Rafael Hoekstra

An Enzyme-based Potentiometric Redox Biosensor for Glucose

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Rafael Hoekstra
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rafael.hoekstra@estudiants.urv.cat

Supervisor: Francisco J. Andrade.
Department of Analytical Chemistry and Organic Chemistry. Universitat Rovira i Virgili (URV)
Campus Sescelades, c/. Marcel·lí Domingo, s/n Tarragona, 43007, Spain.

ABSTRACT

A potentiometric sensor for the determination of glucose at clinically relevant levels is presented. This approach uses an electropolymerized membrane containing glucose oxidase immobilized on gold nanoparticles deposited on platinum or platinized paper. The paper-based biosensor showed a linear dependence with the logarithm of the glucose concentration in the range $10^{-4.5}$ M to 10^{-3} M and a sensitivity of -44.5 ± 3.7 mV/log[glucose] in dilute artificial serum at pH 7.4. Under these conditions, a response time of 22 s and a limit of detection (LOD) of $10^{-4.9}$ M were obtained. Experimental evidence suggests that the working principle of this sensor is based on monitoring changes in the local redox potential produced as a result of the enzymatic reaction. This approach offers a simpler, and more cost-effective alternative to traditional amperometric-based glucometry.

INTRODUCTION

Historically, analytical chemistry and healthcare have evolved hand-in-hand as increasingly sophisticated analytical tools have emerged to improve the diagnosis and monitoring of diseases. Currently, a paradigm shift is taking place in certain areas of medicine to decentralize diagnosis to the point-of-care (POC) or even to the comfort of the one´s home.

This paradigm shift has largely been started and driven by the success story of managing diabetes mellitus, a serious, chronic disease that affects more than 382 million people worldwide, 80 % of whom live in low-income and middle-income countries.[1] Because its complications can best be mitigated through tight monitoring and management of blood glucose levels,[2] glucose has become the most commonly tested analyte,[2] most notably in portable self-monitoring of blood glucose devices or ¨glucometers¨. Thus, the glucometer has become the foremost tool for POC analysis and represents a model for other such devices.[3]

Since the first portable way to monitor glucose- the Dextrostix®- was invented in 1963,[4] a variety of glucometers have emerged and continued to improve in ease of use, accuracy and precision.[5] While early glucometers such as the Detrostix® were colorimetric, the current industry standards are electrochemical readers with plastic disposable test strips. Important advantages of electrochemical measurements are insensitivity to light and insoluble compounds, as well as the fact that readings are quantitative and therefore not easily misinterpreted. The quantitative readings are also highly compatible with decentralized healthcare through integration with mobile devices and communication networks.[6–10]

During the fifty years of research and development of glucometers, enzyme-based amperometric detection has demonstrated the best overall performance and

emerged as the leading approach in commercial devices.[2] This method is based on the detection of the hydrogen peroxide that is generated during the enzymatic conversion of glucose (GLU) to gluconic acid (GA) using glucose oxidase (GOx) (1).

$$GLU + O_2 \xrightarrow{GOx} GA + H_2O_2 \quad \textbf{(1)}$$

$$H_2O_2 \xrightarrow{\text{Pt electrode}} O_2 + 2H^+ + 2e^- \quad \textbf{(2)}$$

This system has significantly improved quality of life for millions of people. However, significant problems in precision and accuracy remain unsolved.[5] Furthermore, the system is invasive, requiring the lancing of blood and the increasing cost of test strips is prohibitive for many patients.[5]

Potentiometry is an alternative electrochemical technique that displays a high degree of simplicity, low-cost and excellent performance for the determination of ions in liquid samples.[11–13] Furthermore, this analytical tool is comparatively robust in terms of the physical and chemical environment in which it can reliably operate- an important feature for decentralized determination in complex biological matrices such as blood.[12,13] Although potentiometry is nowadays very popular because of the extensive use of ion-selective electrodes, a traditional application of this technique is the tracking of the redox potential using a platinum indicator electrode to monitor redox active species. In this way, the hydrogen peroxide generated in (1) could in principle be followed potentiometrically at a platinum electrode (2).

Ideally, platforms for POC sensors should be affordable, lightweight, flexible and versatile. Among the different candidates, paper satisfies all these conditions as well as having an established printing industry for rapid mass production. Recent reviews described numerous further advantageous properties of paper as a platform for sensors.[9,14] The Whitesides group has demonstrated use of paper as a platform for electrochemical devices incorporating enzymes in the determination of glucose,[6,7] cholesterol,[6] ethanol,[6] and lactose,[6] including integration with commercial glucometers.[6] These devices were based on chronoamperometry with inherent complexity, sensitivity to temperature and humidity and requirement of microfluidic wicking for the chromatographic separation of serum or blood components.[6,7] As suggested previously, potentiometry could help to overcome many of these issues. Indeed, Willander and coworkers have demonstrated enzyme-based potentiometry for a suite of biomolecules upon gold coated glass substrates.[15–21] Paper-based potentiometric sensors of ions using nanomaterials as transducers were recently reported by our group and shown to have similar performance to traditional devices.[11,12] To the best of my knowledge, the only report using paper as a substrate for an enzyme-based potentiometric sensor for non-ionic biomarkers has been produced by Eggenstein *et al,*[22] who incorporated urease into a polymeric membrane to generate ammonium from urea. This was determined by an underlying ion-selective membrane upon filter paper with a silver coating acting as conducting track. Thus, this method is limited to enzymes that generate ions. These electrochemical sensors are summarized in Table 1. No other paper-based potentiometric sensor for glucose has been reported until now.

While the glucometer is a prototype device for decentralized POC diagnostics, the tight management of blood glucose levels has still not been realized due to technical challenges.[2] For the same reasons, POC devices have yet to be commercialized for medical targets beyond glucose.

Table 1. Electrochemical sensors for biomarkers: Enzymatic paper-based sensors and potentiometric sensors.

Technique	Electrode Substrate	Analyte
Chronoamperometry (enzyme-based)	Graphite on paper	Glucose[6,7,15] Cholesterol[6] Lactate[6,15] Ethanol[6]
Potentiometry (Enzyme-based)	Au coated glass	Uric acid[15] Glucose[16] Uric acid[17] Lactic acid[18] Penicillin[19] Dopamine[20] Creatinine[21]
Potentiometry (ionophore-based)	Paper with CNTs	Li^{+}[12] K^{+}, $NH4^{+}$, H^{+}[11]
Potentiometry (Enzyme + ionophore)	Ag coated paper	Urea[22]

The simplified systems and lower costs of readers and sensors associated with potentiometry and paper are attractive for POC analytical devices. The range of analytes that can be selectively determined by potentiometry is also rapidly expanding from the ubiquitous pH meter to ion-selective electrodes (ISEs) and more recently to important small molecules such as serotonin[23], creatinine,[24] and dopamine.[25,26] The sensors for these targets respectively used molecularly imprinted polymers, ionophores, or sensitivity to the target's polarity. This work describes a paper-based potentiometric glucose biosensor that operates on a different working principle- the monitoring of redox potential- with the vision that this sensing system could be extended to other enzymatically-recognized biomarkers. Optimization of the sensor construction and performance, together with the analytical figures of merit for the determination of glucose in artificial serum at clinically relevant levels is provided. Potential applications of this novel platform are discussed.

EXPERIMENTAL

Reagents

Medium molecular weight chitosan (78-85 % deacetylated) and Poly(vinyl alcohol) (M_w 89,000-98,000) were purchased from Sigma-Aldrich. Glucose oxidase from Aspergillus niger (228,253 U/g) and D-(+)-glucose were also purchased from Sigma-Aldrich. Gold nanoparticles (AuNPs) (35-45 nm) were synthesized using Frens´ method by adding 0.5 mL of sodium citrate solution (1% w/w) to 50 mL of boiling chloroauric acid (0.01% w/w) and stirring.[27]

All solutions were prepared with doubly distilled water (resistance of18.2 MΩ cm^{-1}) from a Milli-Q water system (Millipore Corporation, Bedford, MA). Phosphate buffer saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, and 1.8 mM $KHPO_4$ was used for all experiments unless otherwise stated. Artificial serum was prepared with 112 mM NaCl, 30 mM $NaHCO_3$, 2.2 mM K_2HPO_4, 0.8 mM $MgCl_2$, and 2.6 mM urea in milli-Q water.[28] The serum solution was used diluted 20 times unless otherwise stated. All of these analytical-grade salts were also obtained from Sigma-Aldrich.

Instrumentation and measurements

Platinum sputtering was performed with an ATC Orion 8-HV from AJA International Inc. (MA, USA) at 150 W, 3 mTorr, 2.97 e^{-8} kgs^{-1} Argon as RF magnetron source. Electropolymerization was performed using a CHI660C Electrochemical Workstation from CH Instruments, Inc. (Austin, U.S.A.) using a platinum wire as counter electrode, a single-junction 3M Ag/AgCl/KCl (type 6.0726.100) from Metrohm AG. (Herisau, Switzerland) as a reference electrode, and either platinum wire or platinized paper as a working electrode. Solid-state platinum electrodes were spun using a Dremel300 Workstation from Dremel Europe (Breda, the Netherlands).

Electrochemical impedance spectroscopy (EIS) was performed using a CHI660C Electrochemical Workstation from CH Instruments, Inc. (Austin, USA) using a platinum wire as a counter electrode, a single-junction 3M Ag/AgCl/KCl (type 6.0726.100) from Metrohm AG. (Herisau, Switzerland) as a reference electrode, and platinized paper as a working electrode.

Electromotive force (EMF) was measured with a high input impedance (10^{15} Ω) EMF16 multichannel data acquisition device form Lawson Laboratories, Inc., (Malvern, USA). EMF measurements were recorded in stirred solutions at room temperature (26 ± 4 ^{o}C) unless otherwise stated while making successive additions of analyte. Double-junction 3M Ag/AgCl/KCl (type 6.0726.100) reference electrodes from Metrohm AG containing 1M LiOAc as ionic salt bridges were used for all potentiometric measurements.

Environmental scanning electron microscopy (ESEM) was performed with a Quanta 200 (FEI, Oregon, USA). Secondary electron imaging was carried out at 15 kV at high vacuum (1.17 e^{-4} Torr) or low vacuum (6.85 e^{-1} Torr) as specified.

Glucose Biosensor construction

Sensors were constructed both on solid-state platinum electrodes and platinized paper electrodes. Solid-state platinum electrodes were wires (1 mm diameter) encased by PTFE (known as Teflon) such that a flat circular surface of the 1 mm diameter wire was in contact with solutions. Platinized paper electrodes were made from number 5 Whatman™ filter papers (GE Healthcare Life Sciences) sputtered on one side with platinum to apply a layer of approximately 100 nm. Sensors were constructed as described elsewhere for paper ion-selective electrodes.[11] In brief, 0.5 x 1.5 cm strips of platinized paper were sandwiched and glued between plastic masks with a 2.8 mm diameter circular window exposing the platinized surface (Figure 1A). The plastic mask was 0.13 mm thick polyester with acrylic adhesive on one side (ARcare 8259, Adhesives Research, Inc. Limerick, Ireland). Chitosan solution was prepared by stirring 1 g of chitosan flakes in acetic acid (1 % wt.) and stirring until the solution was clear. Poly(vinyl alcohol) (PVA) solution was prepared by stirring 0.5 mg of PVA in Milli-Q water at 80 ^{o}C until solution was clear. The membrane cocktail was prepared by dissolving 3.9 mg GOx in 1 mL of PVA solution. This mixture was added dropwise to 0.5 mL AuNP solution while swirling. Finally, 2.5 mL of chitosan solution was added and the cocktail shaken thoroughly.

A membrane was electropolymerized upon the platinum surfaces by applying a current of 25 µA across this cocktail (solid-state platinum: 100 s; platinized paper: 300 s) (Figure 1B). Excess cocktail was removed from solid-state platinum electrodes by spinning at 10,000 rpm for 1 minute. Electrodes required conditioning in PBS (approximately 6 h stabilization time) before being ready for use as glucose biosensors.

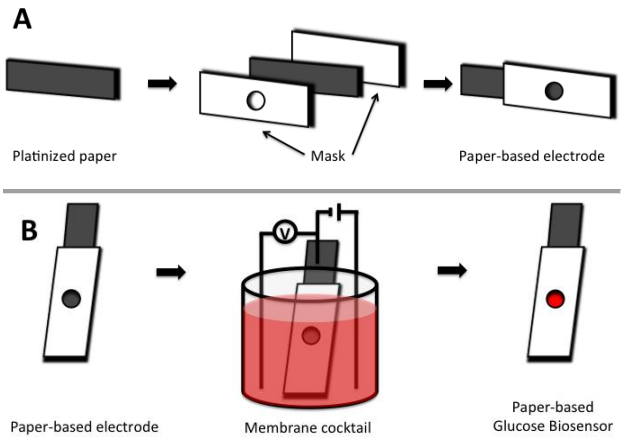

Figure 1. A: Construction of the paper-based electrode by sandwiching platinized paper between plastic masks. B: Preparation of the glucose biosensor by electropolymerization of the membrane cocktail upon the paper-based electrode.

RESULTS AND DISCUSSION

Development and performance of glucose biosensors

Glucose biosensing membranes were first developed and optimized upon solid-state platinum electrodes. Performance was studied in PBS at physiological pH of 7.4 at room temperature (unless otherwise stated) with the motivation of developing a sensor that is viable for real-world decentralized applications. Chitosan has been reported to entrap GOx upon electropolymerization.[29] In our study, it was observed that the inclusion of PVA increased the reproducibility of the sensors´ performance. The film-forming, adhesive, and swellable properties of PVA are probably responsible for this. Both spin-casting and drop-casting approaches were investigated for membrane deposition, however electropolymerization resulted in greater reproducibility between sensors. The consistent potentials required for electropolymerization at the stated conditions reflect the reproducibility of membranes formed (Table 2). Of these, the potential required to generate a current of 25 µA was an order of magnitude lower in the case of the paper-based electrode. This is probably due to the highly conductive, pristine nature of the freshly sputtered platinum surface. The longer time required to achieve a stable electropolymerization potential on the platinized paper could be due to its much greater surface area. Electrochemical impedance spectroscopy studies showed no significant difference before and after electropolymerization, which suggests that membranes were very thin and may not completely isolate the electrode substrate (Pt) from the solution. The glucose biosensor´s (GBs) potentiometric response to glucose was linear with the logarithm of the concentration of the analyte as is usually observed in potentiometric ion-selective electrodes.[30] However, the narrow concentration range of potential change was more resemblant of a potentiometric titration curve with an inflection point at approximately $10^{-3.75}$ M of glucose. The range surrounding this point from $10^{-4.5}$ M to 10^{-3} M was linear with a sensitivity of 40.2 ± 1.5 mV/log[glucose] (n =3) (Figure 2). The LOD obtained was approximately 10^{-5} M.

Table 2. Electropolymerization potentials.

	Solid-state electrode	Paper-based electrode
Electropolymerization potential (V)	-1.02 ± 0.03 (n=3)	-0.10 ± 0.04 (n=5)

The response was established as being contingent upon GOx by comparing the GB to a bare platinum electrode and a platinum electrode with the same membrane omitting the enzyme (Figure 3).

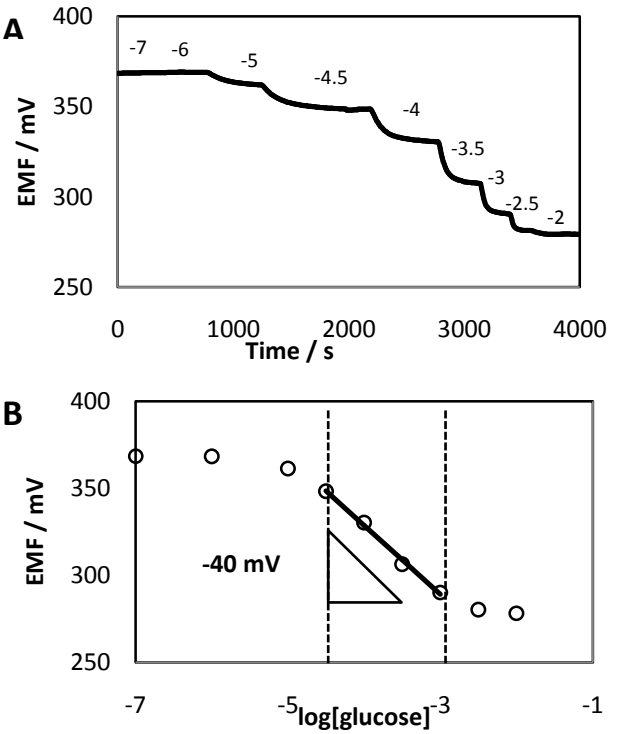

Figure 2. Potentiometric response of the GB to glucose. A: Time trace; and B: Calibration plot. Numbers in the time trace refer to the log of glucose concentration. Dashed lines indicate the linear range.

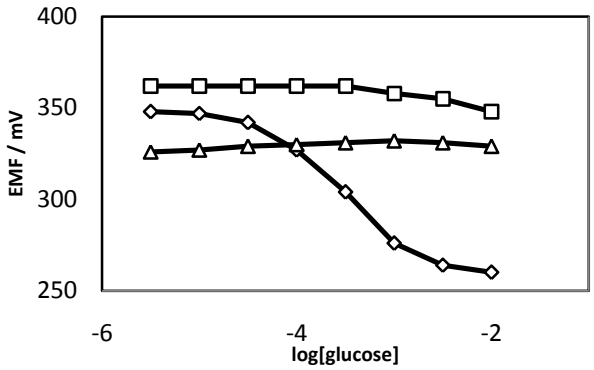

Figure 3. The potentiometric response to glucose of: (◊) the solid-state GB; (Δ) a bare platinum electrode; and (□) the enzyme-free membrane on a platinum electrode.

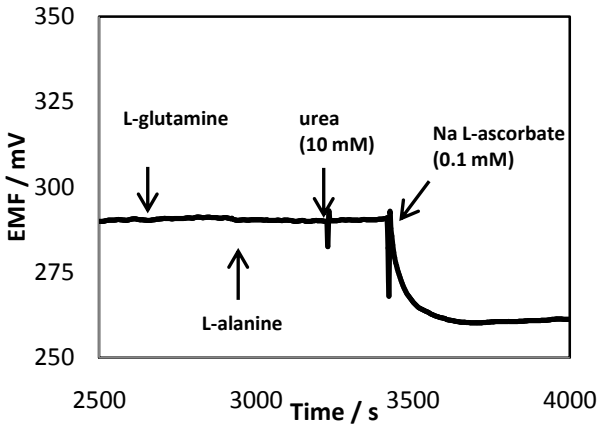

Figure 4. EMF response of the GB to four common interfering compounds (no glucose present).

Within the established working range, the bare platinum electrode exhibited a sensitivity of 2 mV/log[glucose] and the electrode without enzyme a sensitivity of -2.4 mV/log[glucose]. The GB in this experiment showed a sensitivity of -44.2 mV/log[glucose].

The GB showed higher sensitivity at 40 ^{o}C, although reproducibility among sensors was poor (Table 3). This increased sensitivity might be due in part to more optimal activity of GOx at this temperature,[31] however additional experiments will be necessary to evaluate all the factors affecting these changes. Given the poor reproducibility and moreover the aim of developing a sensor for real-world applications, further studies were carried out at ambient temperature.

Interferences study

The influence of four common interfering compounds was evaluated in PBS without the presence of glucose (Figure 4). Of these, L-glutamine, L-alanine and urea did not affect the baseline potential. Sodium L-ascorbate however, demonstrated significant interference. It is probable that the reducing nature of ascorbate is responsible for this response. As serum concentration of ascorbate does not exceed 0.9 mM due to the renal resorption threshold,[32] this interference could likely be overcome by dilution of the sample.

Table 3. Sensitivity of the GB to temperature.

	(27 ^{o}C)	(40 ^{o}C)
Sensitivity (mV/log[glucose])	-40.3 ±1.5 (n=3)	-52.7 ±10.7 (n=3)

Paper electrodes

To move towards disposable low-cost sensors, the membrane was also electropolymerized upon platinized paper electrodes. Imaging by ESEM showed homogeneous platinization of the fibrous paper surface (Figure 5A). Following electropolymerization, the membrane was imaged at low vacuum due to the low conductivity of this surface (Figure 5B).

These sensors showed a similar sensitivity of -41.7 ± 0.9 mV/log[glucose] (n=3) within the same linear range as those upon solid-state platinum. Response times varied between 200 and 600 s. The improved reproducibility of the paper-based GBs' sensitivity may be due to the increased surface area both in the size of the circular platinized area and in the three dimensional topography

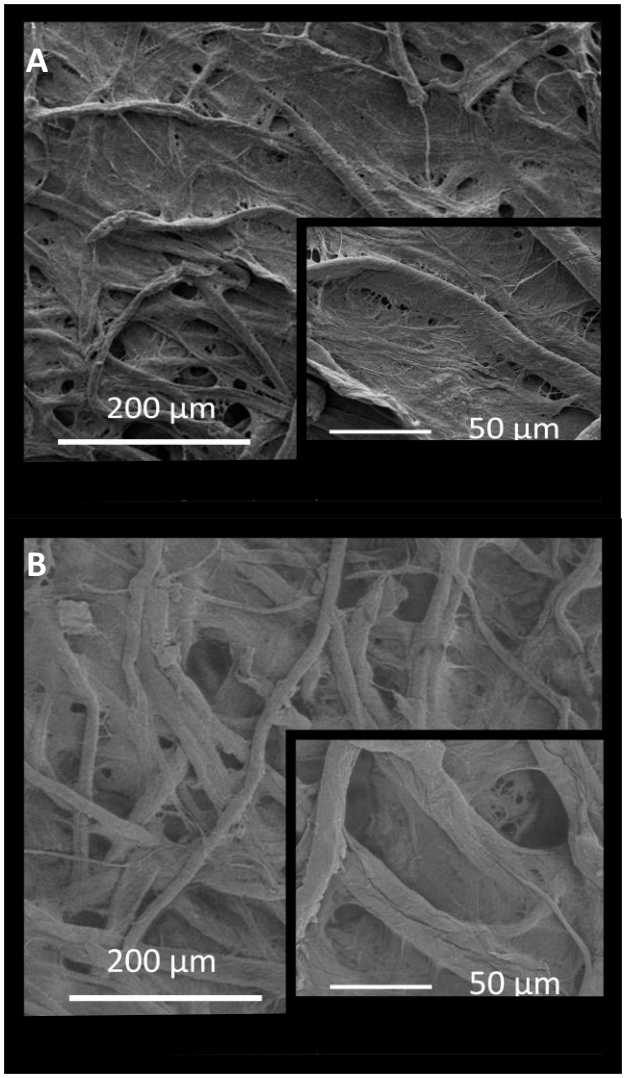

Figure 5. Secondary electron ESEM images of A: the platinized paper surface (at high vacuum); and B: the platinized paper surface with the glucose biosensing membrane (at low vacuum).

of the fibrous paper surface (Figure 5). Another factor is that the sputtered platinum is likely cleaner and more pristine than the platinum wires.

Figure 6 plots the EMF values obtained by calibrating the two types of sensors (solid-state and paper-based) within the glucose concentration range of 10^{-6} M to 10^{-2} M. This demonstrates the transfer of the biosensor from solid-state electrodes to paper-based electrodes.

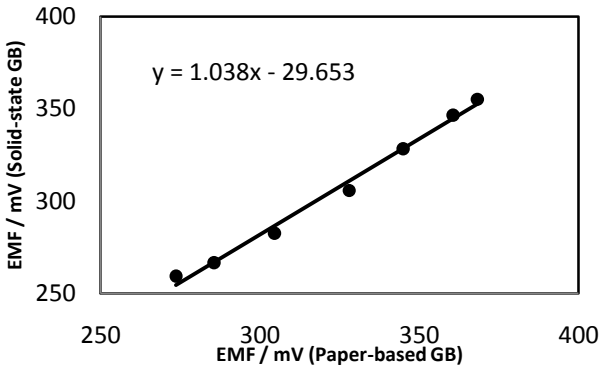

Figure 6. Comparison of EMF values of solid-state GBs and paper-based GBs for glucose concentrations between 10^{-6} M to 10^{-2} M.

As discussed, the paper platform has many advantages for use in disposable sensors. A comparison can be made between this consistent behavior of solid-state platinum sensors and platinized paper-based sensors with results previously reported of ion-selective membranes upon glassy carbon electrodes and single-walled carbon nanotube ink painted paper electrodes.[11,12] Although paper-based sensors only need to be used once before disposal, a degree of reusability is useful for calibration. The paper-based GBs showed same day reusability, after which performance deteriorated.

Performance in artificial serum

As a first step towards validating the paper-based glucose biosensor in real serum samples, it was calibrated in artificial serum diluted 20x. This dilution was made primarily to correlate the sensor´s linear range with the range of glucose found in human serum (3-20 mM), but also to avoid interferences such as that observed from ascorbate. The sensor showed a sensitivity of -44.5 ± 3.7 mV/log[glucose] (n=3) a LOD of $10^{-4.9}$ M and a response time of 22 s in this media. This rapid response is attributed to the low ionic strength of 11 mM (the ionic strength of the PBS used in previous experiments was 211 mM).

Table 4. Performance of paper-based GBs with and without gold nanoparticles.

	GB with AuNPS	GB without AuNPs
Sensitivity (mV/log[glucose])	-41.3 ± 1.3 (n = 3)	-40.5 ± 5.3 (n = 3)

Further studies are required to evaluate this effect conclusively, although the influence of the ionic strength on enzymatic activity is likely to be involved. Although the viability of the sensor within artificial serum is a positive result within the laboratory, the dilution step is impractical for self-monitoring of blood glucose by patients.

Effect of Gold nanoparticles

Gold nanoparticles (AuNPs) were initially included in the membrane cocktail as they are well known to: a) immobilize enzymes in a microenvironment which supports their activity; b) facilitate electron transfer to electrode surfaces; c) provide an enhanced surface area for both immobilization and signal transduction; and d) exhibit electrocatalytic activity.[33] Control experiments showed that AuNPs increased the sensors sensitivity marginally.

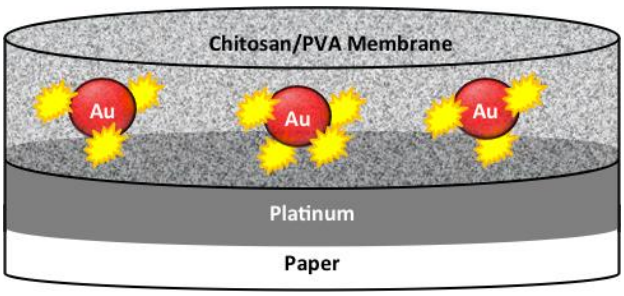

Figure 7. Diagram of the paper-based glucose biosensor. AuNPs and GOx are embedded in the electropolymerized chitosan/PVA matrix upon platinized paper.

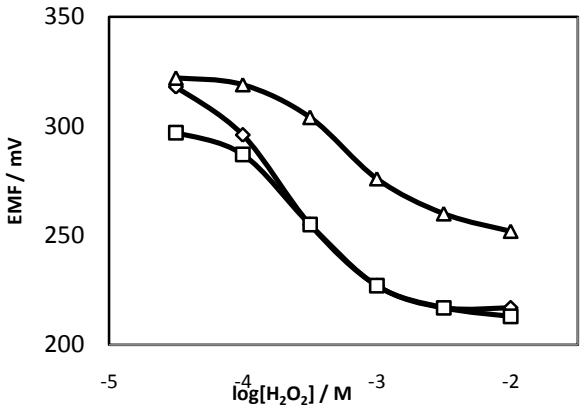

Figure 8. Potentiometric response to hydrogen peroxide. at: (◊) the solid-state GB; (Δ) a bare platinum electrode; (□) the enzyme-free membrane on a platinum electrode.

More significant was the improved reproducibility among sensors when they were included (Table 4). The evidence suggests that the scaffolding provided for enzyme immobilization is probably the most important contribution of the AuNPs in these sensors.

Mechanism of Sensing

Figure 7 illustrates the composition of the GB. The AuNPs and GOx are embedded within the chitosan/PVA matrix that was electropolymerized upon the platinized paper surface.

The interference of the reducing agent sodium L-ascorbate offers a hint that this sensor is redox sensitive. The calibration plots are also consistent with a redox process being monitored at the platinum electrode. Specifically, they resemble the plot of hydrogen peroxide titration shown in Figure 8. The conditions of this experiment were identical to previous calibrations, except that hydrogen peroxide was added instead of glucose. The potentiometric response recorded at the GB was very similar to that of an enzyme-free membrane and indeed that of a bare platinum electrode (Figure 8). These results show that the platinum

electrode might be responding to hydrogen peroxide. The local generation of H_2O_2 in the GB membrane by GOx however, makes the response selective to glucose as illustrated in Figure 3. It is known that the response of platinum to H_2O_2 is pH dependant,[34] thus it must be kept in mind that an acidic environment is being generated within the membrane due to the production of GA.

It has not been established which redox couple is being sensed at the electrode surface, though the similar titration curve of hydrogen peroxide upon a bare platinum electrode might suggest that the species´ involved are a combination of:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \qquad E^o = 0.67\ V \qquad \textbf{(2)}$$

$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O \qquad E^o = 1.78\ V \qquad \textbf{(3)}$$

Both oxidation and reduction of hydrogen peroxide have been reported to occur due to local pH gradients between $3 < pH < 6$.[35] Katsouranos *et al.* reported that adsorbed OH species were produced when H_2O_2 interacted with reduced platinum surface sites in a third possible contributing process, which is a chemical step:

$$2Pt + H_2O_2 \rightarrow 2Pt(OH) \qquad \textbf{(4)}^{36}$$

The proposed mechanism presents a means of 'tuning' the response in terms of linear range and sensitivity by adjusting the redox environment through selection of the membrane´s functional groups.

Evidently, these are so far speculations, since the membrane-platinum interface is complex and might be undergoing several modifications as the local pH and redox potential change. Further studies would be necessary to elucidate the actual mechanisms of this device.

In summary, this sensor utilizes GOx as the biological recognition element for glucose and AuNPs to increase the sensor's reproducibility. Finally, the evidence suggests that the platinum surface both recognizes the redox couple mediator and transduces the potentiometric signal.

CONCLUSIONS

The enzyme-based potentiometric redox biosensors show reproducible sensitivity for determination of glucose within diluted artificial serum. The GBs showed an increased sensitivity at elevated temperature and a faster response time at lower ionic strength. Performance of paper-based sensors was slightly better than solid-state sensors.

This work has demonstrated the possibility of using a simple enzymatic reaction to potentiometrically monitor an important biomolecule at clinically relevant levels. There are two extremely attractive features that must be stressed. Firstly, that it can be easily built as a low-cost sensor without sacrificing the analytical performance. Secondly, it opens the way to use a plethora of other oxidase enzymes to detect alternative species. Thus, in principle, this work is a proof of concept for a wider analytical platform that could be used in the future for decentralized chemical measurements.

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