Amperometric urea biosensor based on immobilized urease on polypyrrole and macroporous polypyrrole modified Pt electrode

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Abstract: Biosensing of urea by a biosensor as a direct detection method at ambient temperature and pressure instead of chromatography leads to a significant reduction in processing costs. Amperometric biosensors based on urease immobilization on macroporous polypyrrole (MPPy) and pyrrole on the surface of a Pt electrode were developed. Applying cyclic voltammetry (CV), we demonstrated the synthesis of MPPy using monodispersed polystyrene spheres (460 nm) as a template. CV and chronoamperometric studies were conducted to evaluate the electrochemical current of the modified electrodes. For the electrode with polypyrrole (PPy), the biosensor response was linear in the range of 1.67–8.32 mM ($R^2 = 0.99$). Sensitivity, detection limit, and response time of this biosensor were 0.0035 mA mM^{-1}, 2.57 mM, and ∼7 s, respectively. For the electrode with MPPy, the linear range was 0.5–10.82 mM ($R^2 = 0.99$). For this biosensor, sensitivity, detection limit (S/N = 3), and response time were 0.0432 mA mM^{-1}, 0.208 mM, and ∼5 s, respectively. The modified biosensor with MPPy showed high stability and desirable selectivity for urea.

Key words: Cyclic voltammetry, electrochemical biosensor, enzyme activity, macroporous polypyrrole

1. Introduction

To improve the quality of human life, control diseases, and evaluate pollution of surface water, it is very crucial to have a low-cost, stable, and selective tool (biosensor) for real-time monitoring of urea. Excess of fertilizers, which contain urea, remains in the soil and contaminates surface water during precipitation and irrigation. The normal range of urea in human blood serum is 2.5–7.5 mM [1,2]. High levels of urea in blood serum can be due to renal failure, shock, dehydration, burns, urinary tract obstruction, and gastrointestinal bleeding. Nephritic syndrome, hepatic failure, low-protein diets, and high-carbohydrate diets cause low concentrations of urea [3–5].

The concentrations of analytes with biosensors were measured by transducers, including potentiometric, amperometric, piezoelectric, thermal, and optical transducers [6–12]. Immobilized urease on various supports as a biocatalyst converts urea to ammonium (NH_4^+) and bicarbonate (HCO_3) ions. These ions change the electrical properties of the sample due to an increase in the pH of the solution according to the Nernst equation, which subsequently gives an electrical signal [2,13–15].

$$CO(NH_2)_2 + 3\,H_2O \xrightarrow{\;Urease\;} 2NH_4^+ + HCO_3 + OH \qquad (1)$$
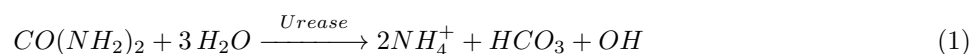
The concentration of the urea in a sample is measured by monitoring the liberated ions using a transducer.

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These ions are not electroactive; thus, they should be oxidized to other species. Some catalytically active supports can interact with these ions [6,7,13,16,17]. A suitable urease immobilization support must have high conductivity, high surface-to-volume ratio, and low enzyme leaching. Nanoscale conductive polymers with high surface-to-volume ratio and good conductivity are a suitable option as support. Polypyrrole (PPy) is a conductive polymer that can be synthesized using chemical and electrochemical methods in aqueous or a nonaqueous media in a wide range of pH [18–20]. Due to biocompatibility [21], conductivity [22], the possibility of synthesis at relatively low potentials (which means less energy consumption) [23], and, most importantly, the ability to interact (reversible deprotonation) with ammonium [24–26], PPy is a suitable option for urea biosensing applications. PPy electropolymerization with cyclic voltammetry (CV) creates a uniform PPy film on an electrode surface with a positive charge [27]. Since at pH values higher than the isoelectric point, urease has a negative charge, it can be adsorbed by electrostatic forces [28]. The enzyme loading on the PPy can be increased by synthesis of the nanoscale PPy on the surface of the electrode as a matrix. Nanoscale PPy (macroporous PPy) provides more sites for enzyme immobilization, preventing loss of activity and leaching of the enzyme. In order to improve the efficiency and stability of the immobilized enzyme on the surface of the electrode, a cross-linking agent (glutaraldehyde (GA)) can be used. Free amino groups of enzymes react with GA and can form a stable and very strong bonding between enzymes.

This work focuses on the development of an amperometric biosensor based on macroporous polypyrrole (MPPy) and PPy. The enzyme was immobilized by GA as a cross-linking agent (GA-Urs) and the electrostatic force on the MPPy (Pt/MPPy/GA-Urs) and PPy (Pt/PPy/GA-Urs). The results of the Pt/MPPy/GA-Urs electrode were compared against those of the biosensor with PPy as an immobilization matrix. The MPPy and PPy film morphology was evaluated using a field emission scanning electron microscope (FESEM). The electrochemical responses of the electrodes were studied by CV and chronoamperometry. The purpose of the present work is to develop urea biosensors based on MPPy and PPy, and to compare their performances. The Pt/MPPy/GA-Urs electrode showed good performance compared to the Pt/PPy/GA-Urs electrode in terms of selectivity for urea, stability, and repeatability.

2. Materials and methods

2.1. Reagents and chemicals

Pyrrole monomer, urease (EC 3.5.1.5) from *Canavalia ensiformis* (jack beans) with activity in the range of 50,000–100,000 U/g, sodium dodecyl benzenesulfonate, monodisperse polystyrene spheres (460 nm diameter), and Nafion (5 wt.% solution in a mixture of lower aliphatic alcohols and water) were purchased from Sigma and used as received. The other reagents, such as urea, Nessler's reagent, Triton X-100 (tetra-octyl-phenoxy polyethoxy ethanol), and GA (50% w/v aqueous solution), were purchased from Merck Company (Darmstadt, Germany).

Phosphate buffer saline solutions (PBS; 0.1 M, pH 7.0) were freshly prepared with sodium phosphate monobasic anhydrous, sodium phosphate dibasic dihydrate, and NaCl in deionized water (resistivity = 18.2 $M\Omega$ cm^{-1}). The enzyme solution was freshly prepared in phosphate buffer (0.1 M, pH 7.0). Urea solution (1 mg/mL) was prepared by dissolving 1 g of urea in 1 L of deionized water. Other concentrations were obtained by serial dilutions.

2.2. Instrumental

All electrochemical measurements were performed in a standard three-electrode cell. A platinum wire electrode as a counter electrode, $Ag/AgCl/Cl_{(sat)}$ as a reference electrode, and a platinum plate electrode (4 mm in diameter) as a working electrode were used both in the chronoamperometry and CV. CV tests, urea sensing, and electropolymerization of pyrrole monomer were conducted with equipment from Bio-Logic Science Instruments Co. (Paris, France), which was connected to a computer for data acquisition. A FESEM (RIRA3, TESCAN, Czech Republic) was employed to study the morphology of the surface of the modified electrodes.

The apparent enzyme activity of the Pt/MPPy/GA-Urs bioelectrode was studied using Nessler's reagent and a UV-Vis SPEKOL 1500 spectrophotometer (Analytik Jena AG, Germany) at a wavelength of 425 nm.

2.3. PPy polymerization

Electropolymerization of pyrrole monomers was carried out by CV in a cell that contained 10 mL of the buffer consisting of 0.1 M NaCl, 50 mM monomers, and 25 mM sodium dodecyl benzenesulfonate [29].

Prior to electropolymerization, the electrodes were polished and rinsed thoroughly with deionized water to obtain a mirror surface. Then the electrodes were conditioned with CV in 0.5 M H_2SO_4 (–0.25 to 1.2 V, 30 cycles, 100 mV s^{-1}).

2.4. Preparation of MPPy

Polystyrene spheres were assembled over the surface of the working electrode by adding 10 μL of aqueous polystyrene suspension (0.5 wt.% aqueous suspension of polystyrene containing 1.0×10^{-6} M Triton X-100). Triton X-100 is a nonionic surfactant that improves the stability of monodisperse polystyrene particles [30]. At high concentrations of surfactant, coagulation rather than stability is induced. Therefore, according to the literature, 10^{-6} M Triton X-100 was used [31]. The aqueous suspension was placed in an ultrasound bath for 5 min before use. For water evaporation, the electrode was left in a saturated-humidity chamber. Then the same volume of the polystyrene suspension was deposited over the electrode. The electrode was then left in the saturated-humidity chamber and was finally placed in an oven at 100 °C for 4 h [31]. Afterward, electropolymerization of pyrrole monomers was performed by CV (20 cycles, 100 mV s^{-1}, –1.0 to 1.0 V) in 10 mL of the buffer containing 0.1 M NaCl, 50 mM monomers, and 25 mM sodium dodecyl benzenesulfonate. After electropolymerization, the polystyrene beads were removed by soaking the electrode in toluene for 24 h under stirring [31]. Then the electrode was rinsed with distilled water, followed by drying at room temperature under flowing nitrogen.

2.5. Enzyme attachment

GA contains an aldehyde functional group; when it is exposed to urease that has an amine functional group, and the aldehyde group will covalently bind to the amine group of urease [32]. The electrodes with the polymer (PPy or MPPy) were placed in a 1% GA solution and kept under stirring for 1 h. Next, the electrodes were rinsed with deionized water and dipped into a 50 mg/dL urease solution for 4 hours at 4 °C. The electrodes were then left at room temperature until dry. The amount of the enzyme on the surface of the electrode was controlled by the exposure time of the electrodes to the urease solution and also by the enzyme concentration in the solution. After preparing the electrodes, they were kept at 4 °C in PBS until use.

2.6. The activity of immobilized enzyme

The activity of the immobilized enzymes was evaluated by using Nessler's reagent and a UV-Vis spectrophotometer [16,33]. The modified electrodes were dipped for 20 min in 10 mL of urea solution (urea in PBS (0.1 M, pH 7.0)) at a certain concentration of urea, which contained 400 μL of Nessler's solution. Ammonia reacts with Nessler's reagent ($Hg^{II}I_4^2$) and forms a colored product according to the following equation [13]:

$$2\,Hg^{II}I_4^2 + 2\,NH_3 \rightarrow NH_2Hg_2I_3 +\ NH_4^+ + 5\,I \tag{2}$$

Absorbance of the colored species ($NH_2Hg_2I_3$) was measured at λ_{max} 425 nm for every 1 min of incubation for a total of 20 min. The activity of the immobilized urease was obtained by evaluating the rate of urea hydrolysis and ammonia formation using Eq. (3) [16,33,34]:

$$\alpha_{app}^{enz}\left(U\,cm^2\right) = \frac{AV}{\varepsilon\,s\,t} \tag{3}$$

Here, A is the difference in absorbance before and after incubation, V is the total volume of solution (10.04 cm^3), ε is the millimolar absorption coefficient of Nessler's reagent at 425 nm, t is the reaction time (1 min), and s is the surface area of the working electrode (0.1256 cm^2).

2.7. Morphological features

The morphological features of the electrodes were investigated using a high-resolution FESEM.

2.8. Electrochemical measurement

Chronoamperometric measurements were conducted to measure the amount of urea in the sample. The urea solution was prepared in 0.1 M phosphate buffer (pH 7.0). To do so, we used the developed electrodes, Pt/MPPy/GA-Urs or Pt/PPy/GA-Urs, as the working electrodes at fixed potential of +0.3 V vs. reference electrode (RE) for chronoamperometry [31]. Polymerization of the pyrrole was carried out with CV within the potential range of –1.0 to 1.0 V vs. RE at the scan rate of 100 mV s^{-1} [15]. The recorded current was directly related to the urea hydrolysis by urease. All experiments were performed at room temperature.

3. Results and discussion

3.1. Preparation of Pt/MPPy/GA-Urs biosensor

Pyrrole was electropolymerized by CV (20 cycles, at 100 mV s^{-1}) on the surface of a Pt electrode (4 mm in diameter). This electropolymerization was carried out in phosphate buffer containing 0.1 M NaCl, 50 mM monomers, and 25 mM sodium dodecyl benzenesulfonate. The cyclic voltammograms of the electrode are presented in Figure 1. This figure shows that PPy is growing on the surface of the electrode by increasing the number of potential cycles.

The second step of the biosensor fabrication was immobilizing the enzyme. The electrode from the previous step was placed in the GA solution, then in the urease solution, and finally allowed to dry. Figure 2 shows a schematic diagram of the fabrication procedure of the Pt/MPPy/GA-Urs electrode.
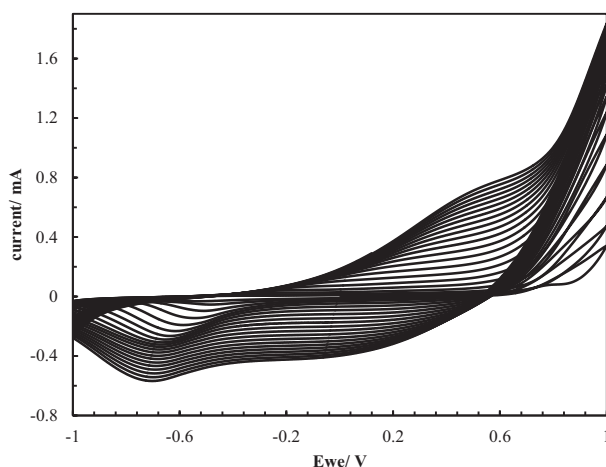
Figure 1. Cyclic voltammograms of the pyrrole polymerization in phosphate buffer (pH 7.0, 0.1 M) containing 0.1 M NaCl, 50 mM monomers, and 25 mM sodium dodecyl benzenesulfonate with scan rate of 100 mV s^{-1}.

3.2. Morphological analysis

A FESEM image of the enzyme is shown in Figure 3. This figure shows nonuniform porous structures. This porosity allows ammonium to diffuse through the surface of the support matrix and then to react with PPy. This reaction transfers electrons to the Pt electrode and results in an increase in electrochemical current response.

Figure 4A depicts a FESEM image of MPPy as a suitable substrate for the immobilized enzyme. For the synthesis of PPy, polystyrene beads (460 nm) were used as a template. Since MPPy has a nanosize structure, the available surface for enzyme immobilization in MPPy is greater than that of PPy as a matrix (compare the surface of the PPy in Figure 4A with 4B). The pore size of PPy in MPPy was directly determined by the diameter of the template particles (460 nm). After immobilizing the enzyme, the pore size decreased to 230 nm (see Figure 4A). Increasing the enzyme loading increased the rate of the urea hydrolysis, which in turn resulted in larger current. Moreover, due to the nanostructure matrix that provided more redox active sites (the electroactive area increased), the response time deceased.

3.3. Activity of the immobilized urease on Pt/MPPy/GA-Urs

The enzyme activity was evaluated using photometric analysis. In this method, the liberated ammonium in the sample was determined by the reaction of ammonium with Nessler's reagent. Figure 5 shows the liberated ammonium and the urea hydrolysis for 20 min. The obtained enzyme activities for the immobilized enzymes on Pt/PPy/GA-Urs and Pt/MPPy/GA-Urs electrodes were 0.64 relative and 0.78 respectively. Figure 5 demonstrates that the enzyme activity decreased after being immobilized on the substrate. The results show a 40% activity loss due to immobilization (on the Pt/MPPy/GA-Urs electrode) compared to the free enzyme. This decrease in activity can be attributed to the toxicity effect of GA. Moreover, covalent binding immobilization can influence the enzyme activity via the active conformation during immobilization [35]. Decrease in activity is lower for the Pt/MPPy/GA-Urs electrode since the MPPy electrode has a nanoscale porosity that provides a larger surface area. Due to the high surface to volume ratio of the MPPy, more enzyme was immobilized; consequently, more enzyme per unit surface area of the electrode was active.
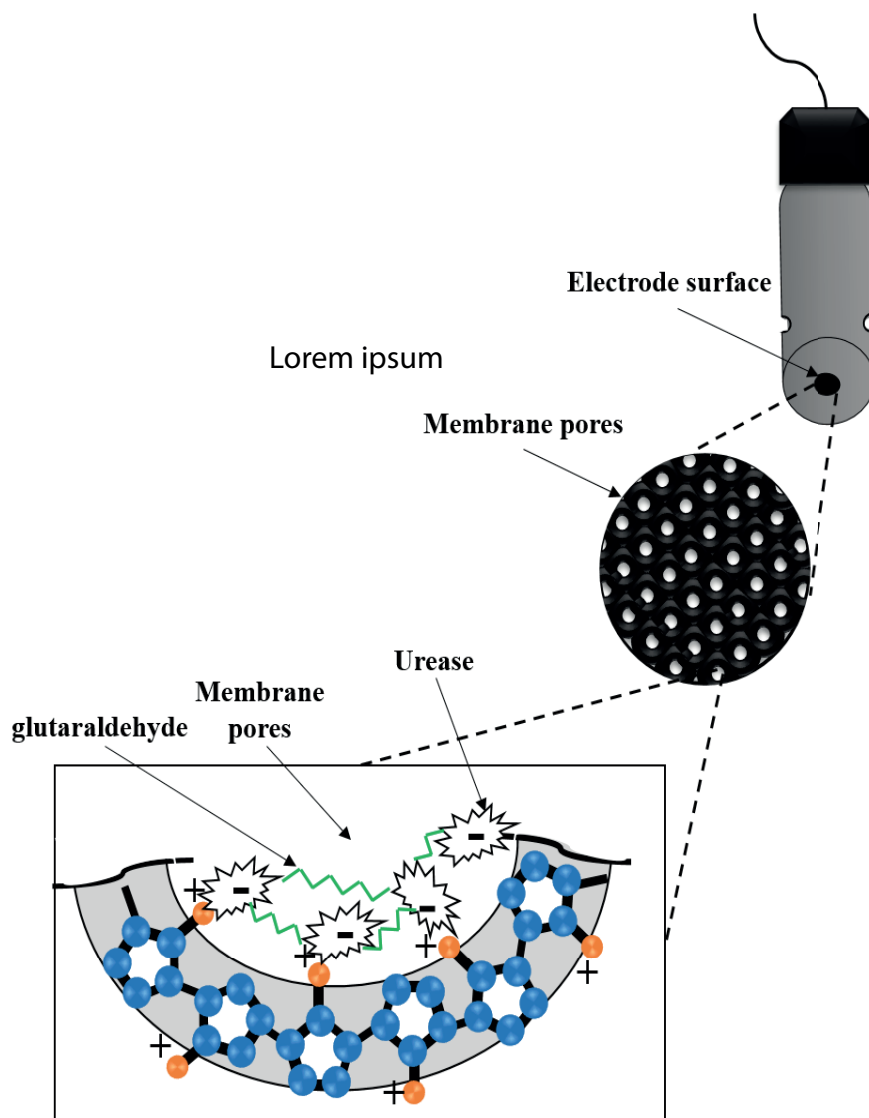
Figure 2. Schematic of the fabrication of Pt/MPPy/GA-Urs electrode.

3.4. Biosensor performance

Figure 6 shows the cyclic voltammograms of the Pt/PPy and Pt/MPPy as working electrodes in 10 mL of PBS (0.1 M, pH 7.0, 0.1 M NaCl). The obtained current and the anodic peak area of the Pt/MPPy electrode were larger than those of the Pt/PPy electrode. The available surface of the Pt/PPy electrode was significantly smaller than that of the Pt/MPPy electrode and the current response of the former was negligible. On the other hand, the porous structure of the Pt/MPPy electrode provides more positions for deprotonation and makes diffusion of the analyte that contains the target material easier. Therefore, saturation state occurs later, and consequently the biosensor can measure a broader range of the target concentration.

To obtain the background current, a chronoamperometry test was performed in the buffer solution. After 120 s, the current reached steady state, and then a certain amount of the urea stock solution was added and the current was recorded versus time. PPy at 0.3 V acts as a transducer for the electrochemical production of nitric
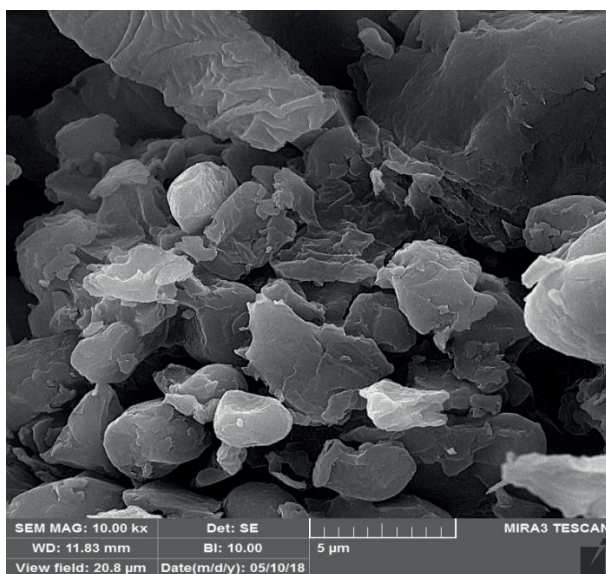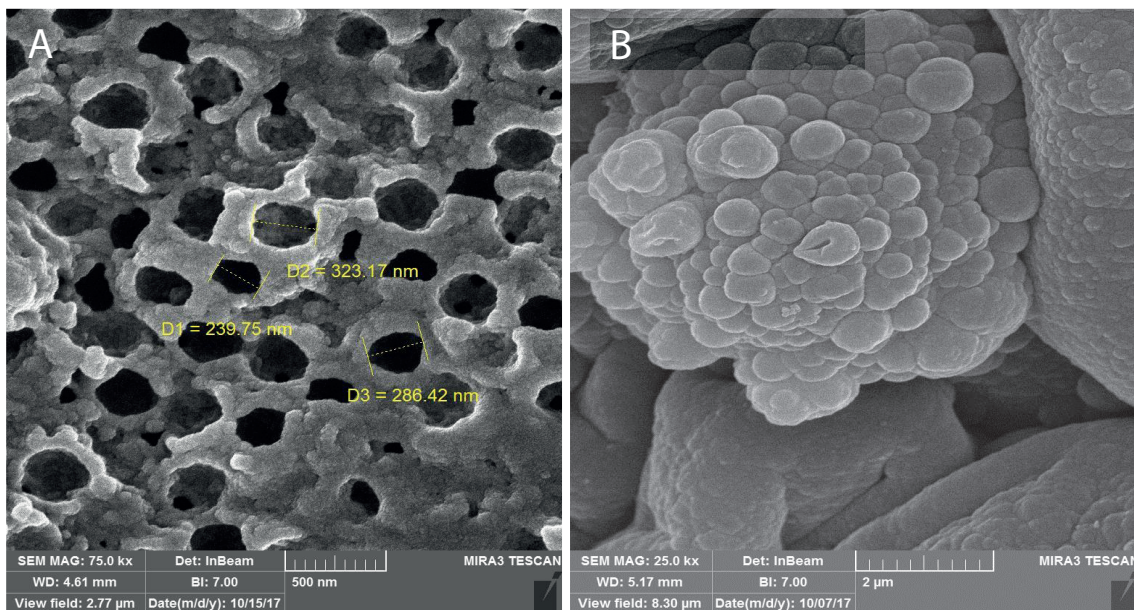
Figure 3. FESEM image of the enzyme.

Figure 4. FESEM image of (A) MPPy coated on the surface of Pt electrode, using polystyrene beads size of 460 nm in diameter and immobilized urease; (B) PPy coated on the surface of Pt electrode by CV at –1.0 to 1.0 V and scan rate of 100 mV s^{-1}.

oxide from ammonia, which is the product of urea hydrolysis by urease. Therefore, the amperometric response current was obtained at 0.3 V using the Pt/PPy/GA-Urs or Pt/MPPy/GA-Urs electrodes. All experiments were conducted at room temperature at applied potential of $+0.3$ V vs. RE and 150 rpm agitation.

As illustrated in Figures 7 and 8, increasing the urea concentration increases the current until it reaches a constant value due to the saturation condition. Linear behavior was observed in the range of 10–50 mg/dL (1.67–8.32 mM) for Pt/PPy/GA-Urs and 3–65 mg/dL (0.5–10.82 mM) for Pt/MPPy/GA-Urs. Sensitivity of
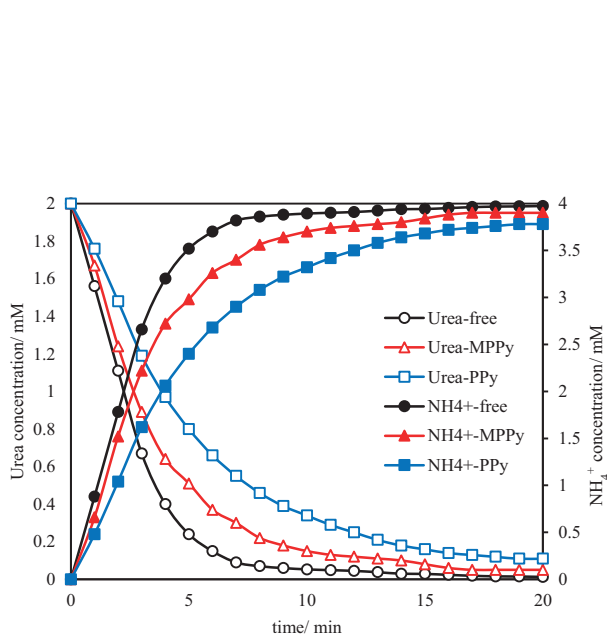
Figure 5. The urea consumption and NH_4^+ liberated with time for free enzyme in the solution and immobilized enzyme on the PPy and MPPy matrixes.
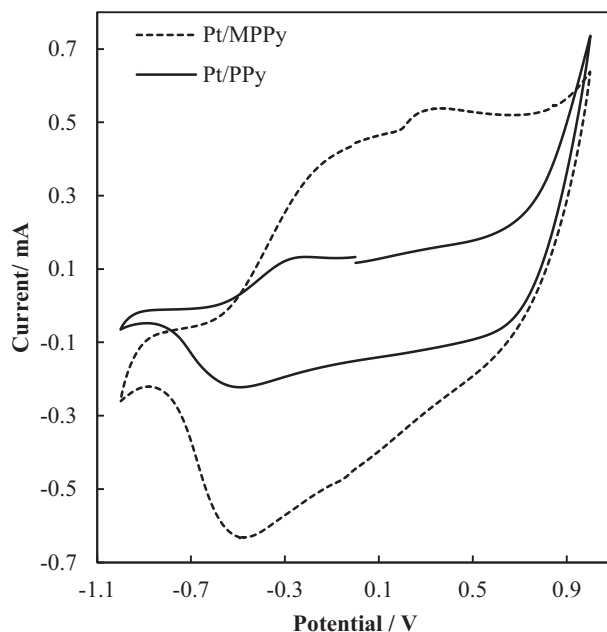

Figure 6. Cyclic voltammograms of the Pt/PPy (—) and Pt/MPPy ($\cdots$) electrodes in a PBS buffer (0.1 M, pH 7.0, 0.1 M NaCl) at the scan rate of 50 mV s^{-1}.

0.0035 and 0.0432 mA mM^{-1} were obtained for Pt/PPy/GA-Urs and Pt/MPPy/GA-Urs, respectively. The detection limits for Pt/PPy/GA-Urs and Pt/MPPy/GA-Urs were 2.57 mM and 0.208 mM, respectively.

The effect of pH on the behavior of the bioelectrode was studied with 0.1 M PBS buffer. Figure 9 shows that the maximum current occurs at pH 7.0 and 7.5 for PPy and MPPy, respectively. Since the maximum enzyme activity for MPPy happens at pH of 7.5, which is closer to the pH of human blood (7.3–7.4), this clearly demonstrates the suitability of MPPy for clinical applications.

3.5. Shelf time, reproducibility, and operational stability studies

The Pt/MPPy/GA-Urs biosensor showed a 4% loss in current response after being used 6 times. Its loss in the current became 7% after a month (at 4 °C in 0.1 M PBS, pH 7.0).

To examine the biosensor for reproducibility, the electrode was modified under identical conditions several times. The modified electrodes were tested in 5 mM urea solution. The achieved results with 4.3% relative standard deviation showed that the biosensor had acceptable reproducibility.

3.6. Interference study

The effect of interferents (glucose, uric acid, ascorbic acid, and triglyceride) was studied to evaluate the behavior of the biosensor in clinical applications. According to the results in the Table, adding the interfering species to 1.67 mM urea solution did not significantly change the response current. The difference in response current of the biosensor in the presence of the interfering species, which is very small, is also provided in the Table.
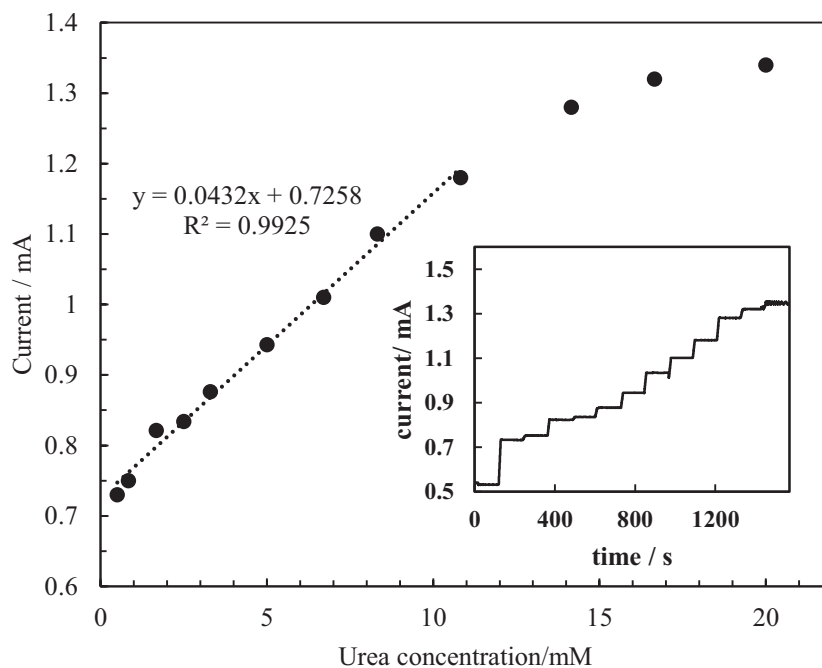
Figure 7. Calibration curve for Pt/MPPy/GA-Urs by continuous addition of urea at constant potential of $+0.3$ V. Inset: Chronoamperometric response of the Pt/MPPy/GA-Urs electrode to successive injection of urea into 10 mL stirring 0.1 M PBS at an applied potential of $+0.3$ V.
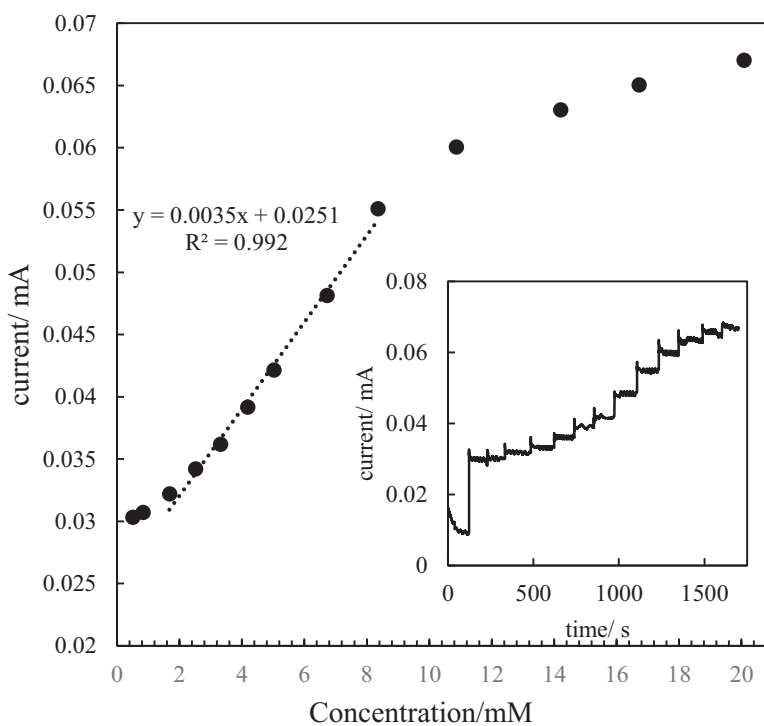

Figure 8. Amperometric response of Pt/PPy/GA-Urs electrode with concentration of urea ranging from 0.83 to 20 mM at working potential of $+0.3$ V. Inset: Chronoamperometric response of the Pt/PPy/GA-Urs electrode to successive injection of urea.
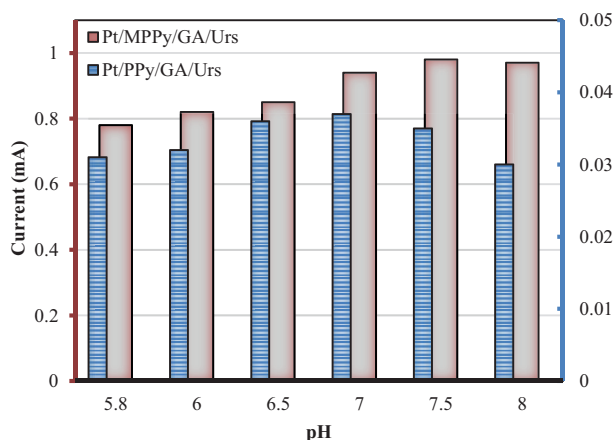
Figure 9. The effect of pH of the cell solution on the peak oxidation current of both electrodes (Pt/PPy/GA-Urs and Pt/MPPy/GA-Urs).

Table. The effect of adding the interfering factors on current responses of different urea biosensors.

Interfering factors	Urea (mM)	I: Current response (μA)		ΔI: Current change in presence of the interfering factor (μA)		Interference percentage [i]	
		With MPPy	With PPy	With MPPy	With PPy	With MPPy	With PPy
Without interferent	1.67	828	32	0	0	0	0
0.1 mM glucose	1.67	828	32	0	0	0	0
0.1 mM ascorbic acid	1.67	831	32.2	3	0.2	0.36	0.625
0.1 mM triglyceride	1.67	834	32.1	4	0.1	0.48	0.312
0.5 mM uric acid	1.67	832	32.3	6	0.3	0.727	0.937

[i]:Interference percentage $= (\Delta I/ I_0) \times 100$, where I_0 is current response without interfering factors.

3.7. Conclusions

In this study, MPPy was electropolymerized on the surface of a Pt electrode, which increased the enzyme loading. The behaviors of the developed biosensors were investigated by CV and chronoamperometry. The biosensor with MPPy and PPy showed linear behavior in the urea concentration ranging from 0.5 to 10.82 mM and 1.67 to 8.32 mM, respectively. Due to the high surface-to-volume ratio of MPPy, the Pt/MPPy/GA-Urs electrode had a wider linear range than the Pt/PPy/GA-Urs electrode. The sensitivities of the electrodes were 0.0432 and 0.0035 mA mM^{-1}, and the response times (at ambient temperature and pressure) were about 5 s and 7 s for Pt/MPPy/GA-Urs and Pt/PPy/GA-Urs, respectively. The maximum enzyme activity for Pt/PPy/GA-Urs and Pt/MPPy/GA-Urs electrodes occurred at pH of 7.0 and 7.5, respectively. The optimum pH value for Pt/MPPy/GA-Urs is closer to the pH of blood. Therefore, this modified electrode is better in clinical applications than the Pt/PPy/GA-Urs electrode. The developed electrodes had a wide linear range, short response time, long-term stability, low detection limit, and insignificant changes in the currents after adding interfering factors. Therefore, these are effective biosensors for urea detection in physiological samples.

Acknowledgment

References

1. Ibrahim AA, Ahmad R, Umar A, Al-Assiri M, Al-Salami A et al. Two-dimensional ytterbium oxide nanodisks based biosensor for selective detection of urea. Biosensors and Bioelectronics 2017; 98: 254-260. doi: 10.1016/j.bios.2017.06.015

2. Jakhar S, Pundir C. Preparation, characterization and application of urease nanoparticles for construction of an improved potentiometric urea biosensor. Biosensors and Bioelectronics 2018; 100: 242-250. doi: 10.1016/j.bios.2017.09.005

3. Lakard B, Magnin D, Deschaume O, Vanlancker G, Glinel K et al. Urea potentiometric enzymatic biosensor based on charged biopolymers and electrodeposited polyaniline. Biosensors and Bioelectronics 2011; 26 (10): 4139-4145. doi: 10.1016/j.bios.2011.04.009

4. vel Krawczyk TK, Moszczyńska M, Trojanowicz M. Inhibitive determination of mercury and other metal ions by potentiometric urea biosensor. Biosensors and Bioelectronics 2000; 15 (11-12): 681-691. doi: 10.1016/S0956-5663(00)00085-3

5. Ali A, Ansari AA, Kaushik A, Solanki PR, Barik A et al. Nanostructured zinc oxide film for urea sensor. Materials Letters 2009; 63 (28): 2473-2475. doi: 10.1016/j.matlet.2009.08.038

6. Ahuja T, Kumar D, Singh N, Biradar A. Potentiometric urea biosensor based on multi-walled carbon nanotubes (MWCNTs)/silica composite material. Materials Science and Engineering: C 2011; 31 (2): 90-94. doi: 10.1016/j.msec.2010.08.001

7. Bisht V, Takashima W, Kaneto K. An amperometric urea biosensor based on covalent immobilization of urease onto an electrochemically prepared copolymer poly (N-3-aminopropyl pyrrole-co-pyrrole) film. Biomaterials 2005; 26 (17): 3683-3690. doi: 10.1016/j.biomaterials.2004.09.024

8. Koncki R, Lenarczuk T, Radomska A, Głąb S. Optical biosensors based on Prussian Blue films. Analyst 2001; 126 (7): 1080-1085. doi: 10.1039/B103044M

9. Yang Z, Si S, Dai H, Zhang C. Piezoelectric urea biosensor based on immobilization of urease onto nanoporous alumina membranes. Biosensors and Bioelectronics 2007; 22 (12): 3283-3287. doi: 10.1016/j.bios.2007.03.006

10. Brownlee BJ, Bahari M, Harb JN, Claussen JC, Iverson BD. Electrochemical glucose sensors enhanced by methyl viologen and vertically aligned carbon nanotube channels. ACS Applied Materials & Interfaces 2018; 10 (34): 28351-28360. doi: 10.1021/acsami.8b08997

11. Çolak Ö, Arslan F. Amperometric biosensing of ethanol based on integration of alcohol dehydrogenase with a Pt/PPy-PVS/MB electrode. Turkish Journal of Chemistry 2015; 39 (1): 84-95. doi: 10.3906/kim-1405-44

12. Kara P, Dağdeviren K, Özsöz M. An electrochemical DNA biosensor for the detection of DNA damage caused by radioactive iodine and technetium. Turkish Journal of Chemistry 2007; 31 (3): 243-249.

13. Hao W, Das G, Yoon HH. Fabrication of an amperometric urea biosensor using urease and metal catalysts immobilized by a polyion complex. Journal of Electroanalytical Chemistry 2015; 747: 143-148. doi: 10.1016/j.jelechem.2015.03.015

14. Tyagi M, Tomar M, Gupta V. Glad assisted synthesis of NiO nanorods for realization of enzymatic reagentless urea biosensor. Biosensors and Bioelectronics 2014; 52: 196-201. doi: 10.1016/j.bios.2013.08.020

15. Soares JC, Brisolari A, da Cruz Rodrigues V, Sanches EA, Gonçalves D. Amperometric urea biosensors based on the entrapment of urease in polypyrrole films. Reactive and Functional Polymers 2012; 72 (2): 148-152. doi: 10.4236/ojab.2013.21002

16. Tyagi M, Tomar M, Gupta V. NiO nanoparticle-based urea biosensor. Biosensors and Bioelectronics 2013; 41: 110-115. doi: 10.1016/j.bios.2012.07.062

17. Meibodi ASE, Haghjoo S. Amperometric urea biosensor based on covalently immobilized urease on an electrochemically polymerized film of polyaniline containing MWCNTs. Synthetic Metals 2014; 194: 1-6. doi: 10.1016/j.synthmet.2014.04.009

18. Carquigny S, Segut O, Lakard B, Lallemand F, Fievet P. Effect of electrolyte solvent on the morphology of polypyrrole films: application to the use of polypyrrole in pH sensors. Synthetic Metals 2008; 158 (11): 453-461. doi: 10.1016/j.synthmet.2008.03.010

19. Cong HN, El Abbassi K, Gautier J, Chartier P. Oxygen reduction on oxide/polypyrrole composite electrodes: effect of doping anions. Electrochimica Acta 2005; 50 (6): 1369-1376. doi: 10.1016/j.electacta.2004.08.025

20. Shanthala V, Shobha Devi S, Murugendrappa M. Synthesis, characterization and DC conductivity studies of polypyrrole/copper zinc iron oxide nanocomposites. Journal of Asian Ceramic Societies 2017; 5 (3): 227-234. doi: 10.1016/j.jascer.2017.02.005

21. George PM, Lyckman AW, LaVan DA, Hegde A, Leung Y et al. Fabrication and biocompatibility of polypyrrole implants suitable for neural prosthetics. Biomaterials 2005; 26 (17): 3511-3519. doi: 10.1016/j.biomaterials.2004.09.037

22. Stempien Z, Rybicki T, Rybicki E, Kozanecki M, Szynkowska M. In-situ deposition of polyaniline and polypyrrole electroconductive layers on textile surfaces by the reactive ink-jet printing technique. Synthetic Metals 2015; 202: 49-62. doi: 10.1016/j.synthmet.2015.01.027

23. Li C, Sun C, Chen W, Pan L. Electrochemical thin film deposition of polypyrrole on different substrates. Surface and Coatings Technology 2005; 198 (1-3): 474-477. doi: 10.1016/j.surfcoat.2004.10.065

24. Scott D, Cooney MJ, Liaw BY. Sustainable current generation from the ammonia–polypyrrole interaction. Journal of Materials Chemistry 2008; 18 (27): 3216-3222. doi: 10.1039/B800894A

25. Vidotti M, Dall'Antonia LH, Cintra EP, de Torresi SIC. Reduction of interference signal of ascorbate and urate in poly (pyrrole)-based ammonia sensors in aqueous solutions. Electrochimica Acta 2004; 49 (22-23): 3665-3670. doi: 10.1016/j.electacta.2003.11.034

26. Gustafsson G, Lundström I, Liedberg B, Wu C, Inganäs O et al. The interaction between ammonia and poly(pyrrole). Synthetic Metals 1989; 31 (2): 163-179. doi: 10.1016/0379-6779(89)90812-6

27. Shul'ga A, Soldatkin A, El'skaya A, Dzyadevich S, Patskovsky S et al. Thin-film conductometric biosensors for glucose and urea determination. Biosensors and Bioelectronics, 1994; 9 (3): 217-223. doi: 10.1016/0956-5663(94)80124-X

28. Gao M, Dai L, Wallace GG. Biosensors based on aligned carbon nanotubes coated with inherently conducting polymers. Electroanalysis 2003; 15 (13): 1089-1094. doi: 10.1002/elan.200390131

29. Massafera MP, de Torresi SIC. Urea amperometric biosensors based on nanostructured polypyrrole. Electroanalysis 2011; 23 (11): 2534-2540. doi: 10.1002/elan.201100239

30. Ma C. The effect of Triton X-100 on the stability of polystyrene lattices. Colloids and Surfaces 1987; 28: 1-7. doi: 10.1016/0166-6622(87)80161-0

31. Gonçales VR, Massafera MP, Benedetti TM, Moore DG, Torresi SI et al. Nanostructured thin films obtained by electrodeposition over a colloidal crystal template: applications in electrochemical devices. Journal of the Brazilian Chemical Society 2009; 20 (4): 663-673. doi: 10.1590/S0103-50532009000400010

32. Okuda K, Urabe I, Yamada Y, Okada H. Reaction of glutaraldehyde with amino and thiol compounds. Journal of Fermentation and Bioengineering 1991; 71 (2): 100-105. doi: 10.1016/0922-338X(91)90231-5

33. Tiwari A, Aryal S, Pilla S, Gong S. An amperometric urea biosensor based on covalently immobilized urease on an electrode made of hyperbranched polyester functionalized gold nanoparticles. Talanta 2009; 78 (4-5): 1401-1407. doi: 10.1016/j.talanta.2009.02.038

34. Kaushik A, Khan R, Solanki PR, Pandey P, Alam J et al. Iron oxide nanoparticles–chitosan composite based glucose biosensor. Biosensors and Bioelectronics 2008; 24 (4): 676-683. doi: 10.1016/j.bios.2008.06.032

35. Zhang D, Yuwen HLX, Peng LJ. Parameters affecting the performance of immobilized enzyme. Journal of Chemistry 2013; 2013: 1-7. doi: 10.1155/2013/946248