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Development of urease based amperometric biosensors for the inhibitive determination of Hg (II)

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ABSTRACT

Enzymatic amperometric procedures for measurement of Hg (II), based on the inhibitive action of this metal on urease enzyme activity, were developed. Screen-printed carbon electrodes (SPCEs) and gold nanoparticles modified screen-printed carbon electrodes (AuNPs/SPCEs) were used as supports for the cross-linking inmobilization of the enzyme urease. The amperometric response of urea was affected by the presence of Hg (II) ions which caused a decreasing in the current intensity. The optimum working conditions were found using experimental design methodology. Under these conditions, repeatability and reproducibility for both types of biosensors were determined, reaching values below 6% in terms of residual standard deviation. The detection limit obtained for Hg (II) was 4.2×10^{-6} M for urease/SPCE biosensor and 5.6×10^{-8} M for urease/AuNPs/SPCE biosensor. Analysis of the possible effect of the presence of foreign ions in the solution was performed. The method was applied to determine levels of Hg (II) in spiked human plasma samples.

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1. Introduction

Hg (II) belongs to those metals known to, and used by, man since ancient times [1]. However, Hg (II) and its compounds result to be extremely toxic, even at low concentrations: they accumulate in vital organs and tissues such as liver, heart muscle and brain, and cause kidney damage, central nervous disorders, intellectual deterioration and even death [2,3]. For this reason the sensitive and selective detection of Hg (II) is important research.

Many analytical methods are described in the literature for the determination of Hg (II), among them electrochemical ones. Nowadays, the use of sensors based in enzymatic modified electrodes as transducers in amperometric and potentiometric techniques has opened important perspectives in the development of numerous biosensors for the determination of many kind of analytes [4,5].

Hg (II), such as other heavy metals, can actuate as an enzyme inhibitor. This phenomenon, when used to determine these hazardous toxic elements, offers several advantages, amongst which figure high sensitivity and specificity [6,7]. Numerous enzymes have been used for inhibitive determination of Hg (II) such as glucose oxidase [8–10], horseradish peroxidase [11], acetylcholinesterase [12], invertase [7,10], and urease that is the most commonly used [6,13–17]. Most of the conventional urea biosensors are based on potentiometric measurements. These measurements are associated to the change of the concentration of hydrogen ions because of the ammonium liberation as a result of the following enzymatic reaction [6,16,17]:

$$\text{Urea} + H_2O + \text{urease} \rightarrow CO_2 + NH_3$$

The presence of Hg (II) inhibits the enzyme that leads to a decrease in enzymatic activity and, as a result, a lower quantity of ammonium is liberated. The inhibitory effect of Hg (II) ions on urease activity is due to their binding to thiol groups of protein amino acids habitually forming the active centre of the enzyme [6,13].

Amperometric biosensors have been less used in the analysis of the inhibitive effect of Hg (II) on urease. However, they are considered a promising tool due to its effectiveness and simplicity. These biosensors are based on measuring the changes in the current of the working electrode due to direct oxidation or reduction of the products of the biochemical reaction [13–15].

The possibilities of the amperometric biosensors can be increased by means of replacing the classical electrodes by disposable screen-printed electrodes (SPEs). SPEs present important advantages such us the elimination of memory effects in the analysis at trace levels and they appear to be particularly attractive for *in situ* determinations. The construction of SPEs involves the printing of different inks on planar ceramic or plastic supports. The great flexibility of SPEs resides in their high number of possible modifications. In fact, the composition of the inks used in the printing

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process can be modified by adding substances of a very different nature, such as metals, enzymes, polymers, complexing agents etc. Furthermore, the possibility of modifying the electrodes also exists once they have been constructed through the deposition of films containing those substances [4,18].

An interesting form of SPEs modification consists of the incorporation of metallic nanoparticles on the working electrode surface. Due to their reduced size, metallic nanoparticles exhibit important physical and electrical properties which make them very useful for the construction of more sensitive electrochemical sensors and biosensors [19].

In this work urease based amperometric biosensors were utilized for the inhibitive determination of Hg (II). To the best of our knowledge this is the first time that a disposable urease amperometric biosensor has been used for the high sensitive and selective determination of Hg (II). The enzyme was immobilized by cross-linking on the surface of screen-printed carbon electrodes (SPCEs) and gold nanoparticles screen-printed carbon electrodes (AuNPs/SPCEs).

2. Experimental

2.1. Reagents

Several inks were used in the fabrication of screen-printed electrodes, namely Electrodag PF-407 A (carbon ink), Electrodag 6037 SS (silver/silver chloride ink) and Electrodag 452 SS (dielectric ink) supplied by Achenson Colloiden (Scheemda, The Netherlands).

All solutions were prepared with water purified with a Milli-Q device which provided a conductivity of 0.05 μS/cm. Nitrogen (99.99%) was used to remove dissolved oxygen.

Urease (EC 3.5.1.5., Sigma, Steinheim, Germany), glutaraldehyde (GA) (Sigma, Steinheim, Germany), bovine serum albumine (BSA) (Sigma, Steinheim, Germany), glycine (analytical-reagent grade, Merck, Darmstadt, Germany) and urea (Merck, Darmstadt, Germany) were used.

Stock standard solutions of Hg (II) were prepared by dissolving the appropriate amount of $Hg(NO_3)_2{\cdot}H_2O$ (analytical-reagent grade, Panreac, Barcelona, Spain) in water.

0.1 M phosphate buffer ($NH_2PO_4{\cdot}2H_2O$, Panreac, Barcelona, Spain) and 0.1 M KCl (Merck, Darmstadt, Germany) solution was used as supporting electrolyte. NaOH (J.T. Baker, Deventer, The Netherlands) was used to adjust the pH value.

Lyophilized human plasma was purchased from Sigma (Steinheim, Germany)

2.2. Apparatus

Hand-made screen-printed electrodes were produced on a DEK 248 printing machine (DEK, Weymouth, UK) using polyester screens with appropriate stencil designs mounted at 45° to the printer stroke.

Electrochemical measurements were made with a μAutolab type III electrochemical system with GPES software (Eco Chemie, Utrecht, The Netherlands).

The pH of the solutions was measured with a Crison Model 2002 (Barcelona, Spain) pH meter.

2.3. Software

Data analysis was processed with a STATGRAPHICS PLUS [20] software package for the experimental design process, PROGRESS [21] for the robust regression and DETARCHI [22] for the detection limit (LOD).

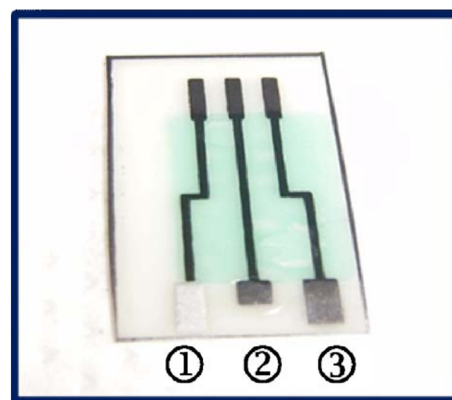

Fig. 1. Hand-made SPCE used in the analysis of mercury. 1. Reference electrode, Ag/AgCl; 2. Working electrode, Carbon; 3. Auxiliary electrode, Carbon.

2.4. Construction of the biosensors

2.4.1. SPEs preparation

Hand-made SPEs (Fig. 1) were used in the determination of Hg (II). For the construction of the SPEs successive layers of different inks were printed onto a polyester strip substrate using four different screens with appropriate stencils to transfer the required design following the printing procedure described in previous works [23,24].

2.4.2. Electrode cleaning

Before utilization the SPCEs, working and counter electrodes were polished with a SiC-paper No 4000 disc (Struers, Copenhagen, Denmark). Then, the working electrode surface was activated by recording 20 cycle voltammograms between 2 V and −2 V, scan rate, 100 mV s-1, in a 0.1 M KCl solution.

2.4.3. Urease inmobilization in SPCEs

Urease was immobilized by cross-linking with BSA and GA. The optimum immobilization process was reached by mixing 10 μL of a 3.32% (w/v) BSA and 1.66% (w/v) urease solution with an identical volume of a 2.5% (w/v) GA solution. Then, 5 μL of this mixture was dropped on the working electrode surface. The electrode was kept at 4 °C for 1 h. The excess of GA in the insoluble biocomponent was thoroughly eliminated by rinsing with 0.1 mM glycine solution in phosphate buffer [25,26]. Finally, it was left to dry at room temperature.

2.4.4. Urease inmobilization in AuNP/SPCEs

With the attempt to improve the performance of the biosensor, SPCEs were modified with AuNPs. Metallic gold nanoparticles deposits were obtained by direct electrochemical deposition on the carbon working electrode surface, using a 0.1 mM solution of $HAuCl_4$ in 0.5 M H_2SO_4. The deposition was performed by applying a potential of 0.18 V during 15 s under stirring conditions [27]. Then, urease was immobilized on the AuNPs/SPCE surface using the same procedure described in the preceding section.

2.4.5. Hg (II) determination procedure

The urease biosensors were placed in the electrochemical cell containing 5 mL of phosphate buffer solution. An adequate potential was applied and, once a steady-state current was set, a defined amount of urea stock solution was added to the measuring cell. A large reduction current was observed due to the addition of urea, and a plateau corresponding to the steady-state response was reached. Then, fixed portions of the Hg (II) stock solution were

Table 1
Experimental analyzed conditions for urease immobilization on SPCE.

	BSA		GA		Urease	
	[BSA] (w/v)	V_{BSA} (μl)	[GA] (w/v)	V_{GA} (μl)	[urease] (w/v)	V_{urease} (μl)
1	1.66%	5	2.5%	10	1.66%	1.66
2	3.32%	5	2.5%	10	1.66%	1.66
3	1.66%	5	2.5%	10	1.66%	3.32
4	1.66%	10	2.5%	10	1.66%	3.32
5	1.66%	10	2.5%	10	1.66%	3.32
6	1.66%	10	2.5%	10	1.66%	3.32
7	1.66%	10	2.5%	5	1.66%	3.32
8	1.66%	10	2.5%	10	1.66%	3.32
9	1.66%	10	2.5%	10	1.66%	1.66
10	1.66%	10	2.5%	10	1.66%	3.32
11	1.66%	10	2.5%	10	3.32%	1.66
12	1.66%	10	2.5%	10	3.32%	3.32

added consecutively, being reached each time a plateau. The addition of Hg (II) solution resulted in a current decrease proportional to the amount of Hg (II) added.

Enzyme electrodes were conditioned in a stirred phosphate buffer solution for 5 min between each calibration setting.

3. Results and discussion

In order to build the urease/SPCE and urease/AuNPs/SPCE biosensors, several experiences were done in order to find the optimum conditions for urease inmobilization. For this purpose, different amounts of BSA, GA and urease were immobilized over the SPCE (Table 1). The amperometric response for a 10^{-5} M Hg (II) solution was taken as response, using a C_{urea} and E_{ap} of 0.25 M and 1.4 V, respectively.

The maximum current recorded was reached using the immobilization procedure described above, that is to say, 10 μL of a 1.66% (w/v) BSA and 1.66 μL of a 1.66% (w/v) urease solution mixed with 10 μL of a 2.5% (w/v) GA solution.

The urease/SPCE and urease/AuNPs/SPCE biosensors produce an amperometric signal, which is sensitive to the concentration of urea, as it is shown in Fig. 2a. It can be seen that, after the reaching of a steady-state current, the addition of urea in the electrochemical cell produces an amperometric signal. The presence of Hg (II) ions produces an inhibition of the enzyme urease which causes a decrease in the urea amperometric signal of the biosensor.

Hg (II) inhibition action was quantitatively evaluated determining the difference between the steady-state current in the absence of Hg (II) (I_0) and the steady-state current in the presence of Hg (II) (I). The parameter ΔI ($I_0 - I$) depends on urea concentration, applied potential (E_{ap}) and pH of the buffer solution. So, it is necessary to optimize all of these variables in order to ensure the quality of the results.

Experimental design has been used as a tool for optimization. In this case, a 2^3 central composite design was applied, with replication in the central point in order to estimate the residual error. The response to be optimized was ΔI obtained for a sample containing a concentration of Hg (II) of 10^{-5} M. The results obtained in the optimization process as the optimum values were the following (Fig. 3):

$$E_{ap} = 1.5\,\text{V} \quad [\text{urea}] = 0.33\,\text{M} \quad \text{pH} = 7$$

Under these optimum conditions no amperometric response was obtained for urea when SPCEs and AuNPs/SPCEs were used. Likewise, no response was obtained for Hg (II) when no enzymatic modified SPCEs and AuNPs/SPCEs were utilized.

The inhibitory effect of Hg (II) ions on the response of the urea biosensors was investigated following the method of

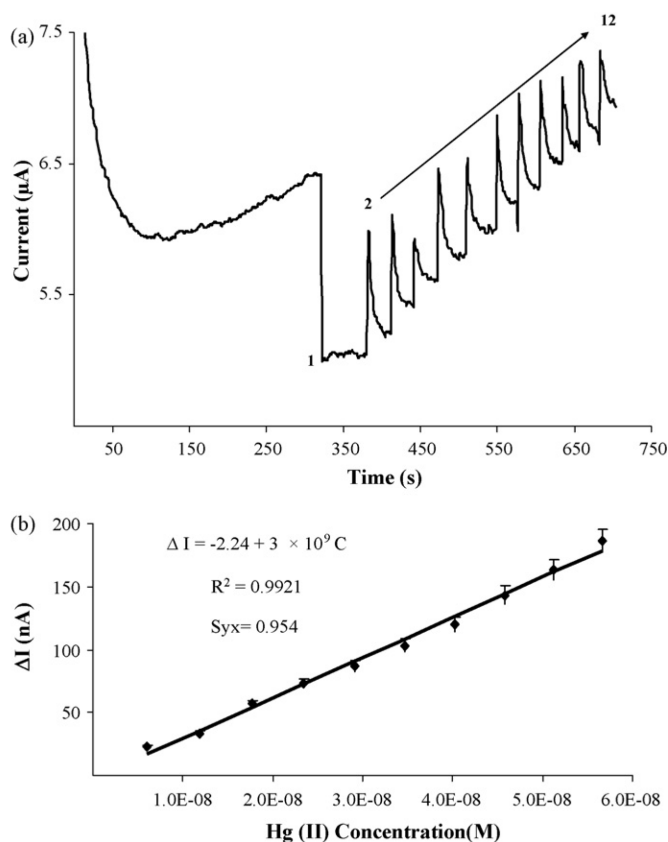

Fig. 2. Amperometric recording obtained at a: (a) urease/SPCE: (1) Addition of urea ((urea) = 0.33 M), (2–12) additions of 100 μL of a 10^{-4} M mercury (II) solution. (b) urease/AuNPs/SPCE: Relation between ΔI vs. [Hg (II)]. E_{ap} = 1.5 V vs. Ag/AgCl, pH = 7

Lineweaver–Burk [12,28]. It can be seen in Fig. 4 the noncompetitive character of the registered inhibition process.

For both types of biosensors, the dependence between ΔI and the Hg (II) concentration is linear as it is shown in Fig. 2. Several calibration curves were carried out in the concentration range from 2×10^{-6} M to 2×10^{-5} M for urease/SPCE and 6×10^{-9} M to 6×10^{-8} M for urease/AuNPs/SPCEs. The parameters of these calibrations and the standard deviation were evaluated. The existence of anomalous points would lead to incorrect adjustments, altering the sensitivity and the detection limit of the method. In order to avoid this problem least median squares regression (LMS) was used [21]. This methodology has been successfully used in relation to calibration problems [29], given that it helps detect outliers and makes it possible to identify a linear range if at least 50% of the data are aligned. The strategy followed consisted of two steps. In

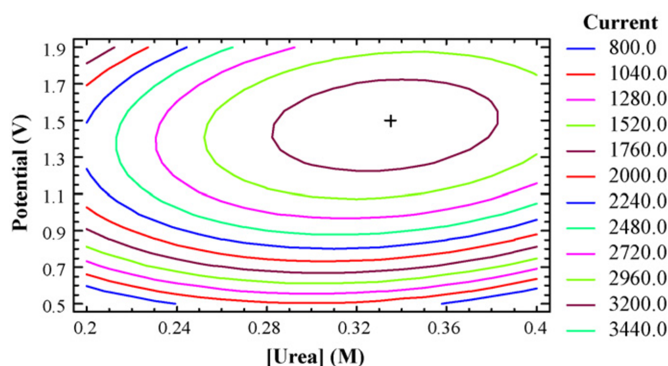

Fig. 3. Level curves for the response variable for the 2^3 central composite design.

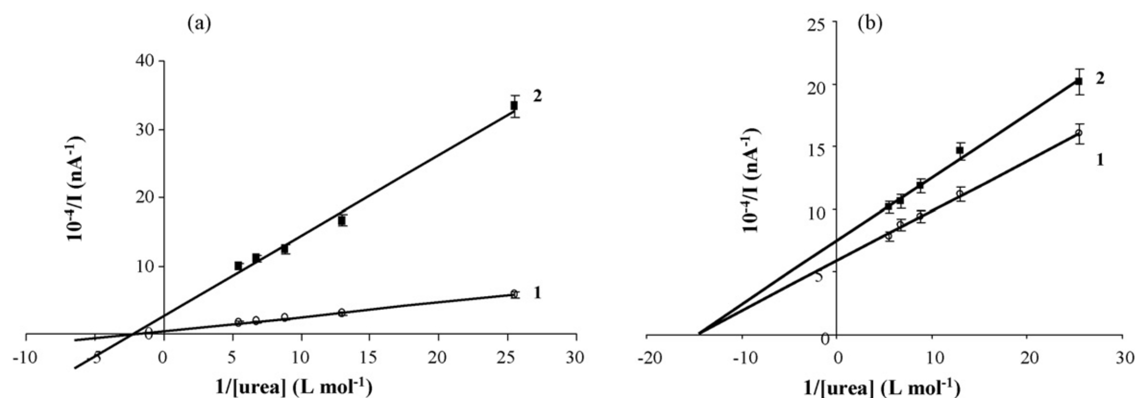

Fig. 4. Lineweaver–Burk plots for (a) urease/SPCE: 1. without Hg(II), 2. with 10^{-4} M Hg(II) and (b) urease/AuNP/SPCE: 1. without Hg(II), 2. with 10^{-5} M Hg(II).

the first, the LMS regression was used to detect anomalous points and, once they were eliminated a regression based on the ordinary least squared (OLS) criterion was worked out, in order to obtain the optimal precision and accuracy of both slope and intercept.

The LOD under the optimum working conditions was determined as $(4.2 \pm 0.2) \times 10^{-6}$ M for urease/SPCE and $(5.6 \pm 0.5) \times 10^{-8}$ M for urease/AuNPs/SPE ($n = 3$, $\alpha = \beta = 0.05$) using the DETARCHI program [22,30]. The value obtained with the urease/AuNPs/SPE biosensor resulted to be much better than the values calculated in previous amperometric urea biosensors where a detection limit of 3.2×10^{-7} M [15] and 7.4×10^{-6} M [13] were found for Hg (II) determination.

The repeatability of successive amperometric measurements with the same electrode surface was tested, conditioned for 5 min in a stirred phosphate buffer solution, pH 7, between experiments. Sets of five successive calibrations for Hg (II) were realized yielding a relative standard deviation (RSD) for their slopes of 5% for urease/SPCE and 6% for urease/AuNPs/SPCE. Likewise, the reproducibility of the amperometric signal was checked using the slopes of five regressions carried out with different electrode surfaces. The RSD values obtained were 3% and 5% for urease/SPCE and urease/AuNPs/SPCE respectively. These results suggest that the fabrication procedure of the urease based biosensors is reliable, and allows reproducible electroanalytical responses to be obtained with different electrodes constructed in the same manner.

3.1. Interferences

The possible effect of the presence of foreign ions in the solution was analysed for both type of biosensors. Zn(II), Cu(II), Cd(II), Fe(III), As(III), Pb(II), Cr(VI) and Ag(I) were studied. Only Ag(I) at concentrations higher than 10^{-5} M was found to have some influence, causing a fall in the urea response.

3.2. Analytical application

Determination of Hg (II) in spiked human plasma samples. From the above described results it can be deduced that the urease/AuNP/SPCE biosensor is more useful for the analysis of Hg (II) in terms of sensitivity. For this reason this sensor has been chosen for the analysis of Hg (II) in real samples.

The determination of Hg (II) concentrations in blood is currently the best way of monitoring individual uptake of Hg (II). For this reason, the proposed method was applied to the determination of Hg (II) in spiked human plasma samples.

The developed disposable biosensor was used for the analysis of Hg (II) in spiked human plasma samples (concentration of Hg (II) 1.0 μM). The concentration found for the spiked human sample was 0.99 ± 0.02 μM ($n = 3$, $\alpha = 0.05$, Recovery = 99%, RSD = 1%). This value closely agrees with the real one.

4. Conclusions

The use of urease based inhibitor biosensors using SPCEs and AuNPs/SPCEs allows selective amperometric determination of Hg (II) [31]. The main experimental variables were optimized by means of a central composite design founding values of 1.5 V, 0.33 M and 7 for E_{ap}, urea concentration and pH respectively as the optimum conditions for the analysis of Hg (II). The biosensors reproducibility and repeatability were studied obtaining values of RSD for the slopes of several calibrations lower than 6% in all cases.

The urease/AuNPs/SPCE biosensor resulted to be more sensitive in the analysis of this heavy metal. The method described in this paper presents several significant advantages, including sensitivity and selectivity, over previous amperometric urea biosensors developed for Hg (II) determination.

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