Paper-based potentiometric biosensor for monitoring Galactose in whole blood

Mohamed Bouri, Julio C. Zuaznabar-Gardona[a], Marta Novell, Pascal Blondeau, Francisco J. Andrade*

[a] current address: Nanobiotechnology & Bioanalysis Group, Departament d'Enginyeria Química, Universitat Rovira i Virgili, Avinguda Països Catalans 26, 43007 Tarragona, Spain

Department of Analytical Chemistry and Organic Chemistry,

Universitat Rovira i Virgili, C/Marcel lí Domingo, 1. Tarragona, 43007, Spain.

*e-mail:franciscojavier.andrade@urv.cat

Abstract

A filter paper sputtered with a layer of Pt and subsequently coated with a Nafion® membrane is used as working electrode. The mixed potential of the Pt electrode allows the detection of H_2O_2 generated by the oxidation of galactose in the presence of the galactose oxidase enzyme. This provides a simple and mediator-free approach method. The system shows sensitivity values of -62.8 ± 9.4 mV/decade of galactose in the range from 0.3 to 31.6 mM, well within the clinical relevant range. MnO_2 nanoparticles were added to decrease the interference from ascorbic acid so that validation of the sensor in whole blood samples was performed with good recovery.

Keywords

Galactosemia, potentiometry, paper-based platform, biosensor, mixed potential

1. Introduction

Galactosemia is a metabolic disorder where the body is unable to transform galactose into glucose [1]. This condition is caused by a deficiency of one or more of the three enzymes (viz. galactose-1-phosphate uridyl transferase, galactokinase and galactose-6-phosphate epimerase) intervening in the galactose metabolism through the Leloir pathway [2]. There are three types of galactosemia, referred to as classic galactosemia (galactosemia type I), galactokinase deficiency (galactosemia type II), or galactose epimerase deficiency (galactosemia type III) [3,4]. Classic galactosemia is due to galactose-1-phosphate uridyl transferase deficiency and is the most common and sever form of the disease[5]. While it is not a common disease, with a typical prevalence in the order of 1:40.000, if undetected, it may have a very negative impact, especially during the first years of the children development. Newborns with either unrecognized or untreated classic galactosemia develop cataracts, liver diseases and kidney problems [6]. Furthermore, high galactose levels in blood (> 1 mM) can cause brain damage leading to newborn death during the first days of life [7–9]. Early detection of galactosemia can prevent developmental disorders and occasional infant deaths. For this reason, in many developed countries it is mandatory to carry out a diagnostic screening of all newborns [10]. However, in most countries with limited healthcare resources, neonatal mass screening programs such as galactosemia are either delayed or ignored.

Diagnosis of galactosemia is commonly performed with methods that are based on either the measurements of galactose or galactose-1-phosphate levels. Alternatively, the activity of 1-phosphate uridyl transferase in blood of newborns is carried out [3]. These methods are based on microbiological tests or enzymatic assays [11]. Among the most representative microbiological tests are the Paigen test and the biological inhibition test [12,13], which are time consuming, require special cultures for incubation (16−20 h), are sensitive to antibiotic treatments and are not suitable for automation. Enzymatic assays that are also used for galactosemia diagnosis include the Beutler's test [14] and the alkaline phosphatase-galactose dehydrogenase assay [15].

However, these assays require special reagents with limited stability, involve multistep sample preparation procedures and are time-consuming (about 3 hours). For these reasons, all current galactosemia diagnostic assays are performed in highly specialized laboratories by skilled personal [9].

Thus, developing an easy-to-use sensing device for the diagnosis of galactosemia may have a significant impact. Additionally, since the treatment of galactosemia consists on avoiding foods that contain galactose in the diet and controlling its blood levels, it is also attractive to develop new, low-cost tools to control galactose levels either in blood or in food outside of labs for dietary management of galactosemia patients.

Electrochemical biosensors are an interesting alternative to develop novel and easily handling tools for outside of the lab diagnosis of different diseases [16]. They effectively combine a high selectivity and sensitivity with portable instrumentation for a simple and low cost diagnosis. Two general approaches have been used to quantify galactose on samples by electrochemical measurements: amperometry and potentiometry. Both methods rely on the measurement of the hydrogen peroxide generated by the oxidation of galactose with the galactose oxidase (GALOx) specific enzyme [8,17].

In the case of amperometric detection, the galactose is quantified by the measurement of the current produced by the oxidation of hydrogen peroxide. However, the results of this procedure are prone to interferences (ascorbic, citric and uric acid) coexisting in biological samples. Different strategies have been used to overcome this issue such as the use of redox mediators to decouple the oxidation of the hydrogen peroxide and the interferences [18] and the use of cyanometallates with peroxidase-like activity that allow the detection of hydrogen peroxide at lower potentials [19]. Additionally, nanomaterials such as metallic nanoparticles (NPs) and carbon nanostructures have been used in order to obtain a faster response time and to avoid intermediate reactions and interferences [20,21]. Regarding the potentiometric detection, there is -to the best of our knowledge- only one report of a biosensor for galactose [22]. GALOx was

immobilized on the surface of ZnO nanorods grown on gold coated glass electrodes. It is claimed that the resulting biosensor responds to the change on local amount of H_3O^+ ions in the vicinity of the electrode resulting from the enzymatic reaction which is correlated with the galactose concentration. However, this potentiometric biosensor requires the thermal growing of ZnO nanorods which makes it unpractical for mass-production.

During recent years, there has been significant work devoted to the development of paper-based sensing platforms. This approach is attractive because it combines many of the inherent properties of paper, compatibility with biological entities and low-cost of manufacturing, among others [23,24]. Our group has pioneered the development of paper-based ion-selective potentiometric sensors [25]. More recently, we have introduced a novel approach for the potentiometric detection of hydrogen peroxide using a platinized paper-based sensor coated with a layer of Nafion®. This approach has been later used for the development of an enzymatic glucose sensors [26,27]. The Nafion® coating indeed stabilizes the potential of the platinized paper and allows for the detection of glucose in blood with enhanced sensitivity [28,29]. We have attributed this enhancement of performance as the stabilization of the mixed potential generated at the platinum electrode interface and the recovery of the original Tafel sensitivity [30].

The present work presents the development of simple, low cost and disposable paper-based potentiometric sensor for the detection galactose in whole blood. The sensor construction, optimization and characterization of the analytical parameters are presented. The detection of galactose in whole blood is demonstrated, showing that this platform may become a valuable tool for galactosemia diagnostics

2. Experimental

2.1. Reagents and materials

Whatman® Grade 5 qualitative filter paper, Nafion® 117 solution (ca. 5% solution in mixture of lower aliphatic alcohols and water), chitosan with 75–85% of deacetylation, and galactose oxidase (GALOx) from Dactylium dendroides (3000 U/g) were purchased from Sigma-Aldrich. Analytical grade salts: potassium hydrogen phosphate (K_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), manganese (II) acetate ($Mn(Ac)_2$), potassium permanganate ($KMnO_4$), sodium chloride (NaCl), sodium hydrogen carbonate ($NaHCO_3$), sodium citrate (Na_3Cit), sodium lactate (NaLac) and ascorbic acid (AA) were purchased from Sigma-Aldrich. BioXtra grade D-(+)-galactose and a commercial galactose enzymatic assay kit (cat. MAK012-1KT) were also purchased from Sigma-Aldrich. Polyvinyl butyral (PVB, Butvar B-98) was sourced from Quimidroga S.A. (Barcelona, Spain). Ag/AgCl ink (113-09) was purchased from Creative Materials, Inc., MA, USA.

0.1 M Phosphate buffer saline (PBS) was prepared and used in all the experiments. The artificial serum used here was based only on the main ions present in body fluids and was prepared with 111 mM NaCl, 29 mM $NaHCO_3$, 2.2 mM K_2HPO_4. pH was adjusted with 1 M NaOH or 1 M HCl solutions. All solutions were prepared using 18.2 MΩ cm^{-1} double deionised water (Milli-Q water systems, Merck Millipore).

2.2. Instrumentation and measurements

Platinum sputtering was performed using a radiofrequency sputtering process (ATC Orion 8-HV, AJA International) operated at 3 mTorr, for 65 s at 200 W. Filter paper strips were placed inside the sputtering chamber to generate platinized paper electrodes with a 100 nm Pt layer.

Potentiometric measurements were performed using a standard two-electrode (i.e., working and reference) cell configuration, using the platinized paper as a working electrode and a commercial reference electrode in a 4 mL cell in 0.1 M PBS (pH 7.4) or artificial serum (pH 7.4) at 25 °C. A double junction Ag/AgCl/KCl 3 M reference electrode (type 6.0726.100, Metrohm AG) containing 1 M of lithium acetate was used in all the experiments. Electromotive force (EMF)

high input impedance EMF16 multichannel data acquisition device from Lawson laboratories, Inc. (Malvern, USA) was used to record the potential.

2.3. Synthesis of MnO_2 nanoparticles

Preparation of MnO_2 nanoparticles (MnO_2 NPs) was conducted following a procedure descried in the literature [31]. In short, diluted aqueous solutions of manganese (II) acetate and potassium permanganate (Eq. 1) were mixed allowing the following reaction:

$$3Mn(Ac)_2(aq) + 2KMnO_4(aq) + 2H_2O \rightarrow 5MnO_2(s) + 2KAc(aq) + 4HAc(aq) \quad \text{(Eq. 1)}$$

Briefly, 250 mL of $KMnO_4$ (25 mM) were added drop wise (about 1 drop per second) to 250 mL of $Mn(Ac)_2$ (37.5 mM) under vigorous stirring at room temperature to allow the reaction to take place. Then, the suspension with MnO_2 NPs was vacuum filtered using 934-AH Whatman® glass microfiber filter. The remaining brown slurry was washed three times with 40 mL of distilled water and then dried at 100 °C for 1 hour.

2.4. Fabrication of the galactose paper-based potentiometric cell

For the fabrication of the working electrode a qualitative number 5 Whatman® filter paper was sputtered on one side with platinum to deposit a layer of approximately 100 nm thickness. For the reference electrode, a filter paper was first painted with a conductive Ag/AgCl ink and cured for 10 minutes at 90 °C. These treated papers were then cut into 0.5 x 2 cm strips and use as the base to build the electrodes, as described elsewhere [32].

To build the miniaturised cell, the working and reference electrodes were placed between a bottom 1.5 cm x 2 cm polyester mask strip and top 1.5 cm x 1.5 cm polyester mask strip containing two circular windows of 3 mm diameter to expose the conductive paper (Figure 1). In this circular windows, working and reference membranes were placed as follows: for the

working electrode, a volume of 7 µL of Nafion® 2.5% solution in 2-propanol:H_2O mixture (1:1 v/v) was drop casted over the platinized paper and dried at room temperature for one hour. Then, the enzyme layer was formed by depositing appropriate amounts of GALOx dissolved in 0.1 M PBS pH=7.0 and left to dry overnight at 4 °C. Finally, 5-µL of 1% Chitosan in 1% wt acetic acid were drop cast on the top of the enzyme layer (Figure 1).

For the reference electrode, a total of 9 µL of a reference membrane consisting of 78 mg PVB and 50 mg NaCl in 1 mL of methanol were drop casted (3 aliquots of 3 µL each, with 10-min drying at room temperature in between) and left to dry overnight. Thereafter, a first (and only) conditioning step of 8 h in 3 M KCl left the reference electrode ready for use. The resulting miniaturised galactose cell was kept at 4 °C when not in use.

2.5. Analysis of real samples

Serum and blood samples of patients used for validation of this method were obtained in a local hospital (Hospital Joan XXIII, Tarragona, Spain). The measurement of galactose levels in serum and blood samples were carried out by using 2 mL of each sample, under agitation, and by adding different concentrations of galactose. All experiments were performed in compliance with the relevant laws and institutional guidelines.

2.6. Enzymatic assay

As a reference method, a commercial galactose enzymatic assay kit (Sigma-Aldrich, MAK012-1KT) was used. The analytical procedure was performed in Nunc® 96-well poly(propylene) plates (Thermo Fisher Scientific, Spain) according to the manufacturer's instructions. Colorimetric measurements were recorded at 570 nm using a multiplate reader Wallac Victor2 1420 Multilabel counter (Perkin-Elmer, USA) (Supplementary Information).

3. Results and discussion

3.1. Electrode response and principle of detection

In previous works, we have shown that a platinized paper electrode coated with a layer of an ionomer, such as Nafion®, can be used as a very sensitive and selective potentiometric sensor for hydrogen peroxide and –upon incorporation of glucose oxidase- also for glucose [26]. The working principle of this system, based on a mixed potential mechanism, has been described in more detail elsewhere [30]. In short, in an aqueous solution that lacks of a well-defined redox couple, the open circuit potential (OCP) of a Pt electrode is determined by the anodic and cathodic exchange currents produced by the surface redox reactions of different solution components. The electrode gains electrons through oxidation (anodic) processes and loses them through reduction (cathodic) reactions. In the steady state, the electrode potential reaches a value so that the rate of these two processes is similar. Because of this electrochemical balance of the two different reactions, the value is called the mixed potential. Under these conditions, the open circuit potential is controlled by the kinetics of the two reactions, which are described by their Tafel relationships. For this reason, these systems based on mixed potential can display exquisite sensitivities and a great versatility, allowing the detection of dissolved oxygen, phosphate, and metal nanoparticles, among others[33]. The major drawback of the mixed potential systems is their extreme susceptibility to the chemical environment. However, we have demonstrated that the use of a polyelectrolyte coating overcomes this problem, stabilizing and enhancing the electrode response [30].

In the case of the Pt electrodes, the mixed potential is created by the oxygen reduction reaction (ORR, cathodic process) and the Pt surface oxidation (anodic processes). Since the ORR is the rate-limiting step, the Pt electrode shows a marked response to dissolved oxygen. Hydrogen peroxide interferes on the reaction pathway of the ORR, and this has been the basis of the detection of this molecule. Indeed, the OCP of a bare Pt electrode shows a dependence with the concentration of H_2O_2, but this response is weak (around 30 mV/decade) and heavily affected

by the presence of matrix components, particularly anions. However, when the electrode is coated with a layer of Nafion®, the response is increased to 120 mV/decade of H_2O_2 and the effect of interfering anions is minimized. It has been shown that the electrostatic effect produced by the sulphonate moieties of the polymer act as a permselective barrier that reduces the concentration of anions on the surface of the electrode, particularly redox-active anions such as ascorbate.

Therefore, analogous to the detection of glucose, the GALOx enzyme can be used to selectively generate hydrogen peroxide (Eq. 2) in the presence of galactose, which will be then measured at the Nafion-coated platinized paper electrode (Pt/Nafion®):

$$Galactose + O_2 \xrightarrow{GALOx} Galacto\ hexodialdose + H_2O_2 \quad \text{(Eq.2)}$$

3.2 Sensor design

To make the system compact and efficient, enzymes are usually immobilized on the vicinity of the electrode. In preliminary experiments, we have found that the direct immobilization of GALOx on the surface of the metal electrode is not effective, since a very poor performance is obtained. This is similar to what has been found in the case of glucose oxidase, and it might be related to charge distribution and enzyme orientation on the metal, which may affect enzyme activity. Thus, the polymeric coating of the electrode surface with Nafion® was also used for the non-covalent immobilization of GALOx. It was found that GALOx can be non-covalently immobilized by direct entrapment on a Nafion® membrane without significant reduction of the enzymatic activity. This might be due to the fact that GALOx is positively charged (isoelectric point ~ 12) under our experimental conditions [34], so it can be easily retained in the Nafion® matrix by electrostatic interactions. Therefore, together with the permselectivity and the enhanced sensitivity towards hydrogen peroxide, Nafion® provides a suitable media for enzyme immobilization. To avoid any chance of leaching and favour the stability, once the enzyme has

been cast on the Nafion® membrane, an additional layer of a polymeric material is added is added on top. In this case, a layer of chitosan –as already described in a previous work [32]- provides a suitable media. In this way, the enzyme is sandwiched between Nafion® (bottom layer) and chitosan (top layer). In summary, the construction of the sensor is based on the following three steps: a) coating with Nafion® the platinized paper electrode; b) non-covalent immovilization of GALOx on Nafion® and c) addition of the chitosan layer, as shown in Figure 1. All these steps are performed by drop casting of a suitable solution. Optimization of the enzyme concentration as well as the reaction conditions were then performed.

Figure 1

3.3 Optimization of the detection of galactose

Preliminary experiments have shown that a volume of 7 µL of 2.5% Nafion® as the first layer and 5 µL of 1% chitosan (in 1% wt acetic acid) for the second one were optimum for the detection of hydrogen peroxide. These results are similar to what we have previously reported for a glucose sensor [27]. Therefore, the effect of the enzyme loading on the sensitivity of the detection of galactose was then performed. To this end, two different approaches were followed. First, sensors were build using a 20 µL fixed volume of GALOx solution in PBS with different concentrations of the enzyme (10, 20, 30 and 40 mg/mL). The results show (Figure 2A) that the initial increase in the amount of enzyme deposited results in a significant improvement, but then the performance decreases (Table S1). The optimum sensitivity was set for the solution of 20 mg/mL, which yields a slope of -49.7 ± 8.4 mV/decade of galactose. Noteworthy, the determination of glucose with a similar type of sensor yield a higher sensitivity, usually close to -120 mV/decade of glucose [32]. This reduced sensitivity may indicate a lower enzyme activity –either due to the nature of the enzyme or due to the entrapment-. Also, it may imply that the effect produced by Nafion® (sensitivity enhancement of peroxide) is somehow affected by the

presence of the enzyme. Alternatively, different volumes of enzyme solution were evaluated (10, 20, 30 and 40 µL) using the optimum concentration of GALOx solution (20 mg/mL). In this case, a similar trend is displayed with an optimum volume of 20 µL (Fig. 2B and Table S1). From the two plots, it can be seen that the response depends on the total mass of enzyme added on this system. The optimum enzyme loading was found to be 400 µg. Higher enzyme loadings yield worst results, probably due to the lower availability of the active sites on the enzyme. This optimum value should be related to the surface coverage (µg/cm^2), so it should be expected that different optimum values were obtained if the electrode surface area (in this case 7 mm^2) is changed. For the purpose of this work, optimum conditions were set at 20 µL of 20 mg/mL GALOx solution.

The GALOx enzyme is a policationic system, with an unusual high isoelectric point (ca. 12) [34]. Therefore, the pH of the drop-casting solution might have an influence on the final activity of the immobilized enzyme. In order to evaluate this point, the pH of the buffer solution (PBS 0.1 M) in which the GALOx is dissolved was varied over the 6.0–7.4 range (Table S1). The results show that the response increases from pH 6.0 to pH 7.0, and then slightly decreased at pH 7.4. Under these optimum conditions, a further increment of the sensitivity, up to -62.8 ± 9.4 mV/decade of galactose, is obtained. This may point towards the increase of the enzyme activity and probably to an improved stabilization of the enzyme based on ionic interactions within the polymeric matrix[35]. Therefore, for the rest of the work, the pH of the enzyme drop casting solution was adjusted to 7.0.

Figure 2

3.3. Analytical performance

Since the activity of an enzyme could be affected by the immobilization process, the performance of two different systems was compared, one with the GALOx dissolved in the bulk

solution and the other, with this enzyme immobilized as described above. Figure 3A and 3B show the time trace of the potentiometric responses and the corresponding calibration curves for the two systems, respectively. When GALOx is in solution, the sensitivity reaches a value of -110.5 ± 4.5 mV/ decade of galactose. Interestingly, this value is close to the sensitivity reported for the detection of glucose (-119 ± 8 mV/decade) [27]. Despite of the high sensitivity of the system with GALOx in solution, its linear range was between 10 to 100 mM of galactose. This does not match the clinical range of interest for monitoring galactosemia, which spans from 0.56 to 1.1 mM [18]. For the system with the immobilized GALOx (Pt/Nafion®/GALOx/Chitosan) a sensitivity of -62.8 ± 9.4 mV/decade of galactose and a limit of detection (LOD) of 0.25 mM (Table 1) was found. Once again, these differences in sensitivities are cueing either that the immobilization process affects the enzyme activity or that the addition of enzyme somehow affects the function of Nafion. In any case, despite of the lower sensitivity, the system with immobilized GALOx shows a linear response from 0.3 – 31.6 mM, well within the clinical range of interest. Additionally, it should be mentioned that the response time for this system was lower (180 s) than the approach with the GALOx in solution, probably due to the slowest diffusion of the hydrogen peroxide to the platinized surface in the former.

Figure 3

3.4. In situ pre-treatment of common interferences

Selectivity towards interferences is a key parameter for optimum biosensor performance. Due to the mechanism of detection, redox-active species may present a severe interference problem. Different kind of electroactive species normally present in real samples are well known to affect the response of electrochemical biosensors [36]. Therefore, the effect of common interferences presented in human blood such as glucose, citric, lactic and ascorbic acid (AA) on the response of the proposed potentiometric biosensor were evaluated. Fig. 3C shows that while

almost none of these substances affect the response, AA is the only one causing severe interference. This is somehow unusual, considering that it has been previously shown that Nafion® acts as a permselective barrier that effectively blocks the interference of redox active anions, ascorbate in particular. One possibility is that the GALOx enzyme is affecting the spatial structure of the Nafion® layer. Nafion® has a crystalline polymeric backbone (poly(tetrafluoroethylene)) and an amorphous side-chain with a pendant negatively charged sulphonate groups. Because of the strong differences between the polymeric backbone and the sulphonate groups, the hydrophilic portions form segregated nanochannels that provide the way to transport of water and polar substances, but rejecting anions due to electrostatic repulsion with the sulphonates. It is this delicate arrangement that leads to permselectivity, which might be altered by the introduction of different materials, such as a positively charged enzyme like GALOx.

From an analytical standpoint, it seems evident that the effect of the AA is a severe interference and the barrier of Nafion® is not enough for determinations in whole blood. Thus, alternative approaches to eliminate the interferences are required. This has been a typical problem in electrochemical techniques. Among the many alternatives, a chemical approach based on the use of a mild oxidant, such as MnO_2 NPs, on the outer layer of the membrane was explored.

MnO_2 NPs were synthesized and characterised prior to their use. TEM image shows particles with an average size of 50 nm with a wrinkled lamellar structure (Figure S1) [31]. Powder X-ray diffraction of these particles yields diffraction peaks (see inset Figure S1) that were indexed to δ-MnO_2 in a poorly crystalline phase [37].

In a first approach, the MnO_2 NPs were incorporated in the polymer matrix to be used as an oxidizing layer. However, any attempts to use different combinations of MnO_2 NPs quantities and different polymers did not yield positive results. Response to AA was still detected, and the presence of the nanoparticles seems to destabilize the polymeric membrane. Eventually, the particles affected the detection and could leach to the solution. As an alternative approach, we

dispersed the particles directly in the solution. Different amounts of MnO_2 NPs were added to the solution under stirring to reduce the effect of the AA (Figure 3D). The results show that for a 100 µM AA solution, the effect of AA is significantly reduced when increasing the MnO_2 NPs concentration up to 20 mg/mL. Thereafter, a smooth decrease is observed. A final concentration of 50 mg/mL was chosen since it produces a minimum and manageable interference. At this level, the signal for the 100 µM AA was reduced more than 10 times, from -127 mV to approximately -8 mV. This successful reduction of the interferences confirms the mild oxidant power of MnO_2 [36]. In addition, the presence of MnO_2 NPs in solution does not alter the sensor performance, i.e. the detection of hydrogen peroxide.

The dispersion of the particles is an effective way to overcome the interferences, though it is far from the ideal solution when looking for decentralized systems. For this reason, further work needs to be devoted to search an effective approach to immobilize the particles. In any case, assessing that this approach to overcome interferences can be applied to real matrices seems the obvious step before taking any further attempt in this direction.

3.5. Prediction of galactose in real samples

In order to simulate the conditions of real sample analysis the designed galactose paper-based potentiometric biosensor was calibrated in artificial serum in presence of 0.1 mM of AA by adding galactose in the range between $10^{-3.5}$ to $10^{-1.5}$ M. The galactose concentrations in ten artificial samples were predicted using the calibration plot obtained and compared with the expected values. The obtained results are depicted in Table S2. The obtained results show that the concentrations added and found were in good agreement and the recoveries were in all cases higher than 85 % with an average of 91.7% (RSD 5.3; N = 10), confirming the ability of this sensor to accurately monitor galactose in spiked artificial serum.

The validation of the galactose paper-based potentiometric biosensor was carried out using eleven independent random real samples (4 blood samples and 7 serum samples). None of the

samples was identified from a patient suffering from galactosemia. Galactose was directly added to the samples at different concentrations within the clinical range. Each galactose biosensor was first calibrated using a two-point calibration and then, the real sample was added to the cell together with 50 mg/mL of MnO_2 NPs under stirring. Potentiometric response was recorded and the signal was taken after 180 s to allow stabilization of the system. All the results obtained were compared with values obtained by a commercial galactose kit (Table S3).

Figure 4

Figure 4 shows the correlation between both methods demonstrating promising results. As it can be seen in Figure 4, the paper-based potentiometric biosensor herein developed shows a good linear correlation with the values obtained by the commercial colorimetric kit, with a slope close to 1 and an intersection close to 0. Noteworthy, the potentiometric biosensor works in whole blood whereas the validation kit used employed diluted samples which may lead to some differences.

Table 1

Finally, the analytical performance of the enzymatic paper-based electrode described in this study has been compared to previously reported galactose biosensors. Table 1 shows that the designed system presents comparable analytical parameters than previous systems although none of these examples reported direct real sample validation in whole blood. Indeed, the linear range of our sensor fits the clinical range and allows for the direct determination of galactose in such samples. Thus, this novel potentiometric biosensor demonstrated a simpler construction together with, for the first time, a validation in whole blood.

Conclusions

Paper-based potentiometric galactose biosensors based on platinum as transducer were prepared by immobilizing GALOx between two polymeric layers. MnO_2 NPs were successfully employed to reduce the interferences presented in real samples. The potentiometric response of these biosensors towards galactose showed a linear response that fits the clinical range. This result suggests that the proposed biosensor can be used for galactosemia diagnosis. Further work is currently being performed in order to develop strategies that allow the integration of the whole set-up, i.e. MnO_2 NPs, into the paper sensor.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability statement

Data are available on request from the authors

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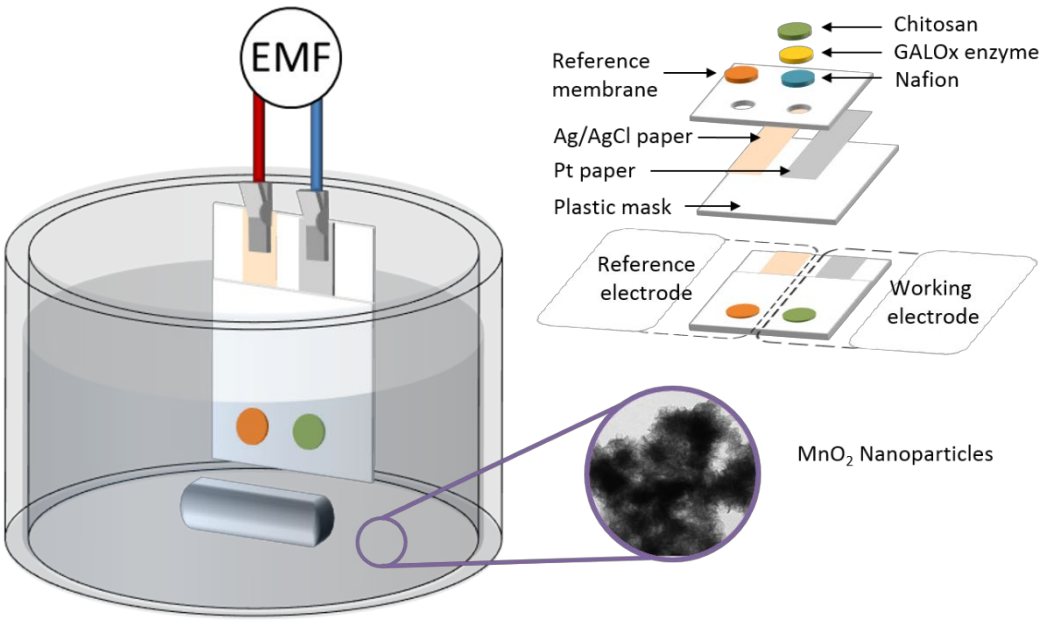

Figure 1. Scheme of the measurement setup, and the construction of paper-based potentiometric biosensor for detection of galactose.

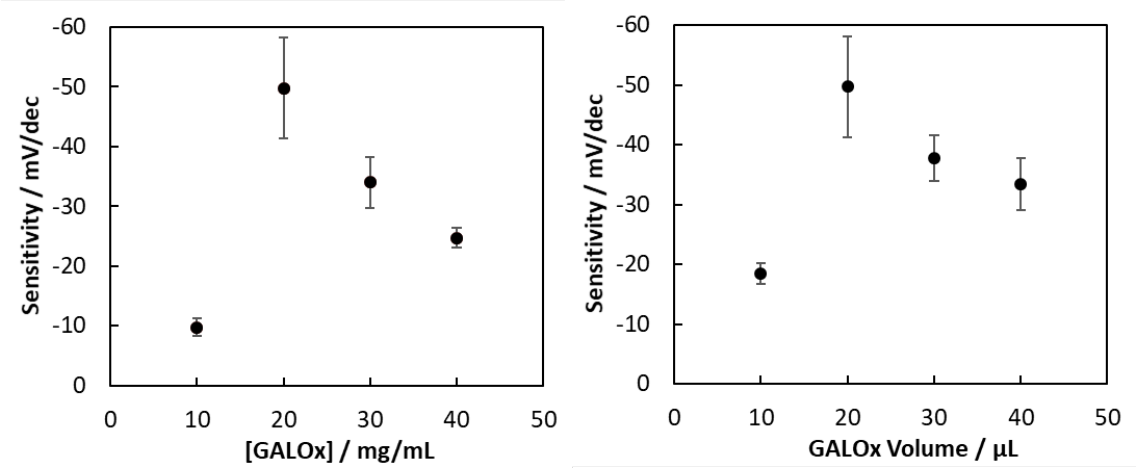

Figure 2. Optimization of the analytical conditions for Pt/Nafion®/GALOx/Chitosan biosensor. (A) Sensitivity for galactose *vs.* concentration of the working GALOx solution. (B) Sensitivity for galactose *vs.* the volume of 20 mg/mL GALOx solution deposited on the Pt/Nafion® surface.

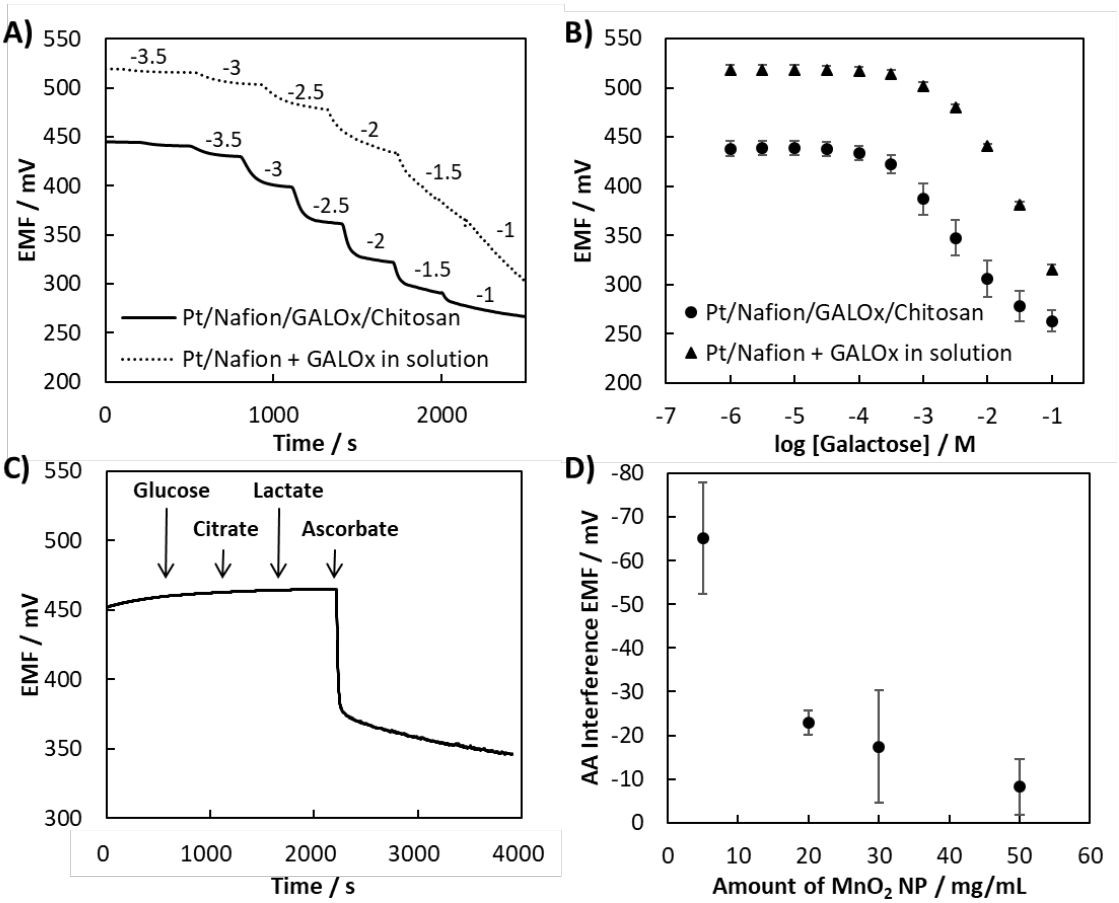

Figure 3. Potentiometric response (A) and the corresponding calibration plot (B) for the system with immobilized GALOx (Pt/Nafion®/GALOx/Chitosan) vs the system with GALOx in solution (Pt/Nafion®+GALOx in solution) (C) Time-trace for the selectivity test to the main interferences added at 0.1 mM level and, (D) response behaviour to 0.1 mM of ascorbic acid interferent in presence of different amounts of MnO_2 NPs at Pt/Nafion®/GALOx/Chitosan biosensor. All the measurements were performed in 0.1 M PBS (pH 7.4) at 25 °C.

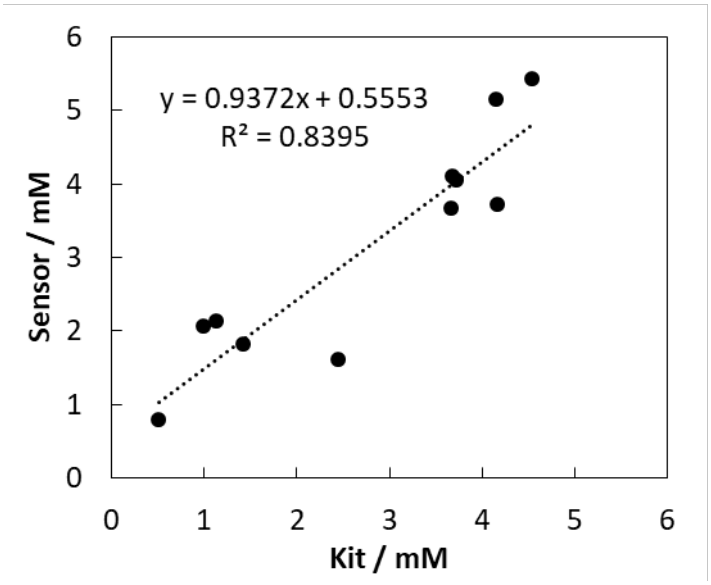

Figure 4. Comparison of galactose determination (mM) in real samples obtained by the Pt/Nafion®/GALOx/Chitosan paper-based potentiometric biosensor and the commercial colorimetric assay kit at 25 °C.

Table 1. Comparison of analytical performances of the reported method with other procedures for the determination galactose.

sensor	Measuring technique	Linear range (mM)	LOD (mM)	Sensitivity (mV/dec)	Response time (s)	Reference
P3HT/SA/GALOx LB	Amperometry	0.3–2.8	–	–	–	[38]
GALOx/PVF	Aqualytic dissolved oxygen meter	27.8–166.5	27.8	–	–	[39]
ITO/PEDOT/GALOx/ PPDA	Amperometry	0.1– 1	0.01	–	30–40	[8]
GALOx/poly(GMA-co-VFc)/Pt	Amperometry	2–20	0.1	–	5	[40]
Gold/ZnO/GALOx with gluteraldehyde	Potentiometry	10–200	0.1	89.1 ± 1.2	≈10	[22]
Pt/Nafion®/GALOx /Chitosan	**Potentiometry**	**0.3–31.6**	**0.25**	**-62.8 ± 9.4**	**180**	**This work**

Note: P3HTfor poly(3-hexylthiophene); SA for stearic acid; LB for Langmuir–Blodgett trough; PVF for poly(vinyl formal); ITO stands for indium tin oxide; PEDOT for poly(ethylenedioxythiophene); GALOx for galactose oxidase and PPDA for poly(phenylenediamine); poly (GMA-co-VFc) for poly(glycidyl methacrylate-co-vinylferrocene).