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Preparation of A New Uric Acid Biosensor with İmmobilization of Uricase Upon Polypyrrole-Paratoluene Sulphonate Film

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ABSTRACT

In this study, a new amperometric biosensor based on immobilized uricase was developed for the determination of uric acid. Polypyrrole-*p*-toluene sulphonate film was prepared on the surface of a platinum (Pt) electrode by electropolymerization of pyrrole in the presence of *p*-toluene sulphonate as an anionic dopant. Uricase enzyme were immobilized in polypyrrole-*p*-toluene sulphonate via the entrapment method. Determination of uric acid was performed by oxidation of enzymically generated H_2O_2 at 0.3 V. Some factors that affect response current were studied such as temperature, pH and substrate concentration. Operation stability and storage stability of the biosensor was determined. The biosensor retained 76.6% of its initial performance after 30 assays and it lost 58% of its initial performance after 60 days. Effects of interferants on the current response of the biosensor were examined. The performance of the biosensor was measured in serum of healthy individuals.

Keywords: *Uric acid, uricase, biosensor, polypyrrole, paratoluensulphonate*

1. INTRODUCTION

Uric acid (UA) is the primary end-product of purine metabolism, which exists in biological fluids, such as blood and urine [1]. The normal level of uric acid in human serum is between 0.13 and 0.46 mM (2.18– 7.7 mg dl^{-1}) [2,3]. An increase in level of uric acid in human serum leads to such disease as gout, chronic renal disease, some organic acidemias, leukemia, pneumonia and Lesch–Nyhan syndrome [4,5]. Therefore, determination of uric acid in serum is very important in laboratory medicine and for routine clinical investigations [6,7,8]. Various methods such as colorimetric [9], electrochemical [10], high performance liquid chromatography (HPLC) [11,12], chemiluminescence [13] and fluorescence methods [14] have been indicated for the measurement of uric acid. However, these methods have certain drawbacks such as time-consuming, labor-intensive, expert handling, pretreatment of sample and also oxidation of certain quantities of uric acid during the processing [9]. Biosensing methods are considered more better than routine analysis, because of their simplicity, quick response time, ease of procedure, higher specificity and sensitivity [5].

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The biosensor is a kind of analytical devices for the detection of analytes which combines a biological component with a physicochemical detector component [15,16]. Enzyme-based biosensors are becoming more important as analytical devices, in that enzymes have a unique ability of recognizing target molecular quickly and accurately in a complicate system. Since the first enzyme electrode was created by Clark and Lyones in 1962 [17], there are a large number of literatures concerning the materials and methods of enzyme immobilization [18,19]. However, the materials and methods have important effect on the activity and the stability of biosensor [20], so it has significant theoretical meaning and practical value to investigate new materials and methods [21].

With the appearance of conducting polymers, the greatest attention had been focused on immobilization of enzyme using conducting polymers such as polyacetylene, polythiophene, polypyrrole, polyindole and polyaniline [22-24] . There are several advantages of preparing biosensors with conducting polymers, including efficiently transfer electric charge [25] and considerable flexibility in the available chemical structure [26,27]. The perceived demand for rapid, inexpensive and reliable determination of analytes which is important clinically and environmentally was resulted with growing interest in the development of biosensors based on conducting polymers [28].

Polypyrrole is one of the most promising conducting polymers for biosensor applications owing to its high conductivity, stability, processability, and ease of synthesis [28-34].

Doping materials such as Nafion, poly (vinyl alcohol), poly (methylmethacrylate), poly (styrene sulphonate), poly (vinyl sulphonate), dodecylbenzene sulphonate, and *p*-toluene sulphonate can enhance the conductivity, stability, and mechanical strength of PPy matrix [34-37]. The incorporation of a large size dopant anion, *p*-toluene sulphonate (pTS) into PPy films during electropolymerization generates PPy films more porous. The porosity is an important factor for the easy immobilization of enzyme [38,39].

In this study, a new uric acid biosensor was created by immobilization of uricase onto platinum/polypyrrole-*p*-toluene sulphonate (PPy-pTS) electrode. The effects of pH, temperature and substrate concentration on the response current of the uric acid biosensor was studied. Effects of the immobilization process on kinetic parameters, storage stability, and reproducibility of the enzyme were investigated. The prepared uric acid biosensor was employed to measure uric acid in human serum.

2. MATERIALS AND METHODS

2.1. Apparatus and Reagents

All electrochemical experiments were performed using a BAS Epsilon-EC electrochemical analyzer with a three electrode cell. The conventional three-electrode system was equipped with a Pt plate (0.5 cm^2) as the working electrode, an Ag/AgCl electrode (3 M KCl) as the reference electrode and a platinum wire (diameter and length, 1 mm and 4 cm respectively) as the auxiliary electrode. The pH values of the buffer solutions were determined with an ORION Model 720A pH-ionmeter. Temperature control was achieved with a Grant W14 thermostat.

Uricase (EC 1.7.3.3. from *Arthrobacter globiformis* and with an activity of 10 U/ml) and uric acid were purchased from Sigma. *p*-Toluene sulphonate was obtained from Merck. The supporting electrolyte sodium perchlorate and pyrrole were procured from Aldrich. All other chemicals were supplied from Sigma. All solutions were prepared using doubly distilled water.

2.2. Preparation of Pt/PPy-pTS Film Electrode

Prior to the electropolymerization, the Pt plate electrode was cleaned mechanically and chemically [40,41]. The Pt plate electrode was rinsed thoroughly with distilled water and dried. The surface of the Pt plate electrode was covered by the electropolymerization of pyrrole in the presence of pTS [42,43]. The electrode was immersed in a 10 mL solution containing 50 mM pyrrole and 25 mM pTS. In order that the oxygen was removed, the solution was purged with argon. The electropolymerization of pyrrole in the presence of pTS upon the electrode surface was carried out through the cyclic voltammetric scans between −0.8 and + 0.8 V at a scan rate of 20 mV/s [44]. Pt/PPy-pTS film electrode was washed with buffer solution after the coating procedure.

2.3. Preparation of Biosensor (Pt/PPy-pTS-uricase)

Immobilization of uricase in PPy-pTS was carried out via the entrapment method. In 10 mL of solution containing 500 µL of the stock solution of uricase, 50 mM pyrrole and 25 mM pTS, the polypyrrole-*p*-toluene sulphonate film was deposited onto the surface of platinum electrode; in the meantime the uricase was entrapped into the PPy-pTS film. The prepared biosensor was washed with borate buffer (0.05 M at pH 9.5) and stored in a refrigerator at 4°C in buffer solution when not in use.

2.4. Amperometric Measurements

The quantity of uric acid can be determined by measuring the anodic current of oxidation of H_2O_2. The anodic oxidation of H_2O_2 is given by the equation:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (1)$$

Firstly, an aqueous solution containing 0.05 M borate buffer at pH 9.5 and 0.1 M sodium perchlorate as a supporting electrolyte was added to the cell. The background current reached stable value at 0.3 V and steady current (i_a) was recorded. Then, uric acid solution was added to the cell and stirred for five minutes. The response of the biosensor against uric acid was recorded after 200 sec. The difference in current values were plotted against the concentration of uric acid (Fig. 5).

3. RESULT AND DISCUSSION

In this study, we prepared a new uric acid sensitive amperometric biosensor. The parameters affecting the performance of the biosensor were investigated.

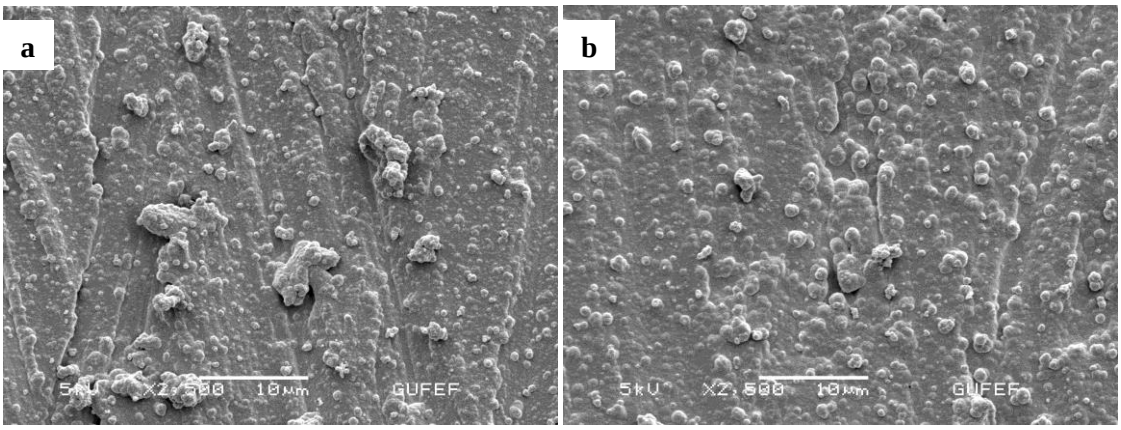
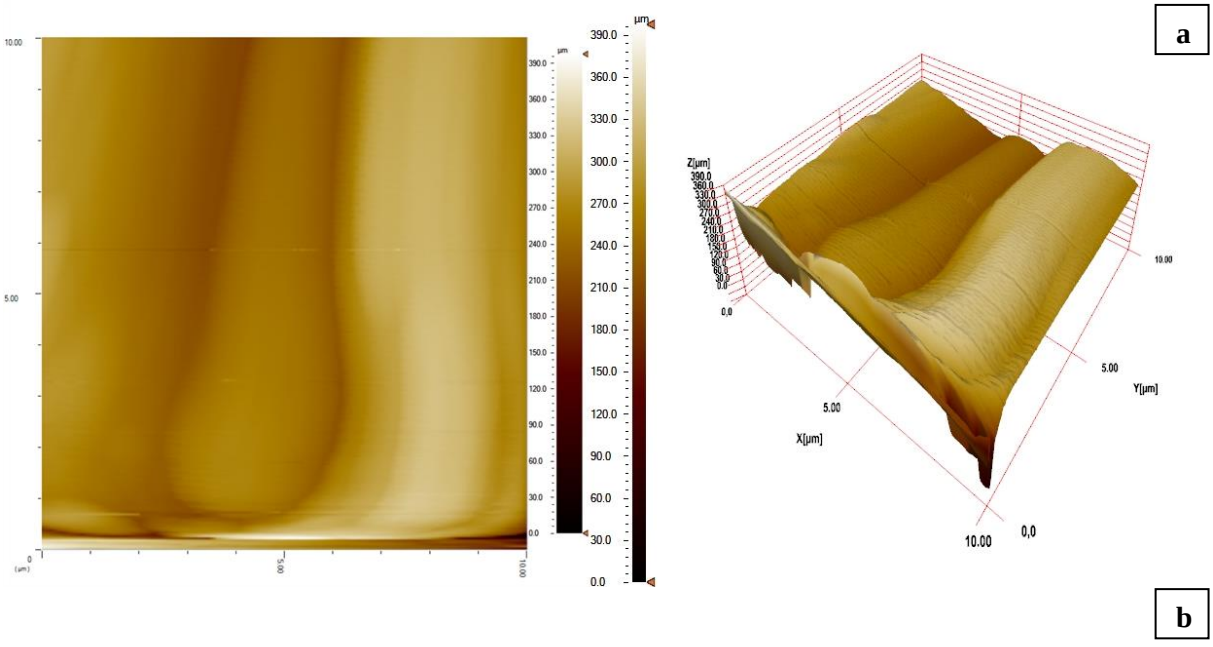


Fig.1 SEM images of **(a)** Pt/PPy-pTS electrode and **(b)** Pt/PPy-pTS-uricase electrode

3.1. SEM and AFM Studies of Biosensor

The surface morphology of electrode in the presence and absence of uricase was characterized by using scanning electron microscope (SEM) and atomic force microscope (AFM). The SEM images of the surfaces of Pt/PPy-pTS electrode and Pt/PPy-pTS-uricase electrode were shown in Fig. 1. The results of SEM showed that the surface morphology of the PPy-pTS film was a cauliflower - like structure, whereas we observed changes in the surface morphology of the PPy-pTS-uricase film.

The AFM images of the surfaces of Pt/PPy-pTS electrode and Pt/PPy-pTS-uricase electrode were shown in Fig. 2. It was observed that as a result of the immobilization of uricase, significant changes was occured in the surface morphology of the electrode without immobilized enzyme.



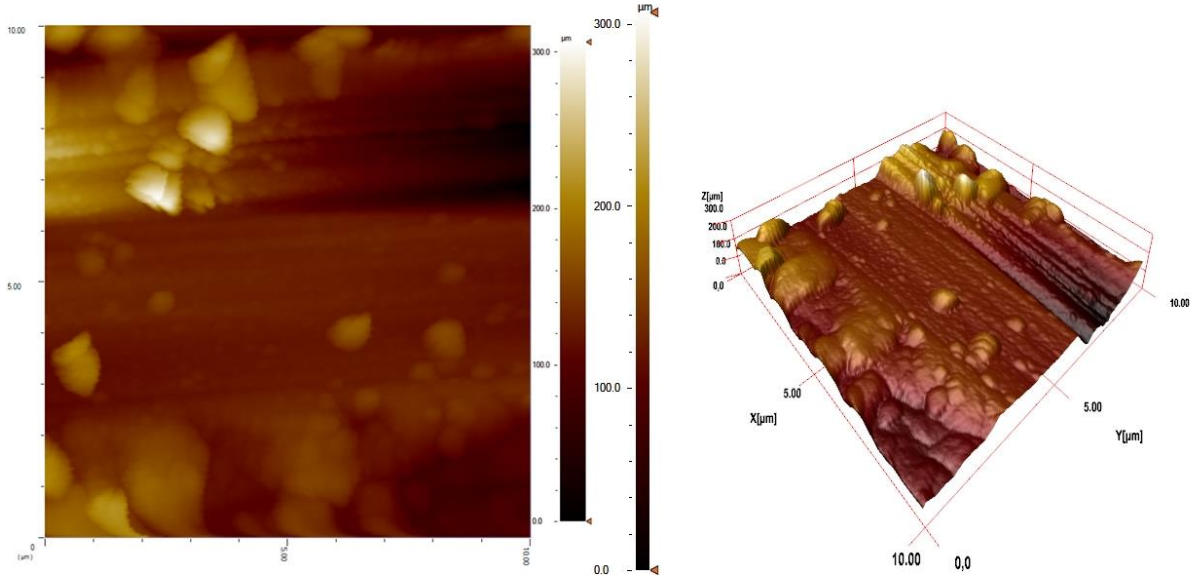


Fig.2 AFM images of **(a)** Pt/PPy-pTS electrode and **(b)** Pt/PPy-pTS-uricase electrode

3.2. The Effect of pH

For the purpose of determining the optimum pH, the influence of pH on the response of the biosensor was studied with 0.05 M borate buffer solutions containing 0.05 mM uric acid. The pH of the buffers were varied between 8.5 and 10.5. The relationship between pHs and the current response were shown in Fig. 3. According to the pH-plot, the optimum pH value was obtained at pH 9.5 and pH 9.5 was used throughout the experiments. pH values of uric acid biosensors which were obtained using different polymer or immobilization methods are available in the literature [27,45,46].

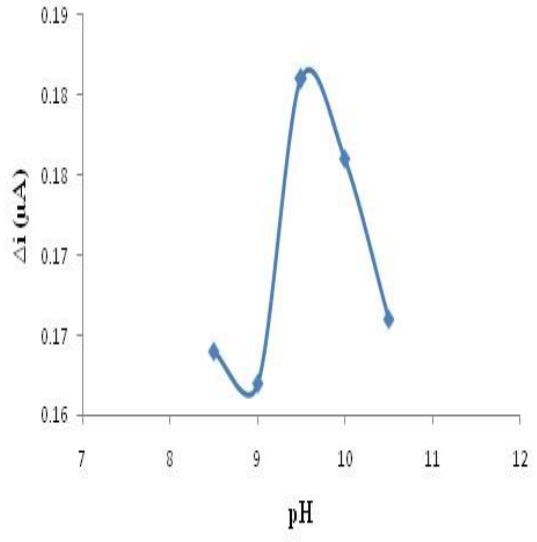


Fig. 3 Effect of pH on the current response of the biosensor (at 25°C, 5.0x10^{-5} M uric acid in 0.05 M borate buffer solution at 0.3 V).

3.3. The Effect of Temperature

The optimum temperature of the biosensor was investigated between 20 °C and 70 °C. The influence of temperature on the biosensor was studied with 0.05 M borate buffer solution at pH 9.5 containing 0.05 mM uric acid. The relationship between temperature and current response of the biosensor at 0.3 V was shown in Fig. 4. According to the temperature plot, the response current of biosensor increased with the increasing temperature but the optimum temperature was not observed. The thermal stability of the biosensor was enhanced enormously after immobilization. Enzymes can be denaturated at high temperatures at long-time; therefore, the rest of the experiments were carried out at 25 °C. Temperature values of uric acid biosensors which were obtained using different polymer or immobilization methods are available in the literature [28,47-49].

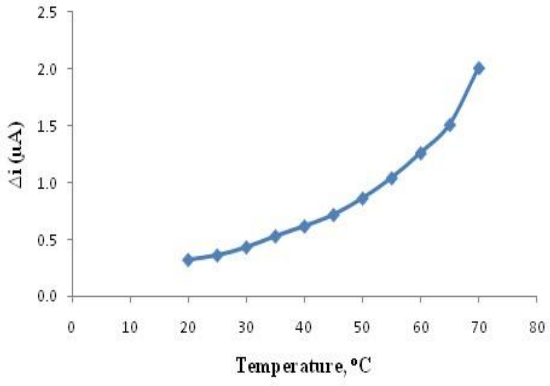


Fig. 4 Effect of temperature on the current response of the biosensor (at 0.3 V in 5.0x10^{-5} M uric acid solution of borate buffer, pH 9.5).

3.4. The Effect of Substrate Concentration

The effect of the substrate concentration on the biosensor response was studied at constant pH (pH 9.5) and temperature (25°C) for varying uric acid concentrations (0,1µM – 1 mM). In the range from 0.5 µM to 0.05 mM, a good linear relationship was observed. The linearity of graph can be used for the quantitative determination of uric acid. According to Lineweaver-Burk plot (Fig. 7), Km(app) and Imax(app) for the biosensor were calculated as $6.75x10^{-3}$ mM and 0.2312 µA, respectively. Other Km values for immobilized uricase found in the literature are 0.17 mM, $5.1x10^{-3}$ mM and 0.238 mM [3,28,48]. Considering these reported results, the variation of Km values could be attributed to the fact that the polymer and the method of immobilization were different.

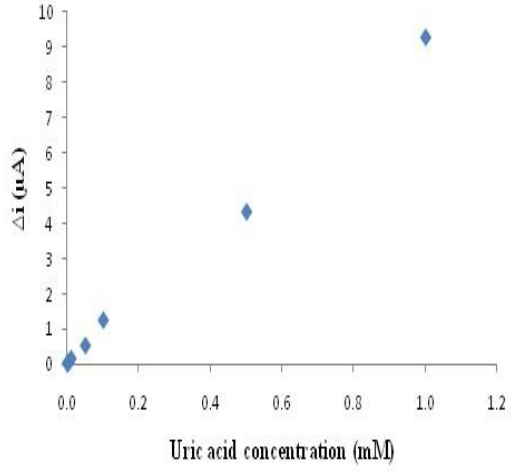


Fig. 5 The effect of uric acid concentration on the amperometric response of the biosensor (Michealis-Menten plot, in pH 9.5 borate buffer at 0.3 V).

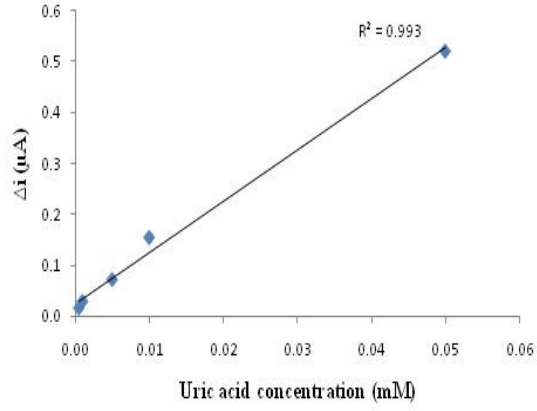


Fig. 6 The calibration curve of the uric acid biosensor (in pH 9.5 borate buffer at 0.3 V).

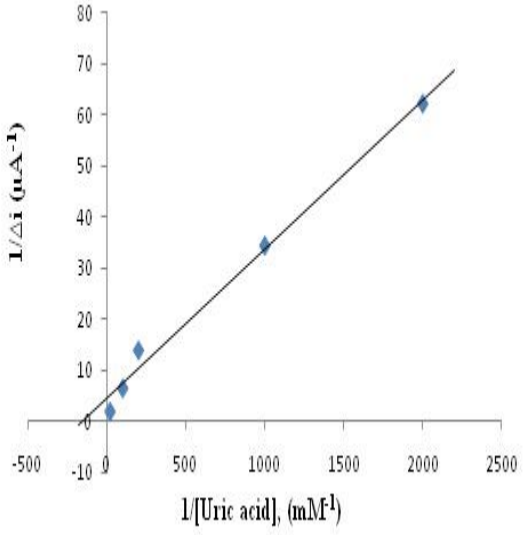


Fig. 7 The effect of uric acid concentration on the amperometric response of the biosensor (Lineweaver-Burk plot, in pH 9.5 borate buffer at 0.3 V).

3.5. Operational Stability

For the purpose of testing the reproducibility of the biosensor, the experiment was conducted 30 times on a single day at constant pH 9.5, temperature(25°C) and uric acid concentration (0.05 mM). The biosensor retained 76.6% of its initial activity after 30 measurements as shown in Fig. 8. Relative standard deviation (RSD) was obtained as 8,67%. As a result, the biosensor showed satisfactory reproducibility.

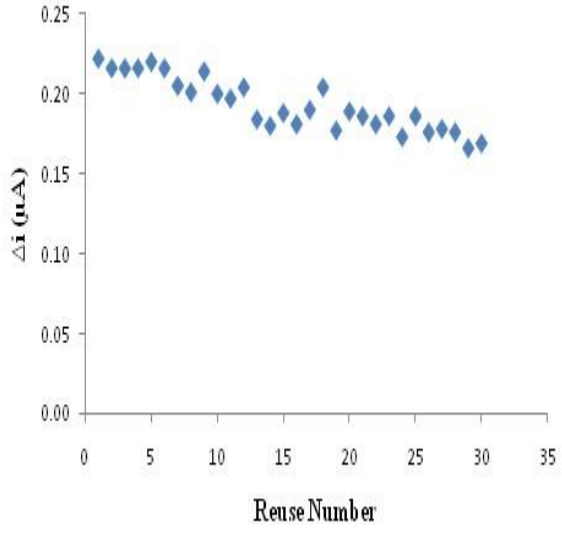


Fig. 8 The reusability performance of the biosensor (0.05 M, pH 9.5 borate buffer at 25°C).

3.6. Storage Stability

The storage stability of the biosensor was studied by measuring activity for a period 60 days under same conditions. It was observed that the biosensor lost 58% of its initial amperometric response after 60 days (Fig. 9). This result indicates that the biosensor showed good stability.

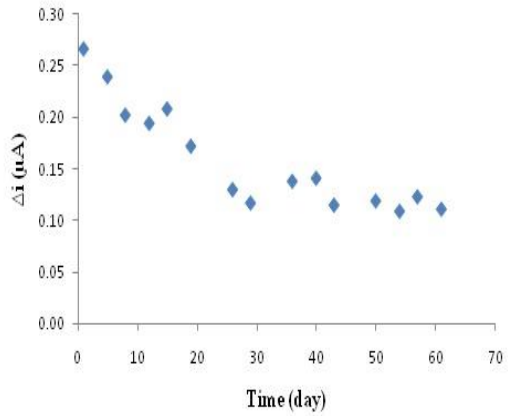


Fig. 9 Storage stability of the biosensor (0.05 M, pH 9.5 borate buffer at 0.3 V).

3.7. Effect of Interferants on the Response of Biosensor

The effect of interfering species on amperometric response of the biosensor was investigated in 0.05 M borate buffer solution at pH 9.5. A few interfering substances such as ascorbic acid, paracetamol (acetaminophen) and glucose were tested at their physiological concentrations. The results were reported in Table 1. It was observed that interference of ascorbic acid and paracetamol were quite high, while interference of glucose was negligible. In order to prevent effect of interference of ascorbic acid and paracetamol, the values of interference were decreased by dilution of solution in cell.

Table 1. Effect of interferants on the uric acid biosensor.

Interferant	Concentration (M)	Uric acid concentration (M)	Interference %
Ascorbic acid	$1.0x10^{-4}$	$5.0x10^{-5}$	83.1
Ascorbic acid	$1.0x10^{-5}$	$5.0x10^{-5}$	42.6
Ascorbic acid	$1.0x10^{-6}$	$5.0x10^{-5}$	10.4
Parasetamol	$1.0x10^{-4}$	$5.0x10^{-5}$	93.3
Parasetamol	$1.0x10^{-5}$	$5.0x10^{-5}$	60.4
Parasetamol	$1.0x10^{-6}$	$5.0x10^{-5}$	16.7
Glucose	$5.0x10^{-3}$	$5.0x10^{-5}$	1.5

3.8. Determination of Uric Acid in Serum Samples

The biosensor was performed to determine uric acid in serum samples of healthy individuals via standard addition method. The results and referenced values were listed in Table 2. The determined values were compared with referenced values obtained by the standard colorimetric-enzymatic technique by spectrophotometri in the hospital. It can be seen that electrochemical measurements showed a good correlation with spectrophotometric measurements.

Table 2. Determination of uric acid in serum samples.

Serum sample	Uric acid (mg/100 mL) using spectrophotometric measurements	Uric acid (mg/100 mL) using electrochemical measurements[a]
1	5.39	5.57±0.26
2	4.38	4.50±0.15

3	3.47	3.77±0.13
4	4.15	4.63±0.44
5	3.69	3.73±0.43

[a]Average of three measurements.

4. CONCLUSION

In this study, an amperometric biosensor for the determination of uric acid was successfully constructed by the immobilization of uricase in a polypyrrole-*p*-toluene sulphonate film. The biosensor is found to be linear in the range from 0.5 µM to 0.05 mM. The Km(app) and Imax(app) values of uricase enzyme immobilized in polypyrrole-*p*-toluene sulphonate film were $6.75x10^{-3}$ mM and 0.2312 µA/min, respectively. After 30 assays, the biosensor retained 76.6% of its initial performance and after 60 days, it lost 58% of its initial performance. Interference of ascorbic acid and paracetamol were decreased by dilution of solution. The performance of the biosensor in serum samples was investigated and it was successfully determined uric acid.

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CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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