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by Dhony Hermanto

Submission date: 30-May-2023 09:02PM (UTC-0500)
Submission ID: 2105653236
File name: B23.pdf (929.52K)
Word count: 3729
Character count: 19342

AIP Conference Proceedings

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Cite as: AIP Conference Proceedings **2609**, 020005 (2023); https://doi.org/10.1063/5.0123946
Published Online: 02 March 2023

Dhony Hermanto, Nurul Ismillayli, Bambang Kuswandi, et al.

View Online Export Citation

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Study Performance of Uricase-Poly (-*O*-Methylaniline) Films as Bio-Optode for Uric Acid Determinations

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Abstract. In the present work, uricase and poly(-*o*-methylaniline) (UOX–P*o*MeANI) films was used as bio-optode development to measure uric acid (UA) in human serum of hyperuricemia or gout patients. UOX has been immobilized onto chemically synthesized P*o*MeANI using the absorption technique. In this developed biosensor, PoMEANI was chosen as a matrix because of its advantages. This polymer acted as a matrix as well as a redox agent and indicator due to its electrochromic effect. UOX–P*o*MeANI films have been characterized using spectroscopic measurement for uric acid (UA) determinations. The experimental parameters of the bio-optode were optimized. Here, the bio-optode showed a maximum response at a wavelength of 590 nm, with the optimum pH at 6 and temperature at 35°C. The calibration curve had a dynamic working range at 0.0 to 0.6 mmol/l of UA with a limit of detection (LOD) of 0.063 mmol/l UA. The bio-optode has a long lifetime in the defined condition (4°C). The bio-optode was successfully used to measure UA in human serum samples, which was shown conformity with the reference method, employing a commercially available enzyme test kit. Thus, the developed biosensor can be used as an analytical tool for uric acid determination.

INTRODUCTION

The 2,6,8-trioxopurine (UA) as the final product of purine metabolism is excreted through the kidneys and the intestinal tract. Typically, UA concentration in urine is in the range 2 mM, while in the blood, its concentration is lower, around 120-450 µM. Higher concentrations in urine and blood indicate certain diseases such as gout and hyperuricemia [1]. Measuring UA for diagnosis and treatment of disease is routinely required in clinical medicine. Therefore, an analytical tool with accurate and low detection limit measurement of UA is needed for medical applications and biomedical chemistry.

Different methods have been reported for the detection and estimation of UA levels, such as the chemiluminescence method [2], colorimetric-fluorometric method [3], voltammetric method [4], and amperometric methods [5,6]. Among these methods, the enzymatic–spectrophotometric biosensors provide advantages, for instance, low-cost instrumentation, simple preparation, high sensitivity, and selectivity. The remarkable ability of enzymes to recognize target molecular accurately and quickly in a complex system causes enzyme-based biosensors to become critical analytical tools. Uricase (urate oxidase, UOX: oxygen oxidoreductase, EC 1.7.3.3), as an enzyme that plays a role in the final step of purine degradation, catalyst UA *in vivo* oxidation reaction in the presence of oxygen as an oxidizer. UA oxidation is carbon dioxide and allantoin, while the oxygen reduction product is hydrogen peroxide [7].

Creating new materials to improve biosensor performance, especially its stability and sensitivity, has been done in biosensor research [8,9]. Recently, significant research efforts have been concentrated on preparing to conduct polymer films chemically for the matrix of enzyme immobilization. Among them, polyaniline (PANI) became the focus of the research [10-12], whereas PANI has unique characters include good stability in aqueous solutions and air, good conductivity and redox reversibility, quick change of color with pH change, simple doping process, and

The 6th International Conference on Science and Technology (ICST21)
AIP Conf. Proc. 2609, 020005-1–020005-8; https://doi.org/10.1063/5.0123946
Published by AIP Publishing. 978-0-7354-4335-8/$30.00

excellent redox recyclability. Those characters enable polymers with significant differences constructed using simple acidic or basic treatments. Thus, polymers of the PANI family become potential promising materials for biosensors [13,14]. Supports coated with PANI have been used for immobilization of UOX [15,16].

Besides the advantage of PANI as an immobilization matrix, there is a limitation to use PANI in a specific condition. It has been known that at pH above 4, PANI has low electrochemical activity and conductivity, which affect the ability of electron transfer or redox activity during the enzyme catalytic reaction process [17]. A possible way to overcome this problem is to use PANI derivative with a wider pH working range such as P*o*MeANI, which becomes the widest area of research to be studied [18]. P*o*MeANI contains the alkyl group in the ortho position of the aromatic ring of the aniline monomer. In this study, a fast and easy immobilization method was explained based on chemical P*o*MeANI synthesize by using the adsorption method. In order to get good performance of the bio-optode toward UA detection, the experimental parameters of the bio-optode were optimized. The performances of the bio-optode were studied as well as its analytical characteristics.

EXPERIMENTAL

This study's used UOX was isolated from *Aspergillus Niger* (type I, EC 1.7.3.3, 35 kDA, 250 units from Sigma). *O*-methylaniline (99%) was obtained from Aldrich and was stored at 4 °C. UA, citric acid ($C_6H_8O_7$) (≥99.5%), ammonium peroxodisulphate $(NH_4)_2S_2O_8$, hydrochloric acid (HCl) 37%, sodium hydroxide (NaOH), potassium dihydrogen phosphate (KH_2PO_4) and sodium tetraborate anhydrous (NaB_4O_7) were received from Merck. UA's stock solution (1000 µg/ml or 6 mmol/l) was prepared in an aqueous solution. The sample solution was prepared by appropriate dilution. UA test kit used as reference method was purchased from QuantiChrom™ Uric Acid Assay Kit (DIUA-250) (USA). All reagents used were analytical grade, and the deionized water was used for solution preparation.

Preparation of PoMeANI Film

The P*o*MeANI film synthesis procedure was used in previous work as described by Sahoo [19] through oxidation reaction by reacting both *o*-methylaniline and ammonium solutions per-sulphate at room temperature, and the reactants solution was kept stirred. P*o*MeANI film can be formed during the polymerization of *o*-methylaniline on the rectangular glass. These glasses were stuck into an adhesive tape and introduced into the reaction vessel before the polymerization started. Once the reactants were introduced to the vessel, the film grew into the glass support simultaneously. After 30 min during the polymerization process, the glass supports were removed from the reaction mixture and was rinsed with 0.1 M HCl; then it was dried. The transparent mica coated with P*o*MeANI film was used for UOX immobilization.

In this work, fabrication of P*o*MeANI film was conducted by mixing *o*-methylaniline monomer and 0.1 M ammonium peroxodisulphate oxidant with a mole ratio of oxidizer/monomer 1.62. The solution was kept stirred (30 min) at a temperature of 30 °C. The printing process was done by coating a technique to produce a thin green layer of P*o*MeANI film on the transparent mica surface as a supporting matrix. Ammonium peroxodisulphate 0.1 M in 1 M HCl solution is a potent oxidizing agent expected to oxidize all *o*-methylaniline monomers in polymer solutions.

Enzyme Immobilization

The immobilization of UOX was conducted using the droplet coating method. Enzyme solution (0.5 ml, containing 2 mg/ml UOX in 5 mM phosphate buffer at pH 6.0) was added to the P*o*MeANI film. The formed film was stored at 4 °C overnight; then, it was washed out with the same buffer solution to remove non-attached UOX. The absorption spectra of the UOX–P*o*MeANI film as bio-optode were recorded on a spectrophotometer. The absorbance of fixed wavelength was measured by using Hitachi (U-1800) UV-VIS spectrophotometer. A homemade cell holder was used with a size of 9x40 mm for the UOX–P*o*MeANI film measurement.

Procedure of Measurement

The UOX–PoMeANI film was added into UA in phosphate buffer solution (pH 6.0) at reaction time (5 min). The film was rinsed off with water then dried. The absorbance of the film was measured using a spectrophotometer at maximum wavelength. This procedure allows the absorbance measurements in colored or turbid samples, such as

urine and whole blood samples. Thus, this measurement will only measure the dried reacted film using un-reacted film as a reference.

RESULTS AND DISCUSSION

UOX-PoMeANI Film

Using polymers for immobilizing enzymes in biosensors has several advantages, such as efficient transfer electric charge and considerable flexibility in the available chemical structure [20, 21]. The enzyme immobilization on conducting polymer that was mainly using adsorption technique can be carried out under moderate conditions and has a low influence on enzyme activity. The stability of immobilized enzymes will be influenced by the distance between the conducting polymer particles. Suppose the diameter of enzyme molecules is longer than particle distance. In that case, the enzyme is only adsorbed on the surface of the conducting polymer film so that it is easily leached from the film during the determination.

The success of UOX immobilization into P*o*MeANI film was indicated by the change in color of the UOX–P*o*MeANI film from pale purple to bluish green when reacted with UA, as shown in Fig. 1. This film's color change indicates the increasing pH in the film and decreasing pH in the environment caused by the proton formation reaction as UA oxidation product catalyzed by immobilized UOX. The UOX–P*o*MeANI film does not require additional agents as an indicator to detect the increasing/decreasing pH. Hence, the UOX–P*o*MeANI film can reduce the possible toxicity and other undesirable effects of the reagents. The formation of enzyme-substrate complexes can be detected directly by the physical-chemical method, namely through a change in the enzyme's absorption spectrum that specifically showed when the substrate is added. In addition, the use of PoMeANI as a matrix polymer in the biosensor can act as a redox agent. This polymer undergoes oxidation (protonation) in the reduction of the hydrolysis product of uric acid. Thus, minimizing the use of double enzymes in the fabricated biosensor.

FIGURE 1. The color change of the UOX–P*o*MeANI film from pale purple to bluish green when reacted with UA

Bio-Optode Scheme

The uricase-P*o*MeANI film used the immobilized uricase as a bio-catalytic element for the selective substrate (UA) into the P*o*MeANI film bio-optode layer. In the presence of oxygen as an oxidizer, UOX catalyzes the oxidation of UA to produce allantoin and CO_2 (as the oxidation products of UA) and hydrogen peroxide (as the reduction product of the O_2), according to Eq. 1

$$\underset{\text{Uric Acid}}{C_5H_4N_4O_3} + O_2 + 2H_2O \rightarrow \underset{\text{Allantoin}}{C_4H_6N_4O_3} + CO_2 + H_2O_2 \quad (1)$$

The hydrogen peroxide resulted as given above, can be easily reduced in the presence of the P*o*MeANI film, according to Eq. 2. While the P*o*MeANI film, in vice versa, can be easily oxidized and protonated.

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

Protonated forms of P*o*MeANI film is indicated by a change in color from pale purple to bluish green and can be detected using a UV-Vis spectrophotometer. The intensity of the color change that occurs can be correlated with the concentration of UA. Figure 2 shows visible spectra of the UOX–P*o*MeANI film before (curve 1) and after reaction with 0.6 mmol/l UA (curve 2) in 5 mM phosphate buffer solution (pH 6.0). It can be seen from Fig. 2 that the presence of UA solution caused the color change of the film and affected the spectra, and the largest difference value of intensity was found at 590 nm. Therefore, this wavelength was used for further measurements. In this system, the pH changes due to enzyme-substrate reactions are within the operating range of the P*o*MeANI film so that the bio-optode works appropriately. The buffer solution is used to minimize the matrix effect of pH P*o*MeANI. It needs to maintain the stability of the bio-optode response, meaning that the redox changes in P*o*MeANI are only determined by the UA concentration no other parameters (physical or chemical factors) that may change the pH.

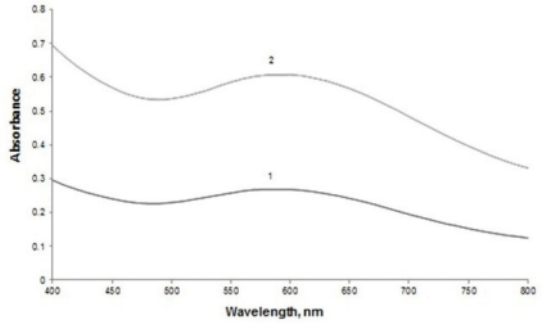

FIGURE 2. Spectra of the indicator from UOX-P*o*MeANI film (1) before and (2) after enzymatic reaction with 0.6 mmol/l UA.

Parameter Optimum

In terms of increased performance of the UOX-PoMeANI as bio-optode, there are many parameters affecting the sensor response, such as wavelength, pH, temperature, and response time. The optimization of the experimental parameters is an essential step in the analytical characteristics of the UOX-PoMeANI as bio-optode performance.

In the phosphate buffer solution containing 0.6 mmol/l UA, the dependence of biosensor response on pH is shown in Fig. 3. At pH range 4.0 to 6.0, the absorbance response increased with the pH increasing. In this pH range, although the conductivity of the polymer decreased, the increase in enzyme activity was more dominant so that the absorbance increased. At pH 6.0 to 9.0, polymer conductivity decreasing was more significant than enzyme activity growing to affect the absorbance. The optimum pH of the PoMeANI film was 6; it is used for further measurements on the biosensor.

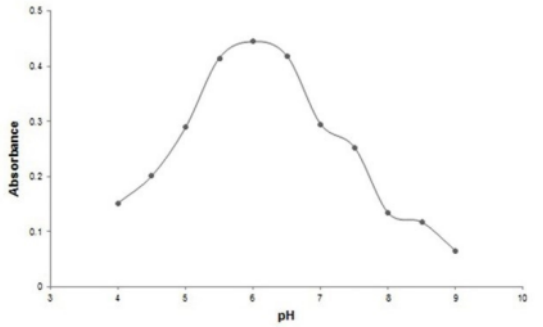

FIGURE 3. The pH enzymatic reaction effect on the absorbance of UOX-P*o*MeANI film.

The effect of temperature on absorbance in bio-optode (at 590 nm in the phosphate buffer solution containing 0.6 mmol/l UA solution) is shown in Fig. 4. Based on the absorbance value, in the temperature range of 25 to 45 °C, the biosensor can work well. A slight increase in bio-optode activity from 25 to 35 °C was observed and then decreased

at temperatures of 35 to 45°C. Bio-optode can still maintain their activity at a temperature of 45°C, indicating that the immobilization matrix increases uricase's thermal stability. Temperature 35°C was used as the working temperature in the next measurement.

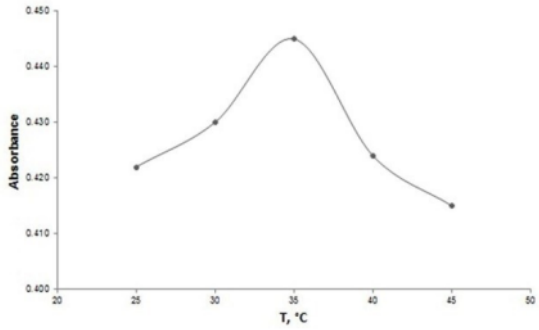

FIGURE 4. The temperature enzymatic reaction effect on the absorbance of UOX-P*o*MeANI film

At pH 6, optical biosensors showed spectra changes in the presence of UA. The response time of the UOX-PoMeANI biosensor to various concentrations of UA solutions at wavelength 590 nm is given in Fig. 5. As a reference, it was used UOX-PoMeANI film that was not treated with UA. It was observed that the 5 minute response time was the optimal time to reach 90% absorbance, so it was used for further measurements.

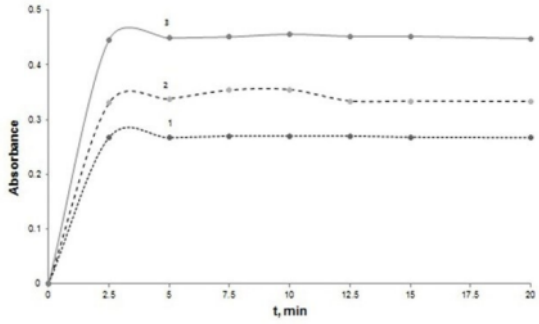

FIGURE 5. Response time of the UOX-P*o*MeANI film in enzymatic reaction with various concentrations of UA (1) 0.06, (2) 0.3, and (3) 0.6 mmol/l)

Analytical Characteristics

A change in the UOX-PoMeANI film's absorbance was observed at various UA concentrations at a wavelength of 590 nm at optimum parameters. A linear relationship was obtained for absorbance and UA concentration in the concentration range (linear range) of 0.0-0.6 mmol/L, as shown in Fig. 6. The regression equation obtained was A = 0.3702UA + 0.2311 with a correlation coefficient (r) of 0.991. The limit of detection of the biosensor, the average of the blank signal plus three times of standard deviation (n = 10), is 0.063 mmol/L UA. The sample volume effect on biosensor sensitivity was also studied. It was obtained that the change in the volume of the sample solution from 5 to 10 mL did not affect the absorbance signal for a certain time (5 min).

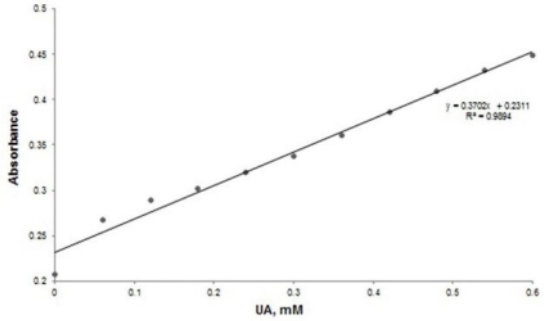

FIGURE 6. The UA optical biosensor showed linear responses to detect standard UA solution from 0.0 to 0.6 mmol/L (at wavelength 590 nm)

The reproducibility and repeatability of biosensors as two important characteristics were studied in this work. The reproducibility of the UOX-PoMeANI biosensor was determined by doing 10 measurements using 10 films. The results showed that the reproducibility was at least 97%, and films that were made on different days have no significant difference according to the ANOVA test. Whereas, biosensor repeatability is determined as relative standard deviation (RSD) in determining the standard solution of UA (n = 6, UA1 = first film to UA 6 = sixth film) using the same film in the concentration range of 0.0-0.6 mmol/L, was 0.0 to 0.26%, which indicate that the biosensor has excellent repeatability, as shown in Fig. 7. The film can be used up to 15 times of the measurements since no significant difference (≥5%) was observed during these measurements. This biosensor has better analytical performance, lower detection limits and RSD than previous work [22], optical fiber based on enzymatic biosensor using gold nanoparticle and graphene oxide as matrix. But its tested linear range is wider than this proposed biosensor.

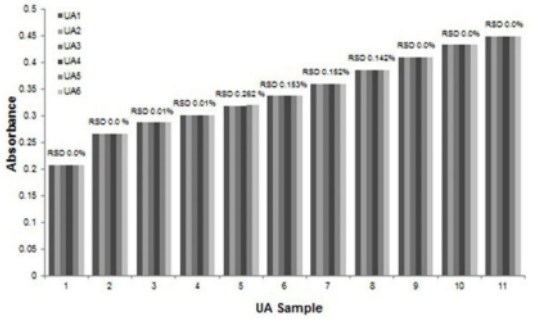

FIGURE 7. The UA optical biosensor response toward reproducibility at various concentration of UA (1= 0 to 11 = 0.6 mmol/l)

Determination of selectivity is done by adding some compounds that have the potential to be interference. In this work, the UOX-PoMeANI biosensor's selectivity was evaluated by adding bilirubin, ascorbic acid, EDTA (ethylenediaminetetraacetic acid), NaF, sodium citrate, glucose, bovine albumin, glutathione, and hemoglobin to a sample solution containing 0.3 mmol/l UA. The tolerance limit is determined as the concentration causing ± 5% error in the UA measurement. The only primary interference was ascorbic acid since the ascorbic acid can also protonation the PoMeANI film. Therefore, the UOX-PoMeANI film as bio-optode is suggested as a method that should be used to determine UA without the use of ascorbic acid.

The biosensor stability is carried out every 7 days by observing UOX-PoMeANI film absorbance to UA. During the stability test, the film was stored at 4°C. It was found that for 35 days, UOX-PoMeANI film response decreased 11%, indicating that UOX-PoMeANI film was a good stability film. Thus, the biosensor has a lifetime of more than 35 days when stored at a defined condition (4°C).

To determine the performance of biosensors in real samples, bio-optode was used to measure UA's concentration in clinical samples, namely serum of gout sufferers. By using standard addition, the number of samples added to UA

with a certain concentration was analyzed. The measurement results given in Table 1, show that the developed bio-optode has measurement results that are in accordance with the measurement results using a reference method that is often used for clinical analysis, UA test kit (QuantiChrom™ Uric Acid Assay Kit (DIUA-250)). The test kit used uricase and peroxidase as a bio-enzymatic system, combined with 2,4,6-tripyridyl-s-triazine as indicators that form a blue colored complex specifically with iron the presence of uric acid. The intensity of the color, measured at 590nm, is directly proportional to the uric acid concentration [23]. It shows that UOX-PoMeANI film as bio-optode has excellent characteristics to determine UA in human serum. Hence, UOX-PoMeANI film as bio-optode can be used as an analytical tool for UA determination.

TABLE 1. Determination of UA in human serum samples

Patient Samples	The uricase-P*o*MeANI biosensors, mmol/l*	QuantiChrom™ UA Assay Kit, mmol/l
1.	0.91 ± 0.01	0.89
2.	0.92 ± 0.02	0.93
3.	0.86 ± 0.02	0.87
4.	0.73 ± 0.01	0.75
5.	0.70 ± 0.02	0.69

*average of triplicate measurements.

CONCLUSION

The UOX-P*o*MeANI films as bio-optode development are easily prepared, stable, and a simple method for UA's determination. The biosensor (5 min) was reproducible and had a linear dynamic range (0-0.6 mM of UA) with a limit of detection is 0.063 mmol/l UA. The biosensor has good stability as far as the UOX-P*o*MeANI film was stored in the defined condition (4°C). The UOX-P*o*MeANI film as bio-optode can be used as an analytical tool for UA determination indicated by biosensor measurement with the value of UA test kit measurement as a reference method in UA determination human serum samples.

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