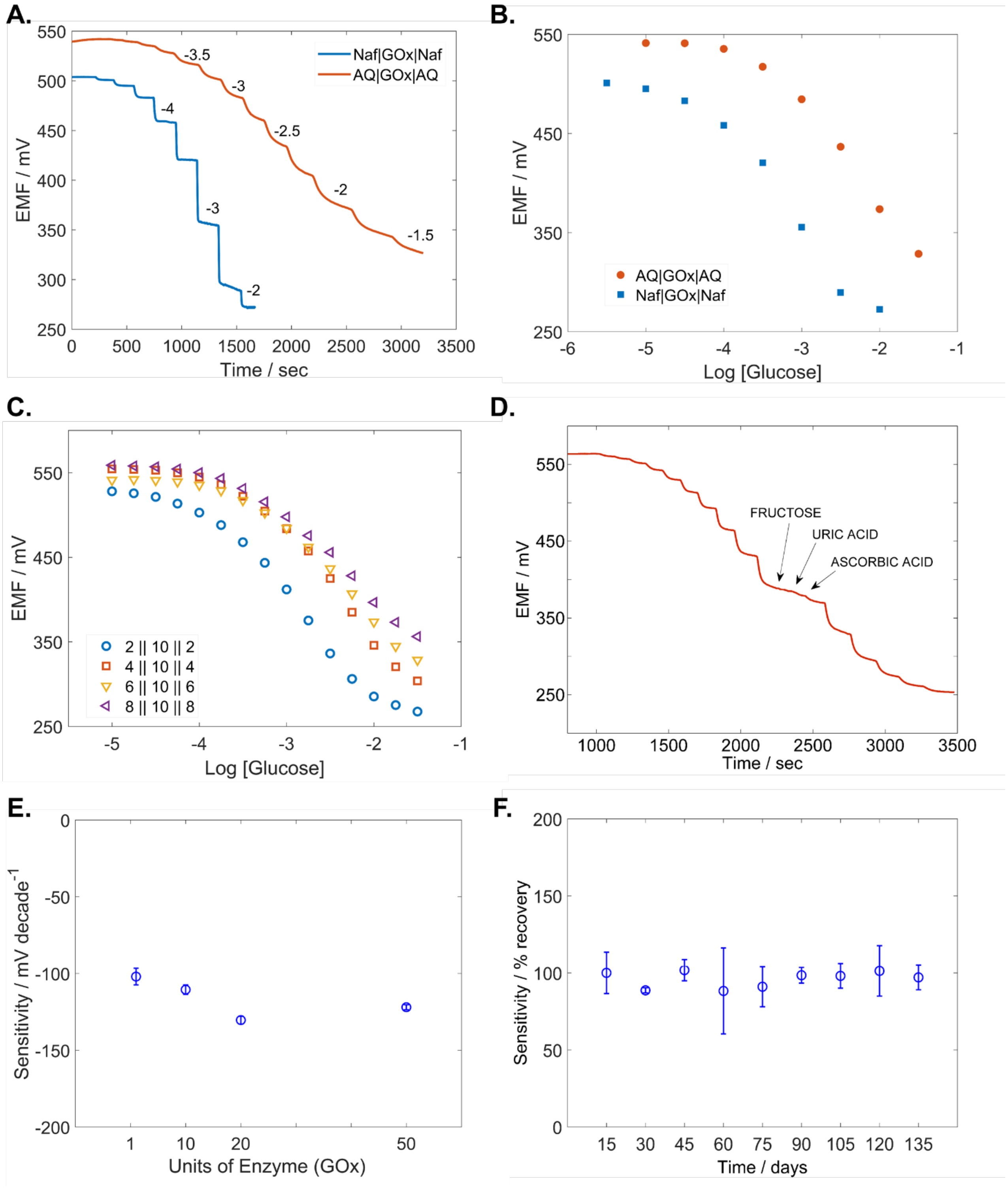


Figure 2. Comparison of the potentiometric response under different conditions and optimization of some analytical conditions of the biosensor. (A) Time trace and (B) calibration curve comparing the response to glucose of electrodes built with different polyelectrolytes Aquivion "AQ|GOx|AQ" (6 µL polymer cast, orange dots) and Nafion "Naf|GOx|Naf" (7 µL polymer cast, blue squares). (C) Sensitivity of Aquivion-based biosensors for glucose vs the volume of solution of polymer cast. (D) Assessment of the selectivity of the enzyme-based electrode to the main redox sensitive molecules (ascorbate and urate) and fructose. All the measurements (N = 3) were performed in 0.1 M PBS (pH 7.4) at 25ºC. (E) Sensitivity for glucose vs. Units of enzyme cast (fixed volume of 10 µL for enzymatic layer). (F) Stability study exhibits values for the recovery of the sensitivity along a period of more than 4 months. All the measurements (N = 3) were performed in 0.1 M PBS (pH 7.4) at 25ºC. EMF means Electromotive force.

3.4 Optimization of polymer coating

Figure 2C displays the results of the optimization of the thickness of the polymeric layer with different amounts of Aquivion drop cast, i.e., the same amount of the polymer sandwiching the enzyme: 2, 4, 6 and 8 μL. Interestingly, the sensitivity with Aquivion is comparable or slightly better than the value previously reported with Nafion. For an optimum volume of 4 μL, sensitivity of -133.5 ± 9.9 mV/dec glucose is obtained (Table S1). The most remarkable effect of changing the ionomer is the shift of the linear range, which is displaced towards higher concentrations with Aquivion. The linear range is a crucial factor for direct, point of need measurement. Thus, a sensor prepared with 4 μL of the ionomer was considered optimum, since it offers highest sensitivity within a suitable linear range. The upper limit of the linear range was moved one order of magnitude, from 0.3-3 mM with Nafion to 1-30 mM with Aquivion. This is of extreme importance since it now allows covering the clinical range of diabetes. Similar analytical parameters were also obtained with larger drop-cast volumes of the ionomer, but response times were unnecessarily increased.

Interestingly, an improvement on the selectivity was also observed, since the response to a 100 μM ascorbic acid solution was reduced from 30 mV for Nafion down to less than 10 mV for Aquivion. This is likely due to the higher coulombic repulsions between the more dense negative sulfonate groups. As the selectivity is crucial for direct real sample measurement, we run a selectivity test with the addition of other sugar such as fructose (no response due to the specificity of the enzyme), and redox species such as uric acid and ascorbic acid in a maximum concentration of 0.1 mM (Figure 2D) which confirmed the enhancement of the permselectivity of this membrane.

3.5 Optimization of enzyme loading and long-term stability

Figure 2E shows the optimization of the concentration of the glucose oxidase (GOx). Different amounts of enzyme (1, 10, 20 and 50 Units) were added to the sensors made with the optimized configuration of 4 μL for Aquivion. As an optimum sensitivity was obtained using 20 Units, this condition was selected for the next experiments (Table S2). Figure 2F exhibits the recovery of the sensitivity values (percentage of sensitivity at each time versus initial sensitivity) for a study during more than 4 months (Table S3). The stability of the electrodes was tested by running calibration curves of increasing concentrations of glucose in a 4 mL cell using PBS 0.1 M at 25 ºC as usual. More than 30 electrodes where prepared at the same time to run the stability test along the long-term period (a total of 27 electrodes where tested every two weeks, N=3 per calibration curve). The reproducibility between electrodes was kept unaltered during all this time. Some increments on the standard deviation were observed on some days, probably due to handling errors during the manufacturing of the electrodes. This result demonstrated the robustness of the developed sensor here so that it encourages us to undertake real sample measurements.

3.6 Measurement of Glucose in real samples

The previous experiments were performed in a 4 mL cell, using a commercial reference electrode. To further prove the validity of the results, a whole integrated potentiometric cell, i.e. paper-based working (WE) and a reference electrodes (RE) (Cánovas et al., 2017) were build and tested. Results are shown in Figure 3. The system resembles a previously develop paper-based cell using Nafion. Nevertheless, with the modifications introduced in this work through the use of Aquivion, prior dilutions were not necessary. The validation of the potentiometric cell was carried out using 15 blood samples provided by different volunteers. Prior to detection, the device was calibrated with three standards of glucose in artificial serum. In order to test the sensors in real conditions, a first calibration in artificial media was performed (Figure 3A, Table S4). As the mixed potential of the platinum coated electrode is known to be affected by several parameters, i.e. pH, ionic strength, convection etc (Meruva and Meyerhoff, 1996), it was not surprising to obtain slightly different analytical parameters when PBS and artificial serum were used for calibration (-117.7 ± 3.1 mV/dec in 0.5 – 10 mM range in artificial serum). The ionic strength of the media may be the main reason for this slight alteration: ionic strength of 0.18 and 0.56 for artificial serum and PBS respectively. Therefore, considering the clinical range of glucose in blood, the above-optimized conditions were suitable for the real sample measurements.

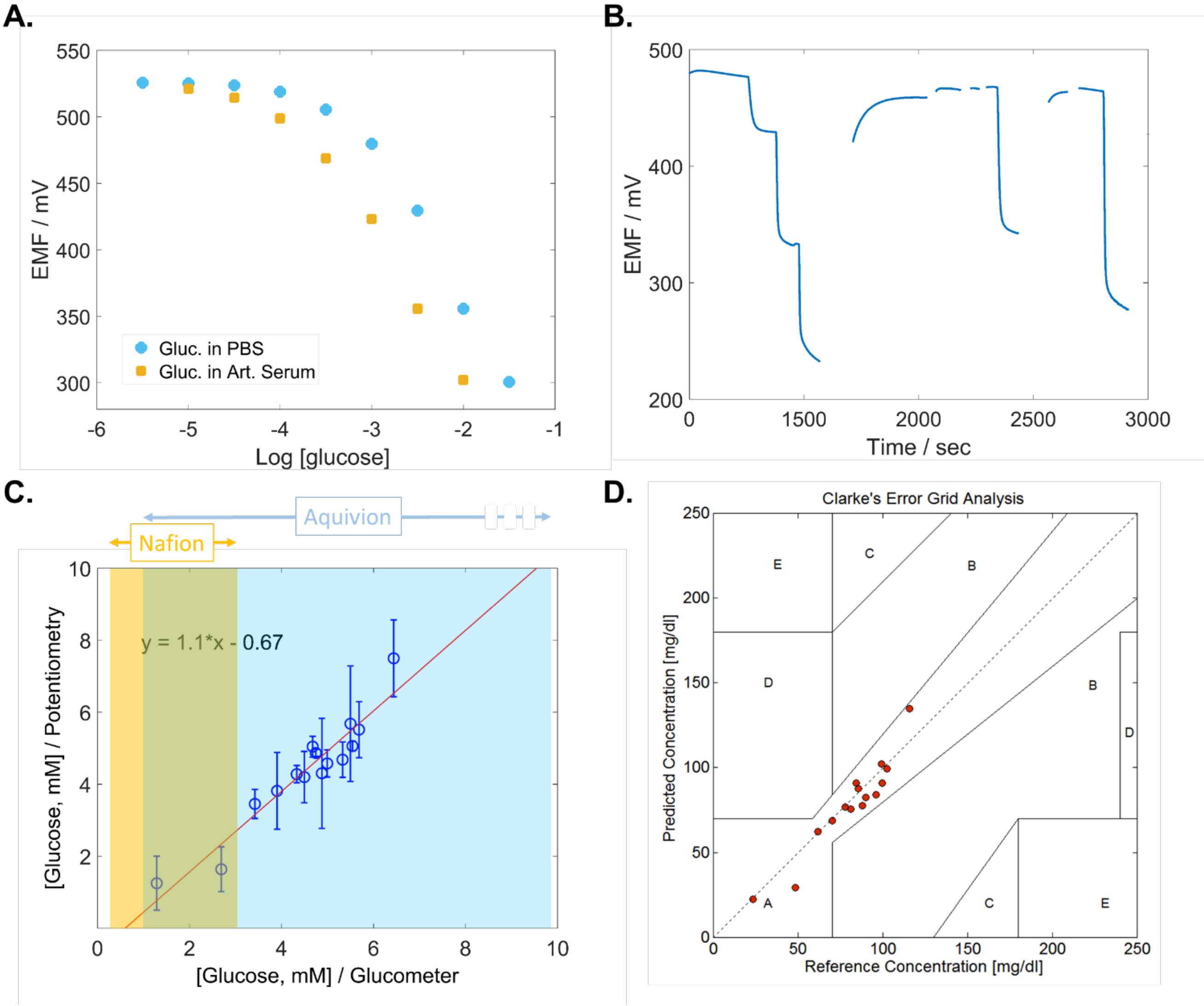


Figure 3. (A) Comparison of the potentiometric response to glucose using different matrices solution such as 0.1 M PBS (blue dots) and artificial serum (yellow squares). (B) Time trace corresponding with a previous three points calibration and two real whole blood samples measurement with washing steps in between. (C) Comparison of glucose determination (mM) in real blood samples obtained by the potentiometric biosensor (paper-based WE and paper-based RE, N=3) and glucometer device at 25ºC. (D) Representation of the well-established Clarke error grid.

Figure 3B exhibits the calibration performed with three different concentrations of glucose in artificial serum, washing steps to recover the signal, the measurement of glucose in a whole blood sample and a second recovery of the signal by washing to repeat the measurement with a different whole blood sample using the same sensor. Here, and in agreement with similar studies (Grygolowicz-Pawlak et al., 2013), it is important to highlight the lack of fouling and drift in complex matrix such as blood. Figure 3C displays the validation between 15 values of glucose obtained using a commercial glucometer versus our potentiometric biosensor (Table S5). Theses result represent a good linear correlation between both methods with excellent recoveries for the assays from blood samples (95.6 ± 12.2%, N=15). This indicates that the blood matrix has no significant effect on glucose determination. It should be mentioned that the same sensor (WE and RE) were used repeatedly for several predictions without changing their performance. This characteristic also indicates the high resilience of the sensors to biofouling. However, as it was discussed in previous studies, this type of sensors is designed to be disposable making them easier to handle and to avoid contamination between different biological samples. Figure 3D displays the values obtained in the validation in a standard Clarke error grid that is used to assess the clinical significance of differences between the glucose measurement technique under test (the experimental development) and the blood glucose reference measurements (in this approach, a commercial glucometer). The diagonal represents the good agreement between both, whereas the points below and above the line indicate, respectively, overestimation and underestimation of the actual values. This plot shows that all values are within zone A showing that the methodology is accurate enough to measure in blood samples for diabetes management (Kovatchev et al., 2004; Maran et al., 2002).

4. CONCLUSIONS

A novel approach for improving the analytical performance of a developed ultra-low-cost potentiometric biosensors was introduced. A platinized paper coated with a new polyelectrolyte matrix -Aquivion- has allowed the direct measurement of glucose in whole blood. The higher ion-exchange and water uptake capacity of this new polymer coating enhances transport properties into the system. As a result, the new sensor does not require any type of sample treatment or dilution, avoiding excessive manipulation, increasing the simplicity of the system, and thus, making it more user-friendly. Besides, an outstanding correlation with the commercial standard method was achieved. Further steps will pursue a calibration free potentiometric sensor as well as the extension of this approach to other oxidase-type enzymes. That will open a new avenue for the development of affordable, simple and robust platforms for decentralized biochemical analysis. All in all, enzymatic sensors are highlighted as a promising technology for highly efficient and economically competent biotechnological processes in several fields such as of environmental monitoring and security, early diagnosis, pharmaceutical and food industries.

Declaration of competing interest

The authors declare no conflict of interest

Credit authorship contribution statement

RC: Conceptualization, Methodology, Investigation, Writing - original draft. PB: Conceptualization, Investigation, Supervision, Writing - review & editing. FJA: Conceptualization, Supervision, Resources, Funding acquisition, Writing - review & editing.

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