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Development of a Urea Bioprobe Based on Platinized Boron-Doped Diamond Electrodes

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Abstract
Urea (CH_6ON_2) is one of the main human nitrogen-based metabolic wastes. The concentration of urea in blood lies between 2.5–7 mM for healthy individuals, and is commonly used as an indicator for several diseases that may alter this value. Spectrophotometric methods are employed for the determination of blood urea concentration during clinical assays. Although these methods are sensitive, they make use of toxic reagents and complex reaction schemes. Therefore, in this research we present the bioelectrochemical determination of urea by the use of the protein urease (E.C.3.1.1.5) along with a nano-platinized boron-doped diamond electrode. This approach has been proven to be efficient and sensitive providing a platform with detection limits of 1.79 mM ($S/N=3$). The linear range resulted from 1 mM to 25 mM for the determination of urea, and response time of five minutes.

Keywords: Urea detection, Urease, Boron-doped diamond, Bioprobe

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1 Introduction

The human body produces a variety of biological wastes as a result of its numerous metabolic pathways. Protein metabolism is among the most common processes performed by the human body and in general, the majority of proteins are metabolized in the liver, where urea (CH_6ON_2) is produced as one of the main nitrogen-based wastes. The normal levels of urea in blood lie between 2.5–7 mM [1,2] for healthy individuals, and is commonly used as an indicator for several diseases. Low urea levels are mainly related to liver diseases or malfunctioning whereas high levels of urea are mainly associated to renal and coronary diseases [3]. Therefore, the monitoring of urea levels is directly related to kidney function. When chronic kidney malfunctioning occurs in patients, dialysis treatment is necessary and urea concentration should remain approximately in a range of 3–16 mM after such treatment [1,4].

Indeed, spectrophotometric techniques are mostly employed for the determination of urea levels. These techniques involve the degradation of urea via the enzyme urease (E.C. 3.5.1.5) to produce ammonia via hydrolysis [5]. The released ammonia from the enzymatic reaction should undergo several reaction steps in order to produce spectrophotometrically active species. The Nessler reaction (Equation 1) [6,7] and the Berthelot reaction (Equation 2) [8] are among the most accepted methods for sensitive and accurate urea determination.

$$NH_3 + 2K_2[HgI_4] + 3OH^- \rightarrow OHg_2(NH_2)I + 7I^{-+}2H_2O \quad (1)$$

$$NH_3 + OCl^- + 2OH^- + 2\text{Phenolate} \rightarrow \text{Indophenolate} + 3H_2O + HCl \text{ (with } [Fe(CN)_5ONO]^{4-}) \quad (2)$$

However these methods are far from providing fast and point-of-care testing since the methods are complicated and require highly trained professionals. Also, some of the compounds used in these reactions are quite toxic.

Therefore, strategies to provide sensitive and fast measuring while accomplishing point-of-care testing are being proposed from which amperometric biosensors shed a bright future. The most common urea amperometric biosensor is based on the interface of the enzyme urease and a selective electrode. The electrode can be selective to ammonium ion (NH_4^+) [1], ammonia [9,10], or to hydronium ion (H_3O^+) [11–13] depending on the working pH of the system since the amount of ammonia produced and its equilibrium with its conjugate acid will cause changes in the pH. These systems are designed by entrapping the enzyme in a polymer (e.g. polyvinyl alcohol or polyacrilamide) while casting the selective sensor or by microencapsulation of the enzyme [1,10–14]. Therefore, urea has to diffuse through the polymer to reach the enzyme, in order for hydrolysis to occur, and then the product must diffuse to travel through the polymer until the selective membrane to permeate through it. After

that, the analyte is detected and an analytical amperometric signal is measured. Generally, these systems have adequate working ranges to measure urea levels, ranging from 0.03–1 mM to 30–1000 mM [2,10,11,13]. However, a major disadvantage of these biosensors consists in that the detection is completely dependent on the diffusion of the species through the polymer. Also, another disadvantage is that the selective membranes are not fully selective, allowing similar species to the analyte permeate through them causing discrepancies in the measurements, which could provide a false positive result.

For these reasons, in this work we propose a urea bioelectrochemical probe consisting on a boron-doped diamond electrode with platinum nanoparticles deposited at its surface and the enzyme urease free in buffer solution. In this system, urea is hydrolyzed by urease in solution, where the diffusion barrier of urea to urease is expected to be considerably lower than through a polymer, what should improve the sensitivity. Then the ammonia is oxidized at the platinized boron-doped diamond electrode (Pt-BDD), creating an electrochemical signal, as follows:

$$2NH_3 + OH^- \rightarrow N_2 + 6H_2O + 6e^- \quad (3)$$

Therefore, the proposed bioelectrochemical probe should help to improve sensitivity and selectivity, in the measurements of urea levels, due to the high urease enzyme selectivity towards urea and the high sensitivity of the platinum nanoparticles to electroactive compounds, such as ammonia. Other advantage of this construction is that the boron-doped diamond electrodes have been proven to be highly conductive and resistant to nonspecific adsorption. These characteristics are known to minimize any background effects [15].

2 Experimental

2.1 Materials and Apparatus

Urea (ACS reagent, 99.0–100.5 %), urease (E.C.3.5.1.5, from *Canavalia ensiformis* (Jack bean) Type VII; initial activity of 400 000–800 000 units/g solid), potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4), sulfuric acid (H_2SO_4, TraceSELECT Ultra, $\geq$95 % (T)), nitric acid (HNO_3), hydrochloric acid (HCl), potassium chloride (KCl), potassium hydroxide (KOH), potassium ferricyanide ($K_3Fe(CN)_6$), potassium ferrocyanide ($K_4Fe(CN)_6$), ammonium sulfate ($(NH_4)_2SO_4$), phenol-nitroprusside, alkaline hypochlorite, creatinine (anhydrous) and potassium hexachloroplatinate IV (K_2PtCl_6, 99.99 + % trace metals basis) were all purchased from Sigma Aldrich (USA) and used without further purification. An HCP-803 potentiostat/galvanostat in low current mode from BioLogic USA, along with a common glass three-electrode cell system was used for all the electrochemical procedures. The boron-doped diamond (BDD) substrate (Element 6, 0.038–0.105 Ω, [B]: 10^{20} cm^{-3}) was used as the working electrode, Ag/AgCl (0.197 vs. NHE) as the reference electrode and a platinum wire as the counter electrode. A UV-Vis spectrophotometer from Shimadzu Co. was also used along with a 1 cm path length quartz cuvette.

2.2 Electrode Preparation and Characterization

First, the boron-doped diamond electrodes were oxidized by sweeping the cell potential between −1.0 V and 2.5 V vs. Ag/AgCl in a 0.5 M HNO_3 solution for 45 consecutive cycles at a scan rate of 100 mV s^{-1}. Thereafter, the electrode was washed several times with deionized water (Barnstead 18.2 MΩ) and dried under N_2 for further use. Then, the reversibility capacity of the BDD electrode was measured using the cyclic voltammetry (CV) technique. The potential was swept between −0.1 V and 0.6 V vs. Ag/AgCl in a 1 mM $K_3Fe(CN)_6$/1 mM $K_4Fe(CN)_6$/0.1 M KCl for a period of three cycles. This process was performed before and after the electrochemical oxidation. After the oxidation process, platinum was electrodeposited over the boron-doped diamond electrodes. Scans from −0.2 V and 1.0 V vs. Ag/AgCl in a 1 mM K_2PtCl_6/0.5 M H_2SO_4 solution at scan rate of 300 mV $s^{-1}$was performed to achieve electrodeposition. Then, the platinized boron-doped diamond (Pt-BDD) electrode was thoroughly washed with deionized water. Immediately after washing, a cyclic voltammetry was recorded by using a 0.5 M sulfuric acid solution to account for the cleanliness and electrochemical active area of the platinum deposits. Scanning electron microscopy (SEM) images were obtained using a JEOL 5800LV Scanning Microscope with an electron beam of 20 kV.

2.3 Ammonia Oxidation at Pt-BDD Electrodes (pH 8.3 and 7.4)

The electrochemical oxidation of ammonia at pH 7.4 and 8.3 was investigated with a 0.1 M $(NH_4)_2SO_4$. In brief, the linear polarization technique was employed from −0.3 V to 0.7 V vs. Ag/AgCl at 100 mV/s.

2.4 Urea Bioprobe Measurements

In order to measure the analytical amount of ammonia molecules being produced via the hydrolysis of urea from the enzyme urease (E.C.3.5.1.5) a calibration curve was performed (data not shown). The Weatherburn method was used following a similar procedure as published elsewhere [16,17]. In this assay, 50 μL of ammonia solution of different concentrations were mixed with 500 μL of phenol-nitroprusside and then with 500 μL of alkaline hypochlorite after vigorous shaking before each addition. These samples were incubated at 37 °C for 30 minutes and then measured at a wavelength of 630 nm in a UV-Vis Spectrophotometer. After having the calibration curve for ammonia we proceed with the enzymatic assay. For the enzymatic assay a protein final concentration of 0.02 mg/mL was selected as a first approach. For all ex-

periments a 100 mM phosphate buffer solution of pH 7.0 and 8.0 was used. To execute the urease assay 100 μL of a 0.2 mg/mL urease solution was mixed with final urea concentrations of 10, 50, and 100 mM with the vial completed to 1 mL with PBS. Aliquots of 50 μL of this mixture were extracted over time and mixed with the phenol-nitroprusside and sodium alkaline hypochlorite.

Therafter, to test the complete system, a 4 mg/mL stock solution of urease in 100 mM PBS was prepared fresh the day of the experiment. Then, 1.25 mL of the enzyme stock solution and different amounts of urea solutions in stock were poured into the electrochemical cell, completing with 100 mM PBS to a final volume of 5 mL in the cell. Therefore, the final concentration of urease in the cell was around 1 mg/mL, while the urea concentration ranged from 1 mM up to 100 mM. In order to measure only the faradic current (related to the transfer of electrons during an electrochemical chemical reaction) the differential pulse voltammetry (DPV) technique was employed. After letting the enzymatic reaction undergo for 5 minutes, a DPV was carried out from −0.1 V to 0.7 V vs. Ag/AgCl with a pulse height of 50 mV, pulse duration of 50 ms, step high of 2 mV and step duration of 400 ms.

Finally, in order to determine the specificity of the system, creatinine was added as interference due to its similarity. The experiments consisted in two parts, first the creatinine was measured in the system without urea, and then both urea and creatinine were measured together in order to observe the effect of its presence. A stock solution of 100 mM creatinine in 100 mM PBS was prepared to measure 50 mM creatinine in the presence of 0.5 mg/mL urease, 10 mM urea and completing with 100 mM PBS to a final volume of 5 mL in the cell. After letting the enzymatic reaction undergo for 5 minutes, a linear polarization was carried out from −0.3 V to 0.7 V vs. Ag/AgCl.

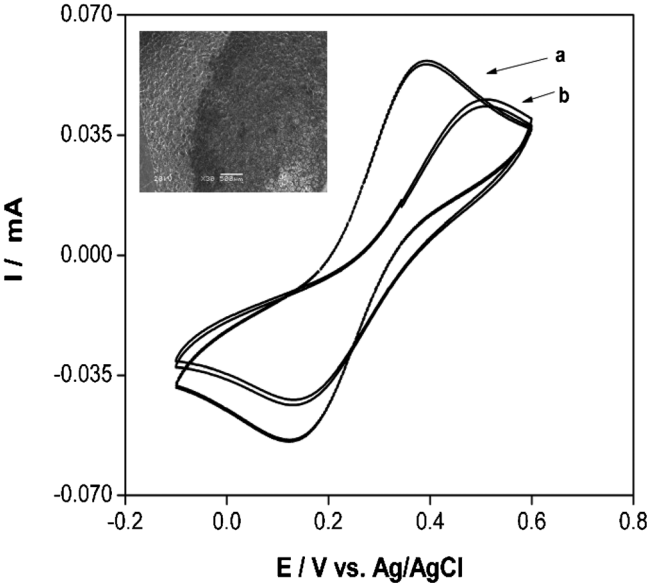

Fig. 1. Cyclic voltammograms of 1 mM $Fe(CN)_6^{3-/4-}$ in 0.10 M KCl at a boron doped diamond (BDD) electrode before and after the oxidation process at a scan rate of 100 mV/s. Inset: Scanning electron microscope image for the oxidized BDD electrode, at 20 kV and 30× magnification.

3 Results and Discussion

3.1 Electrode Characterization

Prior to electrochemical deposition, the boron-doped diamond electrodes employed in this research were anodically oxidized in order to create a hydrophobic layer, which provides a more suitable environment for metal deposition. As shown in Figure 1, after anodic oxidation of BDD the electrode showed an increment in current of 25% and better reversibility (0.51 V (non-oxidized) vs. 0.39 V (oxidized)) when tested with a redox couple of $Fe(CN)_6^{3-/4-}$ in 0.1 M KCl. Also, in this figure an SEM image is shown as an inset where it can be observed the difference in coloration of the oxidized part of the electrode (light circle). This data suggests that the electrode is highly conductive after oxidation and therefore is prepared for further platinum electrodeposition.

Afterwards, platinum was electrodeposited over the BDD previously oxidized by following procedures in Section 2.2. Figure 2 shows an SEM image of the platinized boron-doped diamond electrode (Pt-BDD), where the platinum nanoparticles are visible and it is also noticeable that the platinum nanoparticles showed preference for being electrodeposited on certain facets of the diamond. This preferential electrodeposition behavior has been the subject of investigation in recent years and it is mostly ascribed to the conductivity of the different facets of the BDD [18].

After each electrodeposition the Pt-BDD was accounted for the electrochemical active surface by calculating

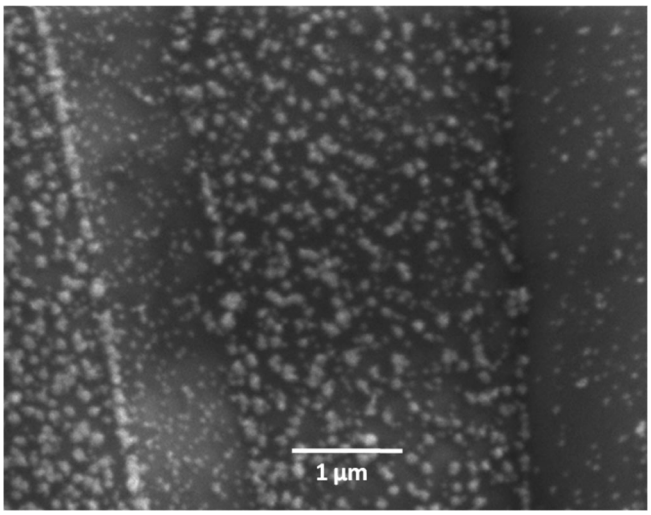

Fig. 2. Scanning electron microscopy image for the electrodeposited Pt nanoparticles at a boron doped diamond electrode, at 20 kV and 20 k× magnification.

the area under the hydrogen desorption peaks after running a 0.5 M H_2SO_4 cyclic voltammetry. Therefore, the results for the proposed sensor are normalized by the effective active area of platinum in the electrode, and shown as the current density, *j*. In general after each platinum electrochemical deposition an approximate area of 0.615 cm^2 is attained.

3.2 Ammonia Electrooxidation at Pt-BDD

As explained in Section 1, the proposed sensor employs the enzyme urease to convert urea to ammonia, and the ammonia molecules are then electrooxidized at the interface of the Pt-BDD. Therefore, in an effort to understand the ammonia electrooxidation profile at different pH's at the Pt-BDD two 0.1 M ammonium sulfate solutions at pH 7.4 and 8.3 were tested. The pH 7.4 was utilized because at this pH the enzyme has the highest activity according to the vendor, while pH 8.3 was chosen because at this pH the enzyme is expected to still have considerable activity and the ammonia/ammonium ion equilibrium is more favorable for the ammonia oxidation since the chemical equilibrium is shifted to the formation of ammonia. Figure 3 shows the linear polarization of the two 0.1 M $(NH_4)_2SO_4$ solutions at pH 7.3 and 8.3.

As can be observed the peak current density was higher for the solution with the higher pH level due to the expected higher concentration of ammonia due to shifting in chemical equilibrium. In addition, in this graph is noticeable that the oxidation potential for ammonia has shifted to lower values as the pH increase, though this is predicted by the Nernst equation. Thus, due to the fact that a higher signal-to-noise ratio can be obtained at pH 8.3 the sensor experiments were executed at such pH. As stated above, this pH value satisfies the necessity of a sensitive sensor without losing all enzyme activity, which is critical for a successful biosensing system.

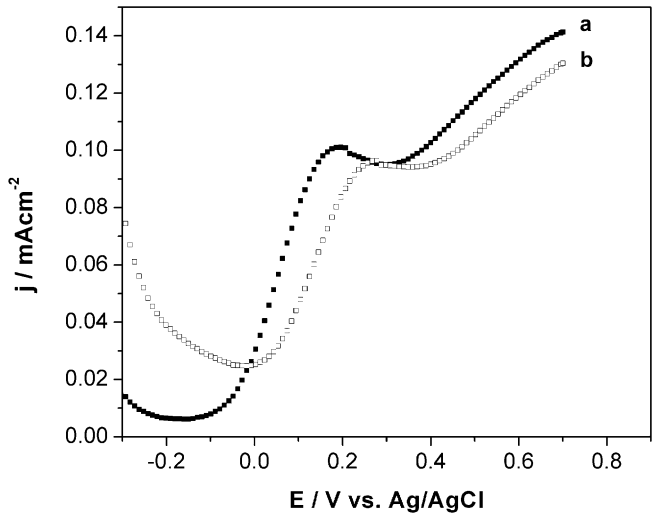

Fig. 3. Linear polarization of the ammonia oxidation at Pt nanoparticles at a boron doped diamond electrode in an ammonium sulfate solution in phosphate buffer solution at pH 7.4 and 8.3. Scan rate of 100 mV/s.

3.3 Urea Bioprobe Experiments

The biomolecule activity and its ability to recognize an analyte is perhaps one of the most critical features of a biosensig device. Thus, the activity of the enzyme urease was determined via the Weatherburn Method. During the assay, the enzyme's activity was measured for two different urea concentrations, 5 mM and 100 mM, and different pH 7.4 and 8.3. As expected, it was found that the enzyme's activity with a urea concentration of 100 mM (13.0 ± 0.3 μmol ammonia/min mg protein at pH 8.3) was twice of the activity of that at 5 mM (6.5 ± 0.2 μmol ammonia/min mg protein at same pH), at any given pH. Also, the higher activity was found for the assay performed at pH 7.4 in comparison to pH 8.3 at both concentrations. Once the biochemical and electrochemical parameters were optimized, the proposed device was tested as a single unit. Here, the urea degradation by urease and the in-situ electrooxidation ammonia was carried out and assessed. For these experiments, all solutions were prepared in phosphate buffer at pH 8.3 and the final concentration of the enzyme in the electrochemical cell was approximately 1 mg/mL after correction by UV-Vis at 280 nm.

Different urea solutions ranging from 1 mM to 100 mM were used in order to test the proposed sensor by means of the DPV technique. In Figure 4 the DPV for the phosphate buffer blank (a), a 100 mM urea solution (b), a 1 mg/mL urease solution (c), and a 100 mM urea-urease system (d) are presented. Before any voltage is applied to the electrochemical cell, the biochemical reaction between the substrate (i.e. urea) and the enzyme was allowed to equilibrate for five minutes for the enzyme to start degrading the urea, producing ammonia in order to have a measurable current.

As can be observed, a different current-potential profile is obtained when comparing the urea-urease system against the blanks. In the graph corresponding to the urea-urease system (Fig. 4d), a distinguishable peak was observed around 0.0 V vs. Ag/AgCl, which belongs to the ammonia oxidation. For the urea and enzyme blanks, this peak was more like a shoulder with negligible current. Also, in these two blanks as in the urea-urease experiments, a broad peak was observed between 0.2 V and 0.7 V vs. Ag/AgCl, therefore it may be related to the buffer, a common specie for all solutions. However, in the buffer blank a strong peak was observed, suggesting that the presences of other species are able to attenuate the buffer signal. After testing the proposed device, two important features of the sensor were determined, *sensitivity* and *selectivity*. First, the sensitivity indicates the minimum measurable urea concentration and the signal variation due to increasing urea concentration. To determine the sensitivity of the sensor the construction of a calibration curve was performed. The differential pulse voltammetry

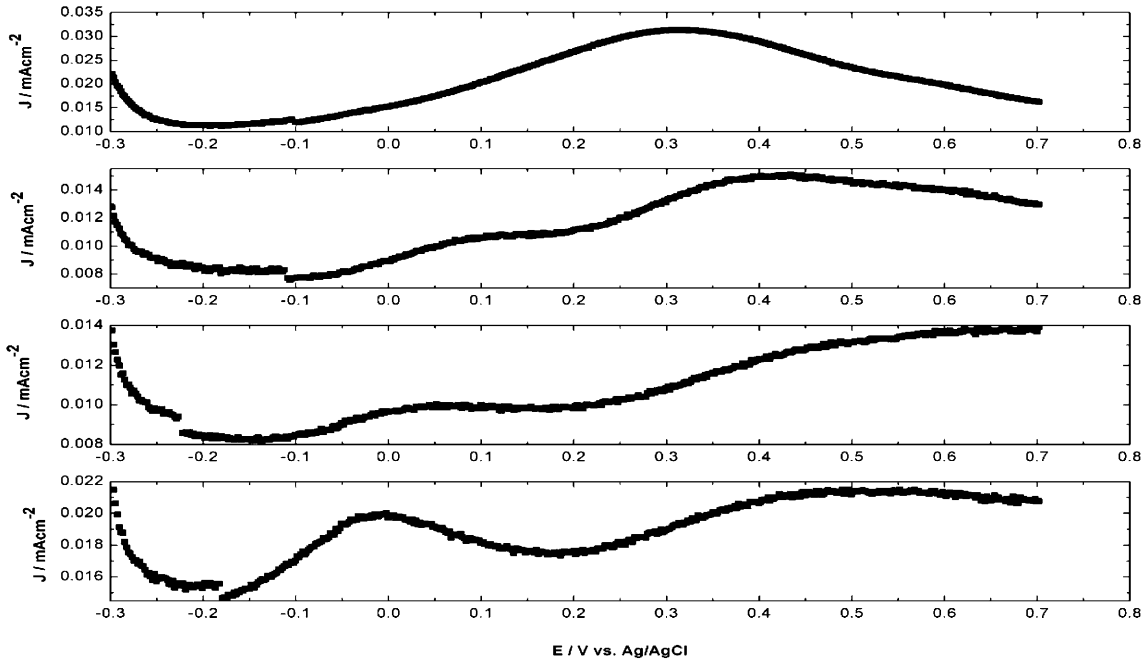

Fig. 4. Differential pulse voltammetry: (a) blank buffer, (b) 100 mM urea solution, (c) 1 mg/mL urease solution and (d) 100 mM urea-urease system at pH 8.3. Pulse height of 50 mV, pulse duration of 50 ms, step height of 2 mV, and step duration of 400 ms.

(DPV) was used as a very sensitive method with sub-micromolar detection limit Thus, for such calibration curve urea solutions ranging from 1 mM to 25 mM were analyzed in the bioprobe device. The results showed an increment in the anodic wave due to increasing concentration of urea as can be observed in Figure 5a.

A linear dynamic range from 1 mM to 25 mM with a detection limit of 1.79 mM ($S/N=3$) is obtained for urea detection with a slope of 0.343 μA cm^{-2}/mM and a correlation coefficient (R^2) of 0.9980, see Figure 5b. The results of the analytical determination of urea by this method are sensitive enough to enable the construction of biosensors for clinical and analytical applications. In fact, the sensitivity of this system is similar to other urea bioprobes and biosensors reported in the literature [19–21]. Although more recent investigations have shown the enhanced urea determination to limits of detection of 10 μM and linear range of 20 mM due to the use of hybrid materials containing nanofiber[22].

3.4 Selectivity Measurements (Interference)

As abovementioned, two important features for any sensor are, *sensitivity* and *selectivity*. The sensitivity of the proposed sensor was determined previously with the calibration curves. Now, in order to determine the selectivity of the sensor, a compound similar in size and composition to urea is measured. From the different nitrogenous based compounds, creatinine was chosen to be studied as interference for the urea sensor, due to its similar size and structure to urea, but also for its presence in blood (i.e. 50–130 μM) [23,24]. Another important argument to use creatinine as an interferent is that this compound is found in concentrations that are ten times higher for patients with kidney problems [25].

Creatinine, as urea, is not an electroactive specie as ammonia. Therefore when a solution of creatinine was measured in the biosensor no significant current was obtained, as with blank urea. Then, when a mixture of creatinine and urease was measured in the sensor, no current was obtained, meaning that the enzyme did not produce any ammonia because its selectivity towards urea. Finally, it was found that creatinine does not inhibit the enzyme's activity, since urease was able to degrade urea in the presence of creatinine, where a significant current was measured.

4 Conclusions

A bioelectrochemical device capable of measuring urea at levels of clinical importance was developed. The biosensor developed is capable of measuring urea levels as low as 1.79 mM which is in the normal range (i.e. 2.5–7 mM) for a healthy individual. Moreover, the linear range presented by this sensor enables measurements of higher urea levels. Another analytical importance feature of the present method is that the presence of other similar molecules does not show considerable effects on the anodic peak current of ammonia. Therefore, the presented biosensor can be considered as a very sensitive and reasonably selective approach for urea determination.

Further investigations will assess the selectivity of the sensor in presence of complex matrixes such as blood.

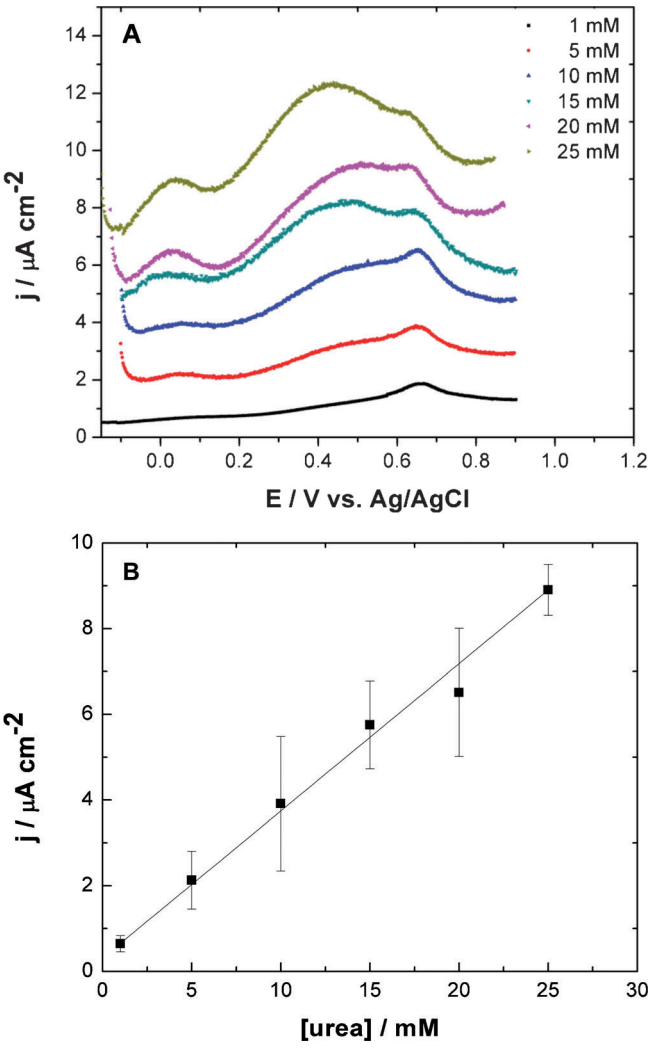

Fig. 5. (a). Differential pulse voltammetry of the ammonia oxidation for urea solutions of increasing concentration. Pulse height of 50 mV, pulse duration of 50 ms, step height of 2 mV, and step duration of 400 ms. (b). Calibration curve for the biosensor, with linear range from 1 mM to 25 mM and detection limit of 1.79 mM ($S/N=3$), slope = 0.343 μA cm^{-2}/mM. The data was obtained from (a).

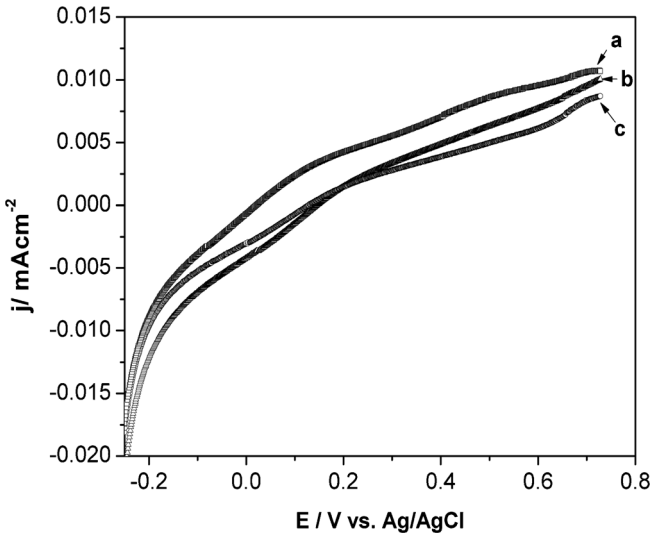

Fig. 6. Creatinine linear polarization: (a) 50 mM of creatinine, 10 mM of urea, and 0.5 mg/mL of urease in 100 mM PBS solution, (b) 50 mM of creatinine 100 mM PBS solution, (c) 50 mM of creatinine and 0.5 mg/mL of urease in 100 mM PBS solution. Scan rate of 5 mV/s.

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