Development of Amperometric Biosensor Immobilized by Entrapment of Urease Enzyme in Polypiroll Film for the Determination of Blood Urea

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Abstract

Accurate determination of blood urea concentration has gained a great clinical interest, especially for diagnosing kidney failure or lever malfunctioning. The biosensor can provide a fast response and accurate measurement about blood urea concentration. In this research, a method for fabrication of a disposable amperometric urea biosensor based on electropolymerization of pyroll was developed. The three electrode configuration fabricated using screen printing method was used namely working electrode made from carbon, counter electrode made from carbon and platinum, and reference electrode made from silver/silver chloride. On the surface of working electrode, urease enzyme was entrapped on a matrix of electrosythesis of polypyrrol film. The urea biosensor was successively verified by cyclic voltammetry. The performance of the urea biosensor had been evaluated with standard urea. The sensor working range was 0.1 mM – 80 mM with a response time less than 10 second and the detection limit of the biosensor of approximately 0.1 mM. The biosensor shows a good stability over a period of 2 months, which is only decreasing to 85% of its initial activity. The influence of the interfering ascorbic acid, uric acid, K^+ ion and Na^+ ions on the biosensor shows a little effect on its performance. The urea biosensor had been tested with the serum and the results have been compared with the measurement conducted with spectrophotometer. A good agreement was observed between both the measurements.

Keywords : urea biosensor, screen printing, cyclic voltametry, blood urea

1. Introduction

Urea is an important biomolecule that is known to play a variety of roles to humans. It is formed as a result of urea cycle in body from ammonia or by oxidation of amino acids. An increasing urea concentration in urine or serum is an indication of an onset of kidney disease. Whereas, its decreased level indicates severe liver malfunction. The normal range of urea in human serum is between 1.7 and 8.3 mM and level increases up to 100 mM under patho-physiological conditions.[1] A conventional method for determining the urea concentration is using colorimetric and spectrometric methods.[2,3] For this reason, the development of new techniques to urea determination is very important, and the biosensors are a very good alternative since it is mainly simple, low cost, and fast response. More recently, biosensors have emerged as a promising technology, especially for applications requiring rapid and continuous monitoring. Biosensors are being applied to a wide variety of analytical problems such as in medicine and food industries.[4-5] Most urea biosensors are based on the catalytic conversion of urea to hydrogen bicarbonate and ammonium in the presence of urease enzyme. It has been observed that ammonium is very easily diffuse in solution. The fundamental reaction is given by the following equation,

$$NH_2CONH_2 + 3H_2O \rightarrow 2NH_4^+ + HCO_3^- + OH^- \quad (1)$$

Enzyme immobilization is one of the crucial steps in the fabrication of a biosensor. Various immobilization techniques for urease encapsulation like adsorption, covalent bonding and cross-linking and sol-gel encapsulation are reported.[3-5] Currently, research in biosensor are focused on resolving the drawbacks of Enzyme instability, fragility of immobilization matrix, difficulty in storage and handling, sensitivity enhancement and higher response range. Some of these drawbacks of enzyme instability were obviated by using a mechanically stable membrane such as of polyvinyl alcohol (PVA) and polyacrylamide (PAA) or electrosythesis membranes such as polypyrrol film, polyaniline (PANi) and polythiophene. Among them,

polypyrrole is one of the most widely used conducting polymers in the fabrication of urea biosensors, since their conductivity and electroactivity do not strongly depend on pH of the electrolyte.[6-8] In previous works it was demonstrated the efficiency of polypyrrole as a mediator to the ammonia detection. And its response could be improved using different dopants into the film preparation.[2]

2. Material and Method

2.1. *Material and Reagents*

Urease (E.C. 3.5.1.5, type III, from jack bean, 100 U/mg) was purchased from Sigma Chemical Co. (St. Louis, USA.). Urea (ACS reagent.99.9 %), uric acid (Purity was 99.8%) and β-D-glucose (99.5%), were purchased from Sigma Aldrich. The materials used for electrode fabrication were ceramic sheet, carbon conductive ink (Electrodag PF-407), silver chloride (AgCl) ink (Electrodag 6037SS), Silver (Ag) conductive ink (Electrodag 427SS), insulator ink (Electrodag 452SS), 1,1'-Dimethylferrocene 97% (Sigma), Platinum powder 99.995% (Aldrich). All the conductive and insulator inks were purchased from Acheson Colloids, USA. The reagents are 10 mM Phosphate Buffer Solution (PBS). It was prepared from Na_2HPO_4 and KH_2PO_4) with sodium cloride in 0.135 mM and the pH was adjusted to 7.4. The solution is used for diluting urea. Pyroll and serum were purchased from Merck Chemical. A stock urea solution was prepared in PBS. All chemicals were analytical reagent grade

2.2. *Electrode Fabrication*

A three-electrode configuration was fabricated using screen printing technique. The printing was conducted on ceramic sheet. The reference, counter and working electrodes were made from Ag/Ag Cl, Carbon+ Pt, and Carbon, respectively. Conductive pad was made from silver ink. The ratio of 1,1'-dimethylferrocene and carbon ink used in working electrode is 10 % : 90% and it is used to enhance the electron transfer. The mass of platinum for counter electrode is 2.5% of the mass of carbon. The insulator layer is printed on the uncovered the silver pad to prevent the crack.

2.3. *Immobilization*

Enzyme immobilization is one of the crucial steps in the fabrication of a urea biosensor. Before the immobilization of urease enzyme on the surface of the working electrode, the electrode was rinsed with PBS. The amount of urease over the transducing membrane affects activity, response time and linear dynamic range due to thickness of sensing membrane and amount of enzyme.

Pyroll electropolymerization was carried out using Uniscan PG 580 Potentiostat – Galvanostat. In the electropolymerizaton procces, 30 µL of freshly distilled pyrrol was dissolved in 0.9 mL phosphat buffer 0.1 M., pH 7.4. To this solution, 100 µL of KCl 0.1 M was added. Then 5 mg of urease was added to the pyrrole solution. The electropolymerization and enzyme entrapment were conducted galvanostatically by applying the current density of 0.2 mA/cm^2 to the working electrode for 10 minutes. Before substance addition began, the response current base lines were established using a known volume of fresh buffer.

After completing these steps, the biosensors were initially checked in different concentrations of urea solutions with an Ag/AgCl reference electrode. The measurements were carried out by a amperometric method utilising three electrodes: modified carbon based electrode coated with enzyme served as the working electrod, an Ag/AgCl electrode served as the reference electrode, and carbon modified electrode served as counter electrode.

2.4. *Instrumentation*

Cyclic voltametry was performed using Uniscan Model PG-580 potentiostat-galvanostat. A pH meter (HI 9124, Hanna Instrument) was used to measure the pH solution. Milton Roy Spectronic 21 was used for comparison in measuring urea concentration.

2.5. *Performance*

The biosensor performance can be expressed by the parameters like reproducibility, measuring range, detection limit, response time and selectivity. The reproducibility is an important characteristic for the performance evaluation of a biosensor.

3. Results

Fig. 1 shows the calibration curva of the urea biosensor. The data were obtaind from cyclic voltametry measurement for urea concentration from 0.05 mM to 200 mM. The applicable measuring range of the sensor

is between 0.1 mM to 80 mM. By extrapolating the linear part of the curve, it shows that the detection limit of the sensor was 0.1 mM.

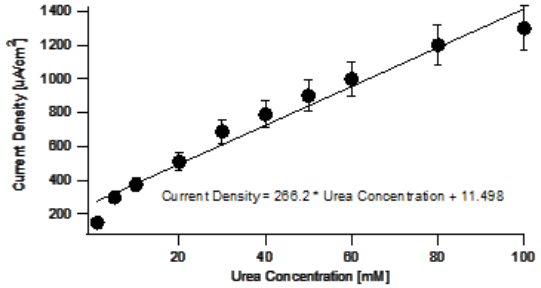

Fig. 1. Calibration curve for the urea biosensor

Fig. 2 shows that the biosensor response in 30 mM urea solution was more than 90 % reproducible untill ten times reused with the relative standard deviation was less than 5 %. The measurements were conducted one hour after the biosensor being fabricated. The biosensors were reused 10 times and the delay between measurements were 1 hour. After each measurement, the biosensor was washed with distilled water.

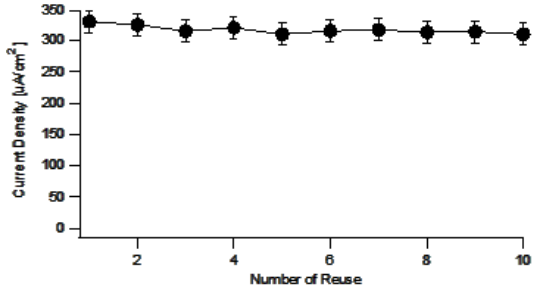

Fig. 2. Biosensor reproducibility (10 times reused).

To evaluate the their stability, the biosensors were stored at 4 0C and periodically tested for more than 2 moths. Fig. 3 shows that the biosensors still retained up to 85% of their original activity and exhibit a good response to urea.

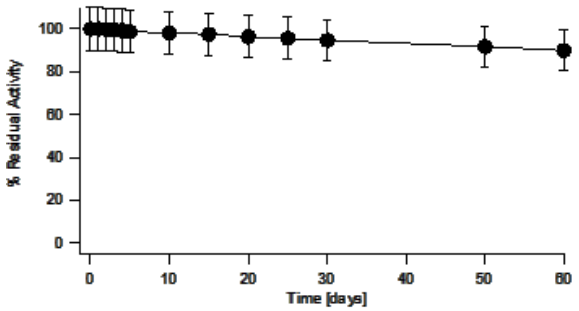

Fig. 3. Biosensor stability

Selectivity is the most important characteristic which describes the specificity towards the target ion in the presence of other ions or interfering ions. In this work, the mixed solution method was used to determine the selectivity of the sensors by checking the output response. The most possible interferences present in blood are uric acid, ascorbic acid, uric acid, glucose and sodium (Na+) and potassium (K+) ions. Hence, such substances were selected to test the selectivity of the sensor. In the experiment, minor signal changes in sensor response were observed when the urea solution of 1 mM was added by 200 µM uric acid , 200 µM ascorbic acid, 5 mM glucose, 10 mM (K+) and 10 mM (Na+) solutions. Such minor change in sensor response was probably due to instability/disturbance caused by the successive addition of the solutions. The similar experiments were conducted several times with other new fabricated biosensors. The results show negligible signal response to interferences which can be ignored in the real application.

The pH dependence of the sensor response was investigated in 10 mM urea solution over the pH range from 6.5 to 9.0. Fig. 4 shows the optimum pH requirement of the biosensor, with the temperature working envirotment of 37 °C. The experimental results indicate that the maximum signal response of the sensor is at pH 7.25. Moreover, it can be noted that at pH value higher than 7.25, the potential decrease due to the decrease in enzyme activity. The broadening of spectrum towards both acidic and alkaline environtment was observed implying that the enzyme becomes less sensitive to pH changes.

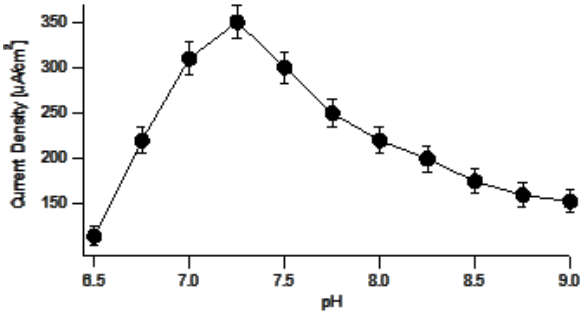

Fig. 4. Effect of pH on sensor response

The effect of temperature on the biosensor response in 10 mM urea solution was also investigated at the temperature range between 15 0C - 70 0C. The applied potential at working electrode was kept constant of 0.6 V with respect to Ag/Ag Cl reference electrode.

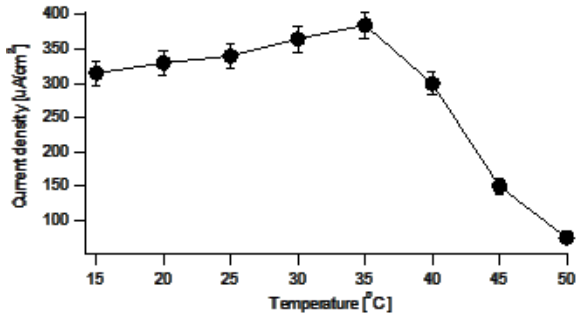

Fig. 5. Effect of temperature on sensor response

Fig. 5 shows the effect of operating temperature on the biosensor responses. It shows that the biosensor response increases with increasing of temperature. After reaching to its maximum value at around 40 0C, the sensor response decreases, because of the denaturation of the urease enzyme at high temperature. Though, at temperature around 36 0C the biosensor response reaching maximum value, however it was not well stable for long term performance compared to the sensor response at room temperature. For the experiment shows that when the sensor was tested at 36 0C, the enzymatic activity degraded drastically and showed poor long term performance. In this work, the room temperature of 18±2 0C was chosen in order to prevent possible enzyme degradation.

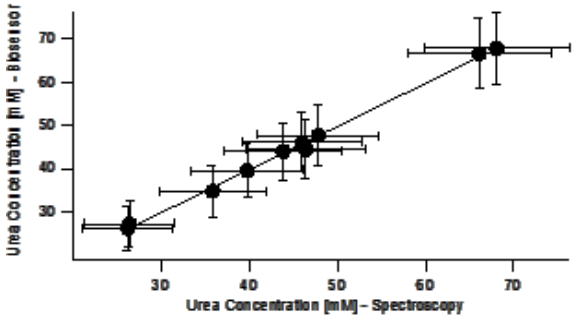

Fig. 6. Comparison between measurement urea concentration in serum using spectroscopy method and biosensor method

Fig. 6 shows the comparison between the measurement urea concentration in serum using spectroscopy method and biosensor method. Close agreements are observed between two measurements. It means that the fabricated urea biosensor is valid.

4. Summary

The urea biosensor showed a fast response with less than 10 s and has a quite wide linear range from 0.1 mM to 80 mM. The urea biosensor also exhibited good performances in sensitivity, stability, selectivity, reproducibility and negligible interference to the common interferents. All these advantageous features can make the urea biosensor applicable in medical or other areas.

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