Biosensors and Bioelectronics 25 (2010) 2200–2204

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Development of a disposable amperometric biosensor for salicylate based on a plastic electrochemical microcell

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ARTICLE INFO

Article history:
Received 18 November 2009
Received in revised form 19 February 2010
Accepted 23 February 2010
Available online 3 March 2010

Keywords:
Screen-printed electrodes
Amperometric biosensor
Salicylate hydroxylase
Salicylate determination
Biosensor
Gold film electrodes

ABSTRACT

The use of an amperometric biosensor for rapid salicylate determination in blood is described. Photolitography was used to make gold electrodes on a polyester film. The plastic microcell was characterized using cyclic voltammetry to demonstrate the electrochemical performance of the system. The biosensor was constructed by immobilizing salicylate hydroxylase onto the working electrode of the plastic electrochemical microcell. The optimized working conditions were 0.1 mol L^{-1} phosphate buffer at pH 7.6 with 0.5 mmol L^{-1} of NADH and 300 mV *vs.* Au as the applied potential. The resulting biosensor exhibited a high sensitivity (97.4 nA/mmol L^{-1} salicylate) and an adequate linear response range (1.2×10^{-4} to 1.0×10^{-3} mol $^{-1}$). The biosensor performance was verified by determining salicylate in spiked blood samples and the results were statistically equivalent to the values obtained from the standard Trinder spectrophotometric method, with a 95% confidence level. This study shows the potential development of a portable, inexpensive and disposable device for point-of-care monitoring.

1. Introduction

Enzyme-based amperometric biosensors have attracted increasing interest in the field of research, but their use in industry, in production or for quality control of bioprocesses, in clinical diagnoses for self-testing or point-of-care diagnoses or even in environmental control are still limited. There are some examples of effective usage of enzymatic biosensors in these areas (Ramsay, 1998; Demir et al., 2008; Montagnana et al., 2009; Dascombe et al., 2007; Nichols et al., 2007; Gill et al., 1998; Thillaivinayagalingam et al., 2010; Valach et al., 2009; Dhall et al., 2008) but, in general, limitations on manufacturing reproducible electrodes on a large scale and problems with the integration of these electrodes into an easy to handle device do not suit the actual commercial needs.

Analytical microdevices represent a very promising trend, especially those amenable to mass production techniques, resulting in low cost (disposable) devices, which have minimized the consumption of energy, space, samples and reagents, as well as waste. The fabrication of electrochemical microcells containing working, reference and auxiliary electrodes in a single dispositive has been considered a key factor for success especially because of its versatility.

Gold is a metal with adequate properties for all-electrochemical technique due to its high stability over a wide potential range, ease in making rods and films and especially as a transducer in electrochemical biosensors because of its biocompatibility. Despite its advantages, the development of biosensors based upon thin or thick-film gold electrodes is gaining importance due to the high interfacial reactivity of those electrodes in comparison with screen-printed ones, even if printed with different ink materials (Ferreira et al., 2008; García-Gonzáles et al., 2008).

The greatest advances and efforts to develop salicylate biosensors were accomplished at the end of the twentieth century. A review made by Kubota and co-workers (Oliveira Neto et al., 1999) compiled the majority of works developed in the area with just a few more recent exceptions (Martín and Domíngues, 1999; Rover et al., 2000; De Carvalho et al., 2001; Campanella et al., 2006). The majority of research into amperometric salicylate biosensors has focused on the development of a rapid and reliable method of analysis by using, in general, the biosensor developed together with a FIA system. But despite the need for a portable device that allows the fast determination of salicylate concentrations to signal cases of accidental or intentional overdosing, a biosensor build on a disposable and portable electrochemical microcell was described just once (Frew et al., 1989).

This work describes the construction of a disposable electrochemical biosensor for point-of-care monitoring of salicylate in blood. It is based on the immobilization of salicylate hydroxylase

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doi:10.1016/j.bios.2010.02.026

with glutaraldehyde and bovine serum albumin (BSA) upon a plastic electrochemical microcell.

2. Materials and methods

2.1. Reagents

Salicylate hydroxylase (SH) from *Pseudomonas sp.* (EC 1.14.13.1), β-nicotinamide adenine dinucleotide (β-NADH), glutaraldehyde and bovine serum albumin (BSA) were purchased from Sigma. Sodium salicylate was supplied by Merck. Reagent grade PIPES (piperazine-N-N′-bis[2-ethanesulfonic acid]), HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) and TRIS (tris[hydroxymethyl]amino-methane) were purchased from Sigma and potassium chloride, citric acid, boric acid, phosphoric acid were from Merck. Purified water was obtained from a Millipore Milli-Q filtering system. All reagents were used as received without further purification.

2.2. Apparatus

Voltammetric and amperometric measurements were performed with an Autolab PGSTAT10 (ECO Chemie, Netherlands) potentiostat interfaced to a computer controlled by Autolab GPES 4.9 Software. The original cell cable connector for indicator, auxiliary and reference electrodes was substituted by a homemade cable adapted by means of a DIN connector on the potentiostat hardware and a slot that allows connection to the biosensor strips.

Spectrophotometric measurements were performed with a Ultrospec 2000 Pharmacia Biotech spectrophotometer using a cell with a 1.0 cm optical path. A JEOL JSM 6360 LV Scanning Electron Microscope (30 kV) equipped with an EDS (NORAN System SIX Model 300) was used to observe the scanning electron microscopic (SEM) images of the screen-printed gold electrodes. A Sartorius BP 211D balance and a 350 Corning pH/ion analyser were also used.

2.3. Gold electrochemical microcell assembly

Gold electrodes were assembled on 100 μm polyester films. The substrate was cleaned and then covered with a positive photoresist in a spinner. The cleanness of the polyester substrate was ensured by immersing it in an ultrasound bath with Extran detergent (Extran MA 02, Merck) for 5 min and then washing with isopropyl alcohol (J.T. Baker). Finally, the polyester film was dried under a nitrogen atmosphere. A conventional photoresist spinner (headway research inc.) was employed to coat the polyester material with positive photosensitive emulsion (S1811, Shipley Co.). The substrate was fixed on a glass support in order to be properly held by the spinner and the process was concluded in 30 s at 4000 rpm. After that, the photoresist solvent was volatilised by placing the substrate on a heating plate at 90 °C for 5 min. The substrate was exposed for 20 s to UV light using a maskaligner (MJB3, Karl Suss) whose lamp intensity is about 9.5 mW cm^{-2}. The photomask was designed to define the traditional three-electrode configuration disposed on the same strip. The revelation step was carried out by putting the photoresist layer in contact with the developer solution (AZ 351, Clariant, basically sodium hydroxide and borate) diluted with water in the proportion of 1:3 (v/v), respectively, for 1 min with constant stirring. A layer of approximately 20 nm of Ti and a second layer of 100 nm of Au upon the substrate was deposited by electron-beam evaporation. All the photoresist under the deposited metallic layer was removed from the substrate leaving the design of the electrode by using the lift-off technique. The design of the microcell is simple: the auxiliary and the reference electrodes are rectangular with an area of 3 mm^2. The working electrode was printed with four different areas 0.3, 0.75, 1.5 and 3 mm^2.

The electrodes were rinsed twice with dilute sulfuric acid and then washed copiously with water and let dry. This pre-treatment step was evaluated and effectively improves the electrochemical reversibility of potassium ferrocyanide upon screen-printed gold interface.

2.4. Working electrode modification

Salicylate hydroxylase was immobilized by dissolving 1.0 mg of the enzyme in 100 μL of phosphate buffer (0.1 mol L^{-1}, pH 7.6) containing 0.5% (v/v) of glutaraldehyde and 1.0 mg of BSA. An aliquot of 5 μL of this solution was disposed on the gold working electrode surface and let dry in air for 1 h. This modified electrode was used as a biosensor for salicylate, without any other treatment of the surface.

The measurements were performed using a drop that is deposited on a confined area of the electrode system. The volume used in measurements was 20 μL. However, volumes of up to 80 μL can be used.

2.5. Electrochemical characterization and optimization of the microcell

The voltammetric behaviour of the developed gold electrochemical microcell was evaluated in a solution of 5 mmol L^{-1} potassium ferrocyanide in 0.1 mol L^{-1} KNO_3, pH 7.0. Cyclic voltammograms (CV) were recorded at different experimental conditions with the scan rate varying from 20 to 50 mV s^{-1}.

As the auxiliary and the working electrode, the reference was made of gold. The gold electrode does not accomplish all the requirements of a standard reference electrode, so it was classified as a pseudo-reference system. The employed pseudo-reference gold electrode was compared with a well-studied calomel reference electrode by performing cyclic voltammetric measurements under the same experimental conditions but employing different reference systems.

The electrochemical surface area (ESA) of the gold film working electrode was determined by integrating the gold oxide reduction peak from the voltammetric curves obtained between −0.3 and 0.9 V (*vs.* Au), at a scan rate of 50 mV s^{-1} in 0.1 mol L^{-1} phosphate buffer at pH 7.0. The ESA is the ratio between the charge of the gold oxide reduction upon the studied surface and the standard reference charge found for polycrystalline Au, 390 ± 10 μC cm^{-2} (Trasati and Petrii, 1991).

2.6. Electrochemical characterization and optimization of the biosensor

The effect of the type of buffer solution was investigated using phosphate, PIPES, HEPES, TRIS, Britton-Robinson (boric acid plus sodium phosphate and citric acid) and Mc-Ilvaine (sodium citrate and sodium phosphate) buffers. The buffer concentrations evaluated were 0.02, 0.05, 0.1 and 0.2 mol L^{-1}. The influence of the applied potential, the electrolyte pH and the amount of NADH on biosensor response was also investigated

The effect of blood, a complex matrix, on the performance of the biosensor was evaluated by comparing the concentration–response relationship of spiked blood samples to that of spiked buffer solution in the range of 1.25×10^{-4} to 1.00 mmol L^{-1}.

2.7. Salicylate determination in blood

Capillary blood obtained from a finger was analysed. The required sample needs to be no greater than 20 μL and did not undergo any pre-treatment before the analysis. If necessary, the samples may be diluted to a suitable concentration range.

Table 1
Voltammetric characterization data of screen-printed gold electrodes carried out in a 5 mmol L^{-1} $Fe(CN)_6^{4-/3-}$ in 0.1 mol L^{-1} KNO_3, pH 7.0.

Geometric area/mm^2	ΔE_p/mV for 50 mV s^{-1}	ΔE_p/mV for 500 mV s^{-1}	i_{pa}/i_{pc} for 50 mV s^{-1}	Electrochemical surface area/mm^2
0.30	288 ± 13	363 ± 4	1.12 ± 0.02	0.70 ± 0.09
0.75	159 ± 26	305 ± 37	1.12 ± 0.01	2.21 ± 0.16
1.50	135 ± 26	257 ± 21	1.07 ± 0.01	3.70 ± 0.11
3.00	92 ± 4	205 ± 22	0.98 ± 0.01	6.91 ± 0.40

A standard stock solution was prepared containing 0.01 mol L^{-1} sodium salicylate in 0.1 mol L^{-1} phosphate buffer, pH 7.6. Five subsequent dilutions were made in Eppendorf tubes by adding an aliquot of standard salicylate solution (20, 50, 100, 200 or 400 μL) to 7.09 mg of NADH. The volume was adjusted to 1.0 mL with 0.1 mol L^{-1} phosphate buffer, pH 7.6. Salicylate-spiked blood samples were prepared by adding 20 μL of diluted standard salicylate solutions to 20 μL of blood. The resulting salicylate concentrations in the blood samples were 0.10, 0.25, 0.50, 1.00 and 2.00 mmol L^{-1}.

The analysis was performed according to the following protocol: 20 μL of a blank buffer solution (containing 0.5 mmol L^{-1} NADH) was dropped onto the biosensor and up to 5 min of amperometric signal was recorded. Subsequently, 5 μL of spiked blood was added and after one minute the amperometric signal was recorded. Next, 5 μL of a standard solution of salicylate (2.00 mmol L^{-1}) was added and after 1 min the amperometric signal was registered.

The salicylate determinations with the disposable biosensor were compared with the Trinder spectrophotometric method (Trinder, 1954). For this latter, EDTA anticoagulant was used for whole blood and the sample was spiked by adding 25, 50 or 200 μL of 0.01 mol L^{-1} sodium salicylate. The resulting salicylate concentrations were 0.25, 0.50 and 2.00 mmol L^{-1}.

3. Results and discussion

3.1. Electrochemical characterization of the microcell

The first task was to prepare a reliable platform for the development of a disposable biosensor. Several substrates were tested, like polyester, glass, kapton and phenolic resins, but the polyester plastic material gives the best reproducibility and electroactivity for the deposited gold. The electrochemical features of the developed gold film electrodes are now described.

Fig. 1 compares the pseudo-reference gold electrode with the well-studied calomel reference electrode and shows that the redox potential of $Fe(CN)_6^{4-/3-}$ shifts toward more negative potentials, about −172 (±1) mV *vs.* SCE. The potential stability of the pseudo-reference gold electrode was attested by monitoring its potential over 1 h in 0.1 mol L^{-1} KNO_3, pH 7.0. The insert in Fig. 1 shows a picture of the metallic electrode with the corresponding SEM image of the gold surface. It was observed that the gold particles are homogeneous in size and shape. This aspect is particularly interesting in order to make reproducible sensing devices, especially when both the working electrode and the reference system depend on it.

The magnitude of the electrochemical surface area is an important attribute of a metallic platform that is going to support an amperometric biosensor. Table 1 indicates that the ESA of the developed gold film electrodes is, on average, 2.5 times greater than the geometric area. The ESA is proportional to the microscopic roughness observed at gold interface and it relates to the immobilization of the recognition layer.

The electrochemical responses of the $K_3Fe(CN)_6$ molecular probe at the gold film working electrodes with different areas were evaluated. As shown in Table 1, the ΔE_p for all electrodes at all studied scan rates are considerably higher than those predicted for a one electron reversible system (59 mV), but it clearly diminishes at small ν and also when the area of the working electrode increases. Additionally, the i_{pa}/i_{pc} ratios for the studied systems increase as the area of the working electrode diminishes. The observed overpotential can be due to residual photoresist resting on the gold electrodes and is caused by the reduction in cross-sectional area as the working gold electrode area decreases.

In general, the auxiliary electrode area must be bigger than the area of the working electrode in order not to suppress the reaction of interest that is monitored by the latter. Screen-printed electrodes smaller than 3.0 mm^2 have a small voltammetric background current. However, electrodes with surface areas of 0.3 and 0.75 mm^2 have poor manufacturing reproducibility (c.a. 50%). So, the electrode of 1.5 mm^2 was chosen as the most adequate working electrode because it presents a satisfactory difference in size compared to the auxiliary electrode, high fabrication reproducibility (83%) and excellent electrochemical behaviour as shown in Fig. 1.

3.2. Salicylate biosensor optimization studies

The sensing surface design was investigated. Initially, the immobilization of salicylate hydroxylase was based on the interaction of thiolated groups of the cysteine residues on the enzyme and the gold of the working electrode. This immobilization strategy gives a biosensor with low robustness and stability. Alternatively, we prepared the biosensor sensing surface by dropping a solution containing salicylate hydroxylase, glutaraldehyde and BSA upon the gold electrode. This strategy preserves the functional stability of the biosensor because glutaraldehyde forms crosslinks between the enzyme macromolecules and BSA favours biocompat-

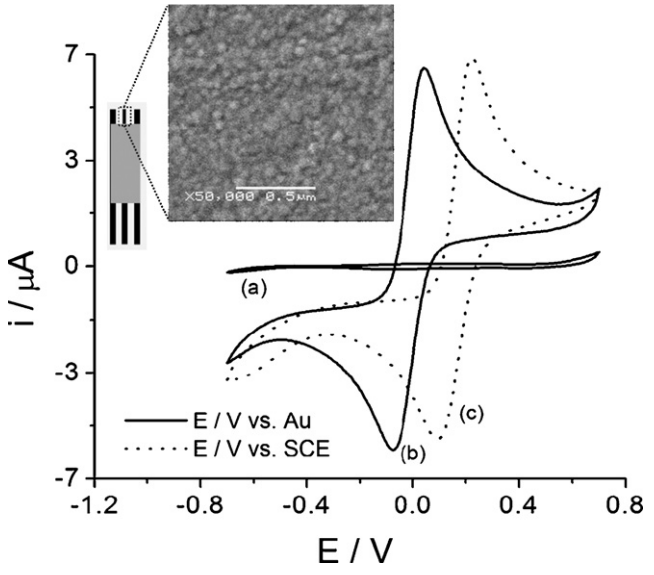

Fig. 1. Cyclic voltammogram of a screen-printed gold electrode considering two different reference systems: saturated calomel electrode (SCE) and pseudo-reference gold electrode (Au). Experimental conditions: (a) 0.1 mol L^{-1} KNO_3, pH 7.0, 50 mV s^{-1}, 1.5 mm^2 of working electrode area; (b and c) +5.0 mmol L^{-1} $Fe(CN)_6^{4-/3-}$. Inserted figure: scanning electron micrography of screen-printed gold electrode (50,000×, 20 kV).

ibility by mimicking the physiological environment of the enzyme. The electrochemical detection comprises the monitoring of catechol oxidation formed by the enzymatic conversion of salicylate, which is chemically amplified by NADH recycling catechol.

The concentration of the phosphate buffer solution employed to prepare the enzyme solution seems to be crucial and two different concentrations were tested: 0.1 and 0.2 mol L^{-1}. The best concentration was found to be 0.1 mol L^{-1} as the dried enzyme layer did not crack, which improves the reproducibility of the biosensor.

The effect of the enzyme concentration on the biosensor recognition layer was studied by testing several biosensors prepared from solutions with enzyme concentrations ranging from 2.5 to 20.0 mg mL^{-1}, assuming that the concentration of the active enzyme in the recognition layer is proportional to its concentration in the casting solution. The best concentration was found to be 10.0 mg mL^{-1} as the voltammetric response (i_{pa}) towards a fixed amount of salicylate and NADH was higher than those found with the other modified interfaces (data not shown).

The influence of the applied amperometric potential on the performance of the biosensor was investigated in the potential range between −50 and +400 mV *vs.* Au. The monitored amperometric current signal (Δi) was the difference of the current before and after the addition of the analyte at 50 s, in the steady-state condition. It was verified that the analytical signal is insignificant until it reaches 100 mV, but after this point, as the applied potential increases the response of the biosensor exponentially increases (data not shown). In order to preserve enzyme activity and to reduce the amperometric signal contribution of interfering compounds from the blood matrix the potential of +300 mV *vs.* Au was chosen.

The effect of the electrolyte pH on the amperometric response of the biosensor was verified for the detection of salicylate. It was observed that, in the pH range from 6.5 to 8.0, the best result was found near 7.5. As the best enzyme activity in solution is reported to be at pH 7.6, that pH was chosen to perform the measurements.

In a previous work it was established that the response of a salicylate biosensor depends not only on the amount of salicylate oxidized by the enzyme in the presence of NADH, but also on a catalytic process involving catechol and NADH, where the quinone is chemically reduced to catechol by NADH (De Carvalho et al., 2001). This way, the amount of catechol that is electrochemically oxidized on the electrode surface effectively relies on the concentration of NADH. For the disposable biosensor developed the best sensitivity was found by keeping the [NADH]/[salicylate] ratio between 2

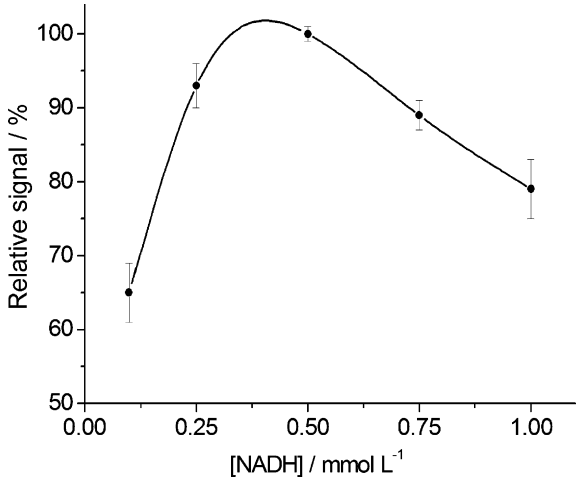

Fig. 2. Influence of the amount of NADH on the biosensor amperometric response. Experimental conditions: salicylate concentration: 2.5×10^{-4} mol $^{-1}$ using NADH concentrations from 1.0×10^{-4} up to 1.0×10^{-3} mol $^{-1}$ in 0.2 mol L^{-1} phosphate buffer, pH 7.6. Applied potential: 300 mV *vs.* Au.

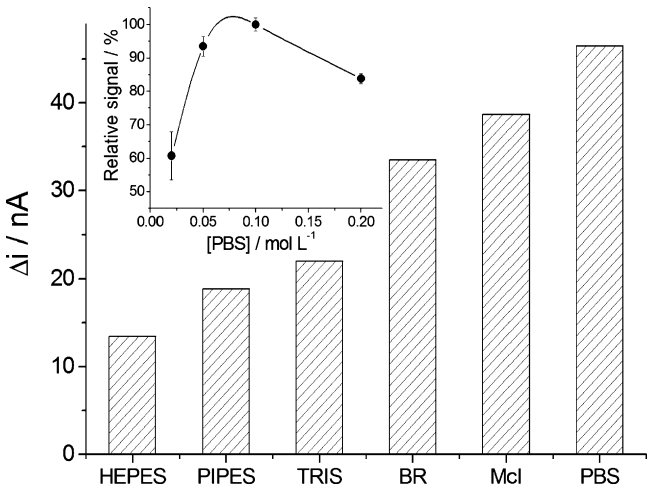

Fig. 3. Effect of the type of the electrolyte or buffer solution on the biosensor amperometric response. Experimental conditions: salicylate concentration: 2.5×10^{-4} mol L^{-1} with 0.5 mmol L^{-1} NADH, at 0.2 mol L^{-1} of specific electrolyte or buffer at pH 7.6. Applied potential: 300 mV *vs.* Au. Inserted figure: effect of phosphate buffer concentration on the amperometric biosensor response.

and 4. Fig. 2 shows a decrease of the response of the sensor for [NADH]/[salicylate] ratios higher than 4, as previously observed in the literature (De Carvalho et al., 2001).

Finally, the influence of the electrolyte and its concentration on the performance of the biosensor was evaluated. The electrolyte that improves the response of the biosensor was the phosphate buffer in the concentration of 0.1 mol L^{-1}, as can be verified in Fig. 3.

3.3. Biosensor performance in salicylate determination in blood

The monitoring of salicylate levels in blood is of great importance in cases of suspicion of acute poisoning and in chronic therapy (Oliveira Neto et al., 1999). When the salicylate concentration in the serum is higher than 2.2 mol L^{-1} it becomes toxic and the side effects are mainly related to the inhibition of the *cyclooxygenase enzyme*, acid–base disturbances and gastric irritation.

During the analytical validation of the biosensor it was verified if the complex blood matrix interferes or not in the performance of the biosensor. This was investigated by comparing the sensitivity obtained for salicylate molecule in the presence and in the absence of the blood matrix (data not shown). As the sensitivities obtained in these media were different, we conclude that there is significant influence of the sample matrix on the amperometric signal of the biosensor. So, the standard addition method was applied to achieve a reliable determination of salicylate content in blood.

A calibration curve obtained for a freshly prepared biosensor showed a linear range of response for salicylate concentration from 0.125 to 1.00 mmol L^{-1}, keeping the [NADH]/[salicylate] ratio between 2 and 4. The analytical curve equation was: $i(nA) = 97.4\ (\pm 4.9)$ [salicylate, mmol L^{-1}] + 15.2 (±2.9), with a correlation coefficient of 0.997 for $n = 5$, is shown in Fig. 4. The addition order of the standard solution and blood sample also influence the analytical performance of the biosensor. It is important to first add the blank buffer solution containing the cofactor, then the blood sample and, last, the standard solution of salicylate.

The biosensor performance was verified by determining salicylate in spiked blood samples. According to the results listed in Table 2, very good recoveries for salicylate determinations were obtained, which indicates the biosensor can be applied in blood analysis with no significant influence from the matrix by using the standard addition method. It was also found that the results obtained with the biosensor were not significantly different, according a paired *t*-test at the 95% confidence level, from those

Table 2
Analysis of salicylate concentrations in spiked blood samples.

Blood samples	Initial salicylate concentration/mmol L^{-1}	Added salicylate concentration/mmol L^{-1}	Biosensor recovery/%	Experimental salicylate concentration[a]/mmol L^{-1}	
				Biosensor	Standard method
1	0.00	0.10	101.9	0.10 ± 0.01	–
2	0.00	0.25	109.1	0.28 ± 0.04	0.26 ± 0.06
3	0.00	0.51	100.6	0.51 ± 0.08	0.51 ± 0.05
4	0.00	1.01	119.3	1.21 ± 0.05	–
5	0.00	2.02	99.4	2.01 ± 0.06	2.02 ± 0.04

[a] Mean ± standard deviation ($n = 3$).

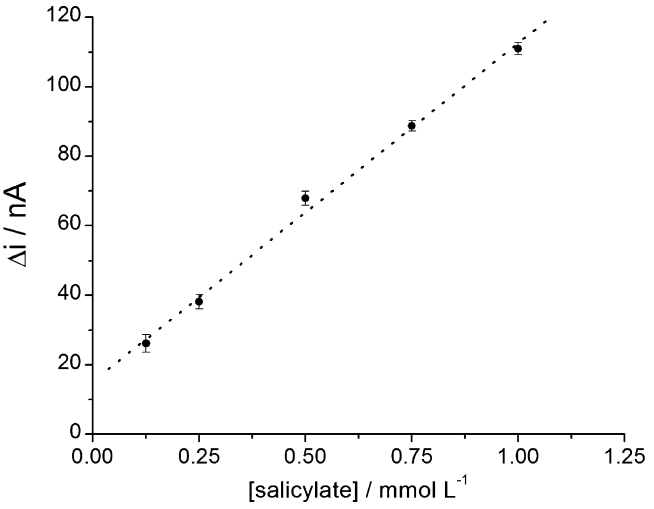

Fig. 4. Standard addition calibration of the biosensor for sodium salicylate in capillary blood. Experimental conditions: media containing 0.5 mmol L^{-1} of NADH in 0.1 mol L^{-1} phosphate buffer, pH 7.6, applied potential +300 mV *vs.* Au at different concentrations of salicylate.

obtained using the well established Trinder spectrophotometric method, and the most used method for salicylate quantification in clinical diagnostics due to its simplicity and sensitivity.

Using the conditions established in the studies described earlier, the biosensors were tested for repeatability. The relative standard deviation for a single batch of electrodes ($n = 6$) was found to be 4.4%.

The storage stability of the developed biosensor was evaluated over a period of seven months, with the devices stored dried and cooled (6 ± 1 °C). The biosensor response for a sample containing 2.0 mmol L^{-1} of salicylate in blood was periodically monitored at room temperature. The biosensor was stable and maintained >90% of maximum response for at least four months. After that period, it is crucial to spend more time rehydrating the recognition layer to achieve an adequate response of the biosensor. Although the biosensor still works after four months, it is no longer possible to perform a rapid analysis.

4. Conclusion

The use of a microcell made of plastic proved to be well suited for the fabrication of inexpensive electrochemical devices that could be applied for the development of disposable biosensors. These devices can solve challenging analytical problems that usually require sophisticated instrumentation, technical staff and some hours to be accomplished. In this paper we describe the development of a disposable biosensor for salicylate determination. The biosensor showed adequate sensitivity and accuracy to quantitatively detect salicylate in human blood. In addition, their good stability, low cost, simplicity and reproducibility of the fabrication of these salicylate hydroxylase biosensors indicate that they will be suitable for commercialization with the aim of point-of-care testing, a fast developing area that leads towards the future in the diagnostics market.

Acknowledgements

The authors are grateful to FAPESP and CNPq for financial support.

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