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Development of an amperometric screen-printed galactose biosensor for serum analysis

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ABSTRACT

The development of a disposable amperometric biosensor for the measurement of circulating galactose in serum is described. The biosensor comprises a screen-printed carbon electrode (SPCE), incorporating the electrocatalyst cobalt phthalocyanine (CoPC), which is covered by a permselective cellulose acetate (CA) membrane and a layer of immobilized galactose oxidase (GALOX). The optimal response of the biosensor, designated as GALOX-CA-CoPC-SPCE, was obtained by systematically examining the effects of enzyme loading, temperature, pH, and buffer strength. The optimal performance of the biosensor occurred with 2 U of GALOX, at 35 °C, using 50 mM phosphate buffer solution (pH 7.0). The sensitivity was 7.00 μA mM^{-1} cm^{-2} and the linear range from 0.1 to 25 mM with a calculated limit of detection (LOD) of 0.02 mM; this concentration range and LOD are appropriate to diagnose galactosemia, i.e., concentrations >1.1 mM in infants. When the biosensor was used in conjunction with amperometry in stirred solution for the analysis of serum, the precision values obtained on unspiked (endogenous level of 0.153 mM) and spiked serum (1 mM added) (n = 6) were 1.10% and 0.11%, respectively, with a calculated recovery of 99.9%.

α-D-Galactose is a nonessential nutrient that provides energy and serves as an important substrate for the synthesis of many biomolecules in the body. In humans, successful utilization of α-D-galactose requires that it is first converted to galactose 1-phosphate by galactokinase (GALK). Then a second enzyme, galactose-1-phosphate uridyltransferase (GALT), reacts galactose 1-phosphate with uridine diphosphate (UDP)-glucose to form UDP-galactose and glucose 1-phosphate (Fig. 1) [1]. The glucose 1-phosphate produced can either enter the glycolytic pathway to yield energy or react with UTP in a reaction catalyzed by UDP-glucose pyrophosphorylase to form a new molecule of UDP-glucose [2]. The other product, UDP-galactose, can be converted to UDP-glucose by the enzyme UDP-galactose-4-epimerase (GALE), or could donate galactosyl for the biosynthesis of glycolipids and glycoproteins [3]. Galactose 1-phosphate can also be dephosphorylated by the enzyme inositol monophosphatase to yield galactose [4]. In GALT-deficient individuals, galactose is reduced to galactitol by the enzyme aldose reductase. Galactitol then accumulates in urine and various tissues (such as in the lens of the eye, leading to cataract formation) [5].

There are three clinically significant metabolic disorders of galactose metabolism, which are due to the deficiencies of GALK, GALT, and GALE [6]. However, the most common disorder is classic galactosemia, an autosomal recessive inherited deficiency of GALT that could be treated by dietary-lactose restriction [5–7]. When the level of blood galactose is high (>1.1 mM) in neonatal infants, it becomes fatal galactosemia [8]. For infants with a deficiency in GALT activity, the ingestion of about 60 ml of cow's milk could result in a sudden accumulation of 10–20 mM galactose in their blood and tissue [9] and if the galactose is not quickly withdrawn from the diet, affected infants tend to suffer from a range of toxicity syndromes, including but not limited to hepatosplenomegaly, bleeding disorders, *Escherichia coli* sepsis, and sometimes death [5,6,8,10]. Consequently, many countries in Europe as well as all 50 states in the United States include screening for this disease in newborns [11]. Despite this widespread implementation of galactosemia screening in newborns, more than 75% of galactosemic infants continue to die [8] and many of those who are saved from neonatal death have chronic, premature morbidity including mental retardation, cataract, growth restriction, dyspraxic speech, learning and intellectual disabilities, ataxia, tremors, premature ovarian failure, and renal Fanconi syndrome [5,7,10].

Additionally, excessive urinary excretion of galactose, usually accompanied by galactitol, is highly suggestive of citrin deficiency in infants, especially those with prolonged neonatal jaundice and conjugated hyperbilirubinemia [12,13]. Galactosuria (galactose > 10 mM), one of the biochemical characteristics of citrin deficiency, may also be indicated by the presence of reducing sugar in urine [16]. Clinical symptoms associated with citrin deficiency

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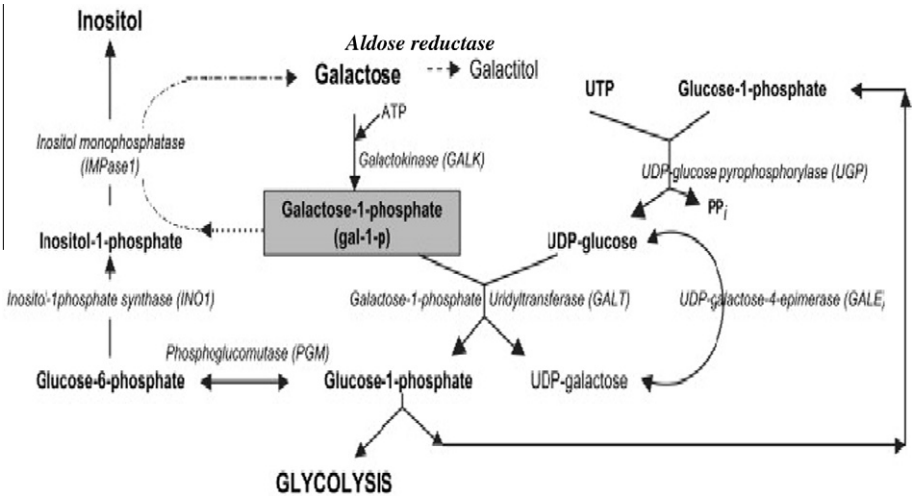

Fig.1. A schematic representation of the pathways that result in galactose metabolism and also prevent the accumulation of galactose and its metabolites in tissues, urine, and blood (adapted from [10,29]).

include hepatomegaly, intrahepatic cholestasis, hepatic fibrosis, hypoproteinemia, hemolytic anemia, and hypoglycemia. Although these symptoms can often be resolved with timely and appropriate treatment, some newborns succumb to infection and liver cirrhosis, while others require liver transplantation [14]. In addition to these, galactose and its metabolism can have a significant impact on the degree of regulation of blood glucose levels in diabetic infants [15,16].

Consequently, an early diagnosis and successful treatment of both galactosemia and citrin deficiency are not only extremely important in preventing these life-threatening disorders, but also help with the effective regulation of blood glucose in diabetic infants. As such, it is imperative that progress is made in developing rapid near-patient-testing technologies for galactose determination and the diagnosis of these diseases. Currently, numerous methods including fluorimetry, polarimetry, chromatography, and spectrophotometry are used to analyze galactose in clinical laboratories [17,18]. However, these methods are costly, cumbersome, and time-consuming [17,19,20]. Electrochemical measurements, especially the use of amperometric biosensors, are most attractive for galactose analysis because they are rapid, simple, economic, sensitive, and selective, which allows for direct real-time and online data analysis as well as excluding preseparation procedures [21,22]. Good biosensor selectivity is based on enzyme catalysis of the substrate: in the case of galactose, galactose oxidase can be utilized. The enzyme reaction is shown below (Eq. (1)):

$$\alpha\text{-D-galactose} + O_2 \xrightarrow{\text{galactose oxidase}} \alpha\text{-D-galactohexodialdose} + H_2O_2. \quad (1)$$

The measurement method is usually based on amperometric monitoring of this catalytically produced H_2O_2 by way of a redox mediator [22]. Typically, the use of cobalt phthalocyanine (CoPC) as a mediator has enabled the detection of H_2O_2 at low potentials during uric acid analysis [23], thus substantially reducing the influence of many possible interferences in biological fluids. These favorable aspects of amperometric enzyme biosensors would allow for the development of a fast, simple, selective, accurate, inexpensive, and reagentless device that allows for near-patient determination of the levels of galactose in the patient's blood and urine, thus helping with the early diagnosis of both galactosemia and citrin deficiency.

In this study, we explore the possibility of developing a novel inexpensive, disposable galactose biosensor. This biosensor is based on the use of a screen-printed water-based carbon ink, incorporating the electrocatalyst CoPC (CoPC-SPCE), with immobilized galactose oxidase (GALOX).

Experimental procedures

Materials and reagents

All chemicals including acetone were of analytical grade, purchased from Sigma–Aldrich, UK. Phosphate buffer (50 mM) was prepared by combining appropriate amounts of trisodium phosphate dodecahydrate, sodium dihydrogen orthophosphate dehydrate, and disodium hydrogen orthophosphate dehydrate to yield the desired pH. D-(+)-Galactose (Lot 021M00041V, Code 1001033250), GALOX (G7400, from *Dactylium dendroides*), uric acid (Lot STBB1642A0, Cat. No. U2625), and cellulose acetate (Cat. No. 3782; Lot 29F0024) were obtained from Sigma–Aldrich, UK. Paracetamol (AC10233-0051; No. 102330051) was obtained from Fisher Scientific Ltd., UK. Ascorbic acid (Product 44006) was purchased from BDH Ltd. (Poole, UK). Urea (Code U0450/53, Batch 34281193) was obtained from Fisons Scientific, UK. Uric acid was dissolved in 50 ml of 50 mM NaOH following up to 15 min sonication. Cellulose acetate (CA) was dissolved in acetone by sonication for up to 2 min. Fetal bovine serum (Cat. No. S1810-500, Lot S06015S1810) was purchased from Biosera (East Sussex, UK) and stored in small aliquots at −22 °C until used.

Apparatus and measurements

All electrochemical measurements were conducted with a three-electrode system consisting of an amperometric biosensor working electrode (WE), Ag/AgCl reference electrode (RE; GEM Product Code C61003P7) screen-printed onto Valox (Cadillac Plastics Ltd., Swindon, UK) (Fig. 2), and a separate Pt wire counter-electrode. The working area of the electrode was defined with an insulating tape (RS, Northants, UK) to a $3 \times 3\ mm^2$ area. The working and reference electrodes were connected to the potentiostat with gold clips. Solutions were stirred when required with a magnetic stirring disk and stirrer (Stuart Scientific, UK) to ensure thorough mixing. An Autolab electrochemical analyzer with General-Purpose Electrochemical Software (GPES 4.9, The Netherlands) was used for the acquisition of data and experimental control, with a 10-ml electrochemical cell inside a water jacket for electrochemical measurements. All weighing was done using a

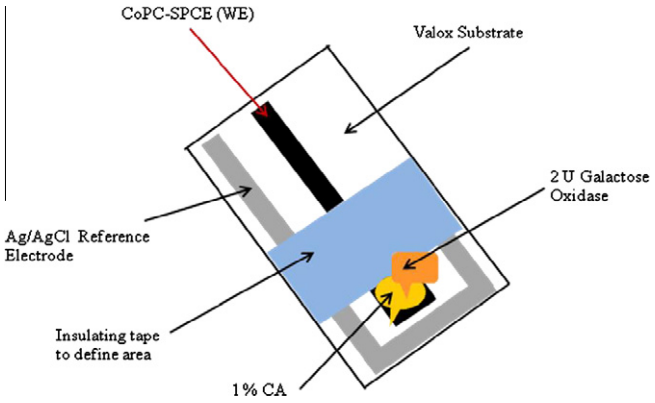

Fig.2. Diagram of proposed biosensor strip. The order of preparation was (a) screen print the CoPC-SPCE (WE) and Ag/AgCl RE, (b) define both WE and RE areas with insulating tape, (c) drop-coat 1% CA and allow to dry, and (d) drop-coat enzyme and allow to dry. All studies were performed using a separate Pt wire counterelectrode.

Precisa 262SMA-FR precision balance purchased from Milton Keynes, UK. Measurement and monitoring of pH were conducted with a Fisherbrand Hydrus 400 pH meter (Orion Research, Inc., USA). Sonication was performed with a Devon FS100 sonicating water bath (Ultrasonics, Hove, Sussex, UK). The temperature was controlled with a thermostated water bath (Thermo Scientific HAAKE DC10-P5/U unit).

Fabrication and surface modification of CoPC-SPCEs

The base unmodified CoPC-SPCE transducer was prepared using a water-based ink (GEM Code C40511D8) and the sensors were screen-printed in groups of six onto Valox as previously described by Crouch et al. [24]. CA solutions of 0%, 1%, 1.5%, 2%, 3%, 3.5%, 4%, and 5% were prepared to coat the electrodes. CA was diluted in acetone. The CoPC-SPCEs were modified by drop-coating 5 μl of various concentrations of CA solution directly onto the exposed working electrode with an area of 9.0 mm^2. Once dry, the electrodes were coated with 5 μl of galactose oxidase solution containing the appropriate enzyme units; the electrodes were left to air-dry. The same procedure was used to obtain the dummy biosensors, by using the same mass of bovine serum albumin (BSA) as that of the enzyme; any responses resulting from these devices were subtracted from the galactose biosensor response. An illustration of the proposed GALOX biosensor strip is shown in Fig. 2.

Once prepared, biosensors were stored at 4 °C in a vacuum desiccator containing silica gel, until used.

Amperometric studies with the proposed biosensor

All the amperometric measurements were performed in stirred solutions by applying a potential of +0.5 V vs Ag/AgCl. Once prepared, the biosensor was immersed in 10 ml buffer solution to allow a steady-state current to be obtained. The amperometric response to the addition of known concentrations of galactose solution was then recorded. Amperometry was used to determine the effects of enzyme loading (1.2–3.0 U), temperature (25–40 °C), pH (5–10), and ionic strength (0.025–0.45 mM) on the analytical performance of the galactose biosensor. The performance characteristics of the biosensor were deduced from calibration studies performed with 50 mM phosphate buffer solution containing 50 mM NaCl, over the concentration range 0.1 to 30 mM galactose.

Application of biosensor to serum analysis

Amperometry, in conjunction with the method of multiple standard additions, was used to determine the concentration of galactose in diluted unspiked serum samples (obtained by adding 2 ml of original serum to 3 ml of 50 mM phosphate buffer solution, pH 7.0). This serum solution was subjected to amperometry in stirred solution using an applied potential of +0.5 V. To determine the original galactose concentration, 5-μl aliquots of 0.2 M standard galactose solution were added to the voltammetric cell. Oxidation currents were measured and the results were used to construct standard addition plots.

This procedure was repeated using serum fortified with 1 mM galactose ($n = 6$) to determine the recovery of the assay. The effects of interference from serum were measured using the dummy biosensor and subtracted from that of the galactose biosensor.

Results and discussion

Principle of operation of the proposed biosensor

The operation of the proposed system is based on the interaction of α-D-galactose with the immobilized galactose oxidase. The enzyme converts α-D-galactose to α-D-galactohexodialdose with the concomitant formation of H_2O_2 (Eq. (1)). This diffuses through the pores of the CA membrane toward the electrode where it undergoes a chemical oxidation by CoPC (Co^{2+}) to produce CoPC (Co^{+}) (Eq. (2)):

$$2Co^{2+} + H_2O_2 \rightarrow 2Co^{+} + O_2 + 2H^{+}. \quad (2)$$

The electrochemical reoxidation of Co^{+} to Co^{2+} (Eq. (3)) occurs using an applied potential of +0.5 V and produces the analytical response:

$$Co^{+} \rightarrow Co^{2+} + e^{-}. \quad (3)$$

Clearly, the measurement of galactose based on H_2O_2 detection has the advantage that the H_2O_2 will pass through very small pore size membranes that would exclude interference commonly present in biological fluids.

Optimization studies

Biosensors were constructed by deposition of 1% CA solution, followed by 2 U of galactose oxidase as discussed earlier (Fig. 2).

The effect of enzyme loading on the amperometric current output of the biosensor was thoroughly investigated with loadings ranging from 1.2 to 3 U using standard additions of galactose solution in 10 ml 50 mM phosphate buffer (pH 7.0) solution at 35 °C. It can be seen in Fig. 3A that the amperometric current response increased with increasing enzyme units, reaching a maximum at 2 U and, afterward, decreased. This decrease in current means that the loadings at 3 U with a concomitant increase in mass of protein could have partially blocked the electrode surface, thus preventing H_2O_2 from diffusing to the underlying electrode surface. Consequently, 2 U loading was used to develop the proposed galactose biosensor.

The effect of pH was investigated over the range 5–10. The amperometric response of the biosensor (2 U) increased gradually from pH 5.0 and reached a maximum response at pH 7.0 and then decreased gradually (Fig. 3B); consequently, the optimum pH for the detection of galactose was considered to be pH 7.0, and used in further studies.

The effect of temperature has a profound effect on the performance of the biosensor. The response of the biosensor was investigated using 1 mM galactose prepared in 10 ml of 50 mM phosphate solution (pH 7.0) at temperatures varying from 25 to 40 °C. The amperometric current response increased with increasing temperature, reaching a maximum at 35 °C, and then gradually decreased

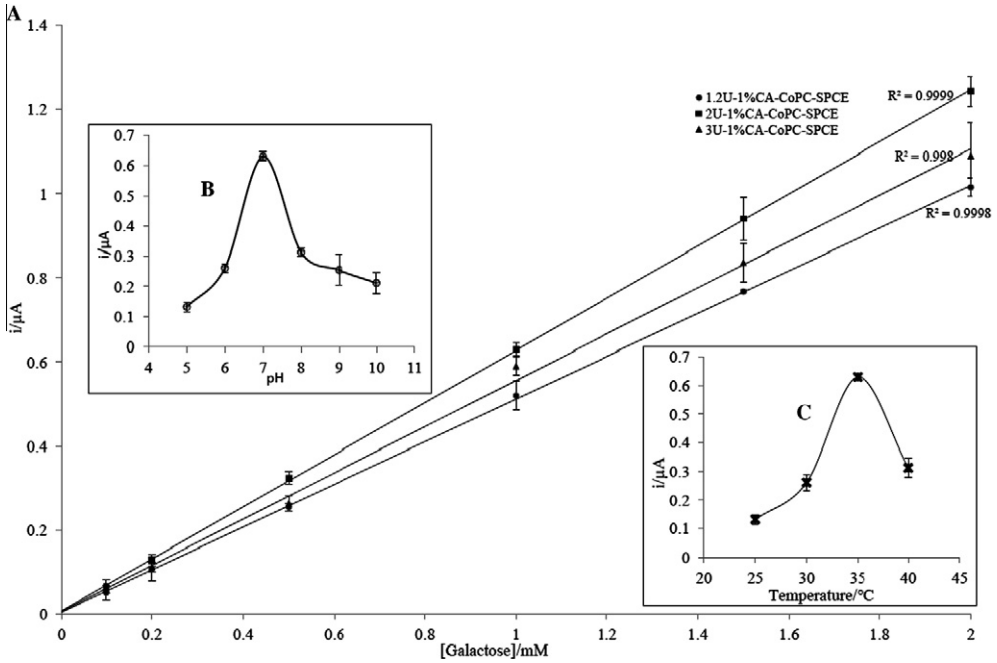

Fig.3. Effects of (A) enzyme loading on the amperometric current response of the biosensor to increasing concentration of galactose, (inset B) temperature on the current response of the biosensor to 1 mM galactose solution; and (inset C) pH on the current output of the biosensor. An applied potential of +0.5 V vs Ag/AgCl in 10 ml 50 mM phosphate buffer solution containing 50 mM NaCl was used.

(Fig. 3C). This decrease in current could be attributed to the denaturation of GALOX at higher temperatures.

Calibration, stability, interference, and reusability studies with biosensor

Calibration studies were performed with the fully developed galactose biosensor using the optimized experimental conditions over the range 0.1–30 mM. A new biosensor was used to obtain each measurement and this was done in triplicate for each galactose concentration. Fig. 4A displays a typical response curve of the optimized galactose biosensor on exposure to various concentrations of standard galactose solutions.

The resulting calibration plot (Fig. 4B) exhibits an apparent Michaelis–Menten kinetics with a linear range up to 25 mM. Each galactose addition elicited a rapid current response. This behavior is particularly important in near-patient tests, because a biosensor response within 30 s is highly desirable. The biosensor response exhibited a slope of 0.63 μA mM^{-1} (7.00 μA mM^{-1} cm^{-2}) and a good linearity range of 0.1–25 mM. The calculated limit of detection of 0.02 mM (based on 3× the baseline noise) is clearly appropriate to detect the elevated levels of galactose associated with galactosemia (>1.1 mM).

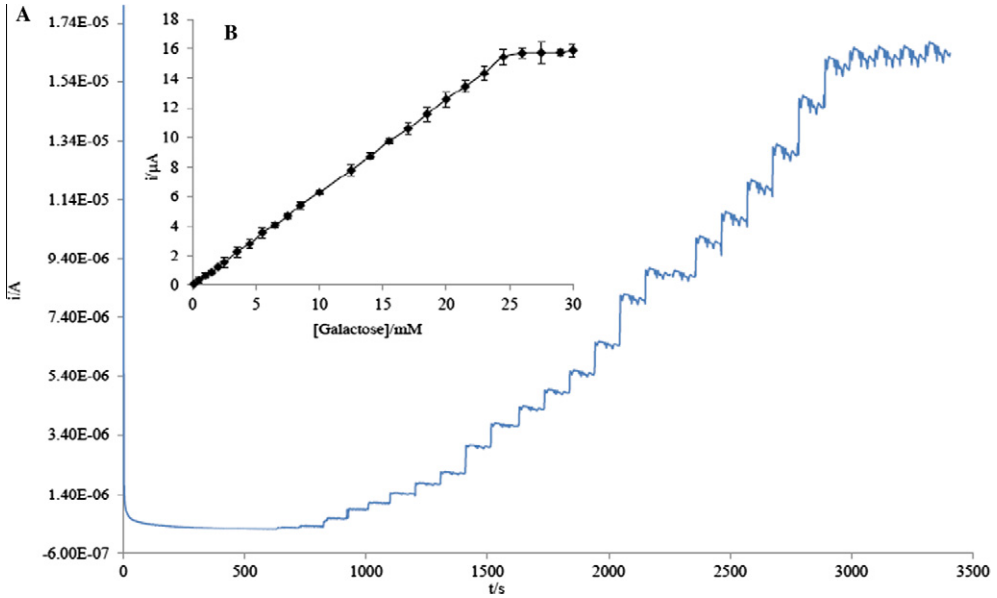

Fig.4. (A) A typical response curve of the optimized galactose biosensor on exposure to various concentrations of standard galactose solutions, 0.1, 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 10.0, 12.5, 14.0, 15.5, 17.0, 20.0, 21.5, 23.0, 24.5, 26.0, 27.5, 29.0, and 30.0 mM, in 10 ml stirred 50 mM phosphate buffer solution (pH 7.0) containing 50 mM NaCl solution at an applied potential of +0.5 V. (Inset B) The calibration plot. Each point represents the mean ± SD for $n = 3$ measurements.

Table 1
Comparison of the analytical performance of galactose biosensors.

Linear range	Detection limit	Sensitivity	References
0.05–10 mM	25 μM	3.75 μA mM^{-1} cm^{-2}	[25]
NS–40 mM	NS	2.4 μA mM^{-1} cm^{-2}	[17]
0–24 mM	NS	0.106 μA mM^{-1} cm^{-2}	[28]
2.0–20 mM	NS	0.162 μA mM^{-1} cm^{-2}	[26]
2.0–20 mM	0.1 mM	23 nA mM^{-1} cm^{-2}	[16]
0.1–1 mM	0.01 mM	6.37 μA mM^{-1} cm^{-2}	[8]
0.44–7.0 mM	0.44 mM	NS	[27]
2.0–16 mM	25 μM	1.75 μA mM^{-1} cm^{-2}	[30]
0.1–25 mM	0.1 mM	7.00 μA mM^{-1} cm^{-2}	This work

NS, not stated.

These performance characteristics are superior to published results obtained from other enzyme immobilization studies (Table 1), which tended to have reduced linear ranges [8,16,25–27], low sensitivity [8,17,25,26,28], and/or poor detection limits [27] compared with our device.

To compensate for the influence of interferences that could not be completely removed by our device, we performed studies (see Supplementary Table A) in fortified serum (0.5 mM galactose) in the absence or presence of 0.5 mM urea, 0.2 mM uric acid, 0.2 mM paracetamol, or 0.2 mM ascorbic acid, with both the galactose biosensor and the dummy biosensor. As can be seen in Supplementary Table A, the excess current due to each of the interfering species tested at the galactose biosensor was fully compensated for by subtraction of the dummy biosensor current.

We examined the reusability and stability of the GALOX-CA-CoPC-SPCE biosensors after storage in a desiccator at 4 °C. The response for a standard 0.5 mM galactose solution did not decrease over a 14-day study period (see Supplementary Fig. A).

Analytical application of biosensor

Amperometry, in conjunction with the method of multiple standard additions (Fig. 5), was used to determine the recovery for serum spiked with 1.0 mM galactose. Six replicate serum samples were analyzed. The determination was performed on 2 ml serum added to 3 ml 50 mM phosphate buffer (pH 7.0) containing 50 mM NaCl solution, and a fresh biosensor was used for each measurement.

Table 2 shows that a mean recovery value of 99.9% was obtained ($n = 6$) and the coefficient of variation (CV) was 0.112% for the spiked serum samples. For the unspiked serum samples, endogenous concentration was 0.153 mM and the CV was 1.10% ($n = 6$). These data demonstrate that the proposed amperometric galactose biosensor holds promise for application for diagnosing galactosemia in infants.

Conclusions

In this work, a galactose biosensor was prepared and the effects of various experimental factors on the analytical performance of the biosensor were thoroughly investigated. The amperometric response of the optimized galactose biosensor on exposure to various concentrations of standard galactose solutions was linear over the range 0.1–25 mM with a sensitivity of 7.00 μA mM^{-1} cm^{-2}. The response time was only 30 s. The influence of several interfering species such as uric acid, paracetamol, urea, and ascorbic acid was successfully compensated for by the use of a dummy biosensor. Clearly, this galactose biosensor exhibits performance advantages over other reported galactose biosensors that use enzyme immobilization methods. It is also less time-consuming and less expensive than the highly sophisticated spectroscopic and chromatographic techniques commonly employed during quantitative measurement of galactose in the clinical laboratory. There is clearly a further developmental step in producing a biosensor that could be used within a hospital environment. This would include testing the device on human sera from a range of individuals under various storage conditions. It should also be mentioned that the galactose biosensors were fabricated in-house using a water-based carbon ink formulation and screen-printing technology, which allows for mass production at low cost. Disposable glucose biosensor

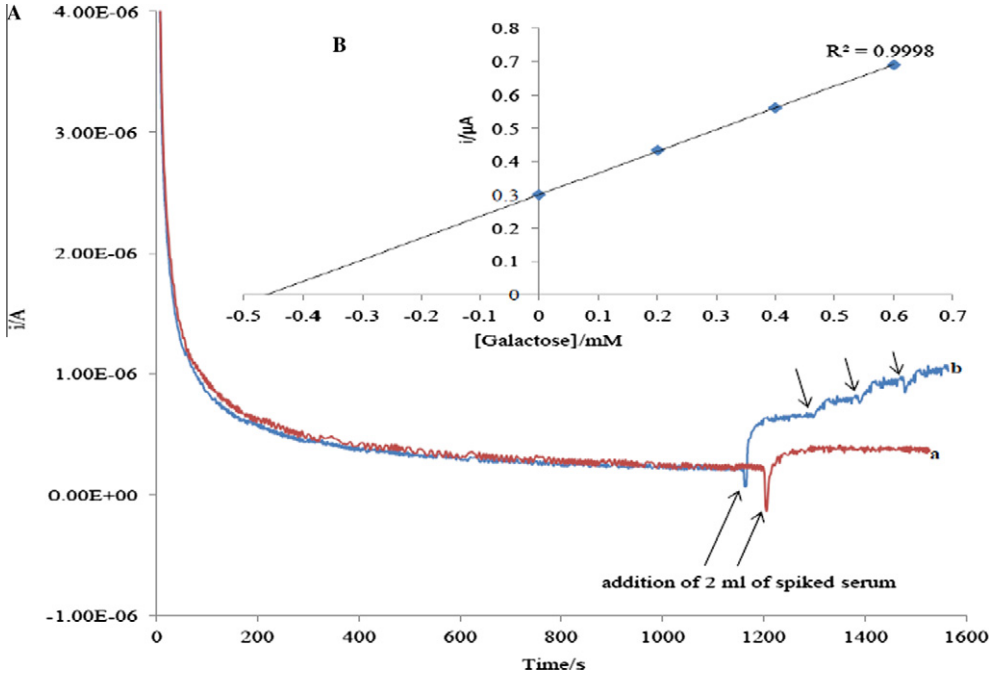

Fig.5. (A) Amperograms obtained with dummy (BSA) biosensor and spiked serum sample (trace a) and with the galactose (GALOX) biosensor plus spiked serum sample and multiple standard additions of 0.2, 0.4, and 0.6 mM galactose (trace b). (B) Multiple standard addition plot ($R^2 = 0.9998$). The dummy biosensor signal was subtracted from the biosensor signals to obtain the plot.

Table 2
Recovery of galactose from fortified serum using the proposed biosensor.

Repeat	Endogenous [galactose] (mM)		Mean (SD)	CV (%)
1	0.156		0.153 (0.002)	1.100
2	0.153			
3	0.154			
4	0.151			
5	0.153			
6	0.152			
	[Galactose] (mM)			
	Amount added	Amount found		
1	1.000	1.153	1.152 (0.001)	0.112
2	1.000	1.151		
3	1.000	1.153		
4	1.000	1.152		
5	1.000	1.153		
6	1.000	1.153		

The endogenous concentration of galactose (top) found using the amperometric biosensor and the concentration found (bottom) following fortification with 1.0 mM galactose are shown. The mean recovery was calculated from the means of endogenous (0.153 mM) and recovered galactose (1.152 mM) as follows: recovery = $(1.152 - 0.153)/1.000 \times 100 = 99.9\%$. The CV was 0.112%.

electrodes for near-patient testing retail for less than €1.00 per test and since they are based on a similar technology, the galactose biosensors would be expected to retail at a similar cost if manufactured on a commercial scale.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2013.01.006.

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