ELECTROCHEMICAL SENSOR FOR URIC ACID ESTIMATION USING URECASE BIOMIMIC

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ABSTRACT

Biomimetic or synthetic enzymes are used as alternatives to enzymes, which are costly and sensitive to changes in pH, temperature, ionic strength etc.. Although the selectivity of a biomimic is less than that of enzyme, it can be used as a detector element in inexpensive but stable biosensors. In this work, an organic compound phthalimide has been used as functional biomimic for the enzyme uricase for the estimation of uric acid. The purified biomimic was immobilized by gelatin on the working electrode of a three-electrode sensor assembly. Amperometry was performed with different concentrations of uric acid at a constant applied potential of 0.462 V. The response of the sensor constructed using the mimic was comparable to that of the pure uricase enzyme electrode. The use of the biomimic can reduce the cost and enhance the stability of the uric acid sensor.

KEYWORDS: *Biomimic; Phthalimide; Uricase enzyme; Three electrode sensor*

I. INTRODUCTION

Pure enzymes are costly and sensitive to change in pH, temperature, ionic strength etc. Researchers have developed a number of alternative synthetic organic molecules that resemble enzymes with respect to their catalytic activities.

Enzymes are organic protein molecules which catalyze reactions of specific substrate molecules. There are different theories to explain the selectivity of the bio-molecules towards the substrates. The classical theory for enzyme selectivity was attributed to the "lock and key" mechanism for binding of a specific substrate. Pauling [1, 2] related enzyme selectivity to the selective binding of the transition state rather than complexation of a substrate. Whatever may be the interpretations, the interactions within an enzyme's active site result from non-covalent forces such as dipole-dipole interactions, hydrogen-bonding, pi-stacking etc [3]. These non-covalent interactions must be substrate-specific and directional to control the geometry of a substrate with respect to the available active sites. The three dimensional configuration of the enzyme-substrate complex can increase or even override the substrate's natural reactivity as dictated by the functional groups.

During the last four decades, much research has been carried out to find alternative synthetic molecules which resemble enzymes with respect to their catalytic activities. These synthetic prototype enzymes are often known as biomimetic enzymes or in short, biomimics. Professor Ronald Breslow used the term "biomimetic" for the first time to explain the selective halogenation of steroids [4]. Researchers working on enzyme mimicry try to design catalysts that exploit non-covalent interactions to achieve similar levels of control.

Biomimics are usually classified into two types. The s*tructural mimics* contain groups common to a particular biological moiety, while the *functional mimics* imitate a particular task performed by a bio-molecule.

Many researchers have developed mimics for various enzymes. Bioimics of enzymes such as acetyl choline esterase [5, 6, 7], cholesterol oxidase [8], horseradish peroxidase [9,10] etc. have been reported in literatures.

Thus different activities in the area of developing mimics for biological reagents are going on. In this paper we report a functional mimic of uricase enzyme, a very important enzyme for the detection of uric acid (UA).

UA is a metabolized product of purine derivatives in human blood [11]. UA in body fluids (e.g. serum and urine) is a clinically valuable diagnostic indicator for gout, hyperuricemia, or Lesch-Nyhan syndrome [12]. Serum UA level may also increase due to alcohol consumption, obesity, diabetes, high cholesterol, kidney diseases [13] and cardiovascular diseases.

In clinical laboratory UA is estimated by different methods using uricase enzyme. The enzyme based sensors for UA have good selectivity, but they are costly and lack stability. The enzyme often gets degraded as it is highly sensitive to change in pH, temperature, ionic strength etc.

A lot of research has taken place to develop sensors for uric acid using uricase as detector element. Tao [14]*et al.* developed uric acid biosensor using uricase enzyme immobilized on glassy carbon electrode modified by titanate nanotubes. The sensor showed a wide linear range of 1 μM to 5 mM uric acid. Conventional and planar chip sensors were used for potentiometric assay of uric acid in biological fluids using flow injection analysis [15]. Automatic detection of uric acid in urine was done in a flow injection analysis system with a tubular amperometric detector [16] .Zhang [17] *et al.* developed a UA sensor using uricase immobilized on ZnOnano rod . Santha [18]*et al.* developed an amperometric sensor for estimation of UA using uricase enzyme.

Many researchers have used various detector elements to replace uricase enzyme to enhance the stability of similar enzyme based sensors. Patel*et al.*[19] fabricated uric acid sensor by pre-anodised sol-gel coated graphite electrode with a molecularly imprinted polymer brush of poly(melamine-co-chloranil) grafted to its exterior surface using cathodic stripping voltammetry . Lakshmi*et al.*[20] estimated uric acid in aqueous and blood serum samples by cathodic stripping voltammetry. They developed molecularly imprinted polymer-modified hanging mercury drop electrode for the measurement. The limit of detection for uric acid was found to be 0.024 μg mL^{-1}. A novel amperometric sensor for uric acid was developed by Ma *et al* [21] based on ordered mesoporous carbon modified pyrolytic graphite electrode. The detection limit for the sensor was 4.0×10^{-7} mol L^{-1}. Fritea et al. [22] developed a β-Cyclodextrin based electrochemical sensor for the estimation of UA and ascorbic acid in urine simultaneously. Oukil et al. [23] reported an electrochemical sensor for the estimation of UA using of polypyrrole films doped by ferrocyanide. The sensor hada sensitivity of 4.46 μA/mM.An amperometric biosensor for the estimation of UA was developed using chitosan-carbon nanotubes electro-spun nanofiber on silver nanoparticles [24]. Nitrogen-doped zinc oxide thin films were used for determination of uric acid by Jindal *et al.*[25]. The sensor had a long shelf-life of 20 weeks.

Nowadays nano-casting is a popular method of estimation of UA [26]. Spectoscopic analysis [26] was used to determine UA by acrylonitrile-acrylic acid copolymer membrane [27] .Bio-thermo chip was also utilized for estimation of UA [28].Thus there are a lot of activities reported in the area of developing sensors for estimation of UA.

In this paper we report the synthesis and use of a functional mimic of uricase enzyme for amperometric estimation of UA. The biomimic was capable of catalyzing the reaction of uric acid to allantoin [29]. The cost of production of the mimic was negligible compared to that of the pure enzyme and gave a response similar to that of the pure enzyme. The method of preparation of the mimic was simple and it can be easily produced in laboratory with simple facilities. The novelty of the method is not only its simplicity and stability but also the ability to detect and estimate uric acid in a sample.

II. MATERIALS AND METHODS

2.1. Materials and Reagents

Phthalic anhydride, urea, acetic acid, ethyl alcohol, uric acid, uricase enzyme (U7128-100UN 15 U per mg) and gelatin were supplied by E. Merck, India. The chemicals were used as received. Double distilled water was used for all the tests.

2.2. Sensor Fabrication

The three electrode sensing assembly was fabricated using a pH electrode (PSAW, India) and a copper counter electrode [30] .The glass membrane of a pH probe was broken and the platinum wire (working electrode in the pH probe) was used as the working electrode. A small loop was made at the end of the platinum wire to help immobilization of the detector element. The built-in reference electrode (silver/silver chloride/0.3 M potassium chloride) of the pH probe was used as it was. A copper wire (purity 99%) of diameter 2 mm and length 52.5mm was used as a counter electrode and was fixed to the side of the probe. The construction of the sensor assembly is shown in Figure 1.The end of the counter electrode was coiled to increase the area.

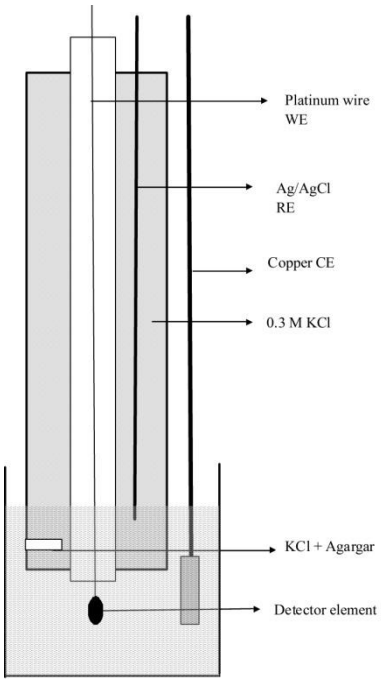

Figure 1. Schematic of the electrode assembly

Measured quantity of the detector element (0.1U for uricase enzyme and 1mg for the biomimic) was immobilized on the loop of the working electrode.

2.3. Immobilization of the detector element

0.8 gm gelatin was melted by heating at 80ºC. Then 2 mL aqueous solution of the detector element was added to it and mixed thoroughly. 10µL of the solution was deposited on the loop of the working electrode of the sensor assembly and dried at room temperature. The final amount of the detector compound immobilized on the working electrode was 1 mg for phthalimide and 0.1U for uricase.

2.4. Instrumentation

All the test procedures were performed using custom made potentiostat (CMG) device (Figure 2). The equipment was able to apply a constant potential across the working electrode and the reference electrode and measure current between the working electrode and the counter electrode. Thus it can be used to do amperometry at constant voltage. The voltage between the reference electrode (RE) and the working electrode (WE) can change during the measurement. The variable voltage source, shown in Figure 2, was adjusted such that the potential across the WE and RE remained constant during the measurement. An ammeter measured the current flowing between the working and the counter electrodes. This setup was fabricated to make a low-cost substitute of the costly potentiostats.
FTIR spectroscopy was performed using FT-IR/460 plus (Jasco, USA).

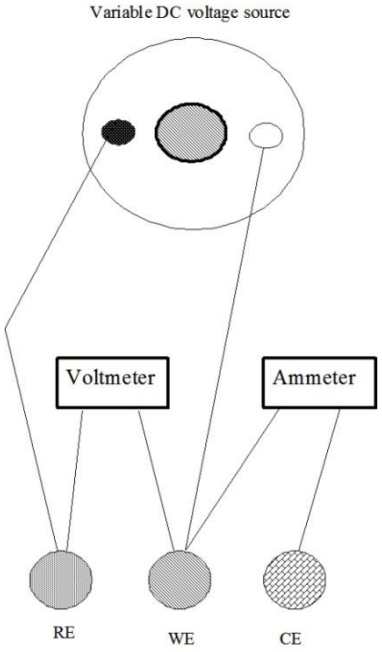

Figure 2. Schematic of the setup for amperometry

2.5. Methodology

Uricase enzyme catalyzes the oxidation of uric acid to allantoin. Thus a biomimic for uricase would be able to catalyze the oxidation of uric acid. To catalyze the oxidation of the substrate, the mimic has to bind to the substrate molecule. To provide selectivity for this binding at first different active sites for the substrate need to be selected. The method described by Bhattacharyay*et al.* [7] was used to design the biomimic

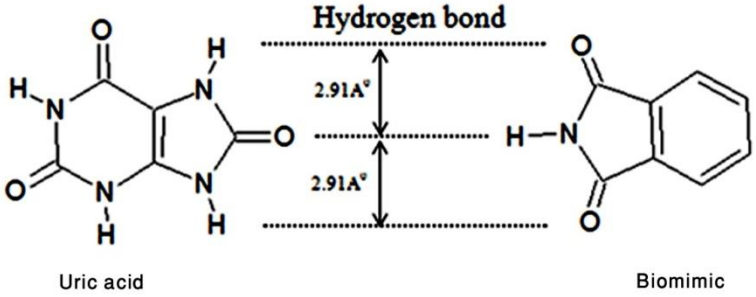

Figure 3. Comparison of position of functional groups in biomimic and uric acid

Corresponding to each active site, the complementary active groups can be identified to provide selectivity. These complementary groups in the biomimic molecule will form weak bond (hydrogen bond, electrostatic bond etc.) with the corresponding functional group. The binding can be ensured when the linear distance between the active sites of substrate and that between the complementary sites of the biomimic are same. On this basis the biomimic molecule was designed using Chemsketch software[7]. In uric acid there are seven active sites, out of them three active sites namely, =O,21; NH,19 and NH,22, were selected to prepare the biomimic. The hydrogen atoms of –NH groups and =O,21 of UA may form hydrogen bonds with two =O of keto groups and one NH group of the mimic molecule respectively. The bond distances between the active sites of the substrate and the bond distances between the complementary groups of the mimic were similar (Figure 3) facilitating binding of the UA molecule to the mimic at a particular orientation. Thus phthalimide was identified capable of mimicking the function of uricase enzyme.

2.6. Synthesis of biomimic using green procedure

Phthalic anhydride, an inexpensive laboratory chemical, was chosen as the starting material for the synthesis of the biomimic. It was treated with urea to undergo nucleophilic substitution in presence of

dilute hydrochloric acid. Then a small amount of water was added and filtered. The solid was washed thoroughly with water until it was free from acid. The solid was then dried on steam bath. It was finally crystallized in ethyl alcohol.

The method of synthesis is shown in Figure 4.

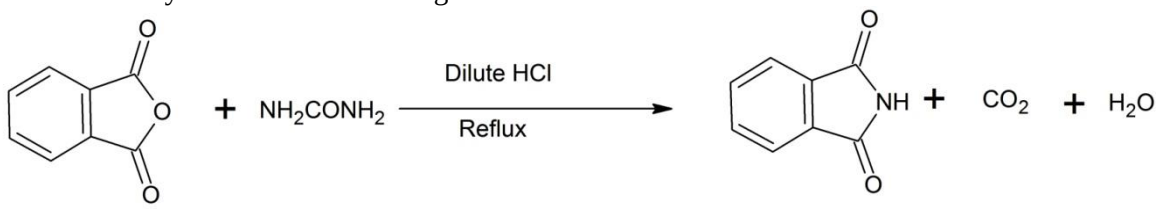

Figure 4. Synthesis of the biomimic

2.7. Measurement

1 mg of phthalimide was immobilized on the WE. The CE, RE and WE of the sensor were connected to the respective terminals of the CMG. 1 mL 3mM KCl solution was taken in a small test tube (diameter 12 mm) and the end of the sensor assembly was dipped in it.

Usually for amperometry cyclic voltammogram is used to determine the voltage for which the current response is maximum. In this work cyclic voltammogram was not used. The voltage for amperometry was identified by measuring the current response for different applied potentials. The applied potential, for which the current response was maximum, was used for the final measurement (Figure 5). On that basis, amperometry was done at a constant potential of 0.462 V applied between RE and WE. After equilibration (when the value of the current was constant) the value of current (I1) was recorded. Then 1 mL of uric acid solution of different concentrations was added. The current responses (I2) were noted after 30 s of addition of uric acid and a calibration curve was prepared by plotting the concentrations of uric acid against the change in current response (I2-I1).

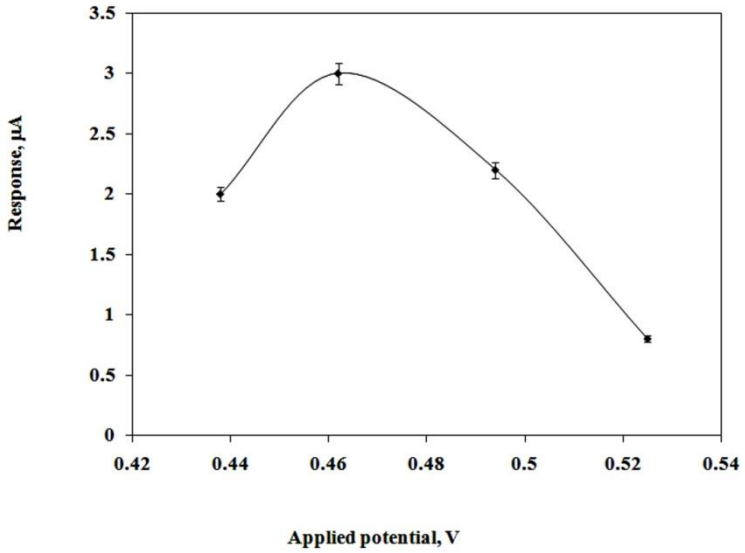

Figure 5. Determination of maximum voltage for amperometry

To confirm whether the mimic was able to catalyze the oxidation of uric acid, gelatin was immobilized on the working electrode without addition of biomimic and amperometry was performed according to the procedure described earlier. It was assumed that if the response of the sensor in absence of the mimic has much smaller response compared to that with the mimic then the mimic definitely has significant effect on catalyzing the oxidation of uric acid.

The catalytic activity of the biomimic was compared with that of pure uricase enzyme by constructing the same biosensor with pure enzyme immobilized on working electrode. In this case 0.1 U of the pure enzyme was immobilized on the working electrode by gelatin and amperometry was performed

following the procedure as described earlier. Each of the measurements was repeated three times and the averages and standard deviations were plotted.

III. RESULTS AND DISCUSSIONS

Figure 6 shows the FTIR spectrum of the biomimic using potassium bromide and the synthesized biomimic. The peaks of Figure 6 proved qualitatively the presence of the desired functional groups in the synthesized product molecule. Table 1 shows the functional groups in the biomimic and their corresponding wavenumbers. All the above functional groups are characteristic of the biomimic. Thus the FTIR result shows that the desired compound was synthesized by the chemical route.

Table 1. List of functional groups in biomimic from FTIR study

Wavenumber, cm^{-1}	Functional group
3200	N-H
1720	C=O
1600	Ar C=C

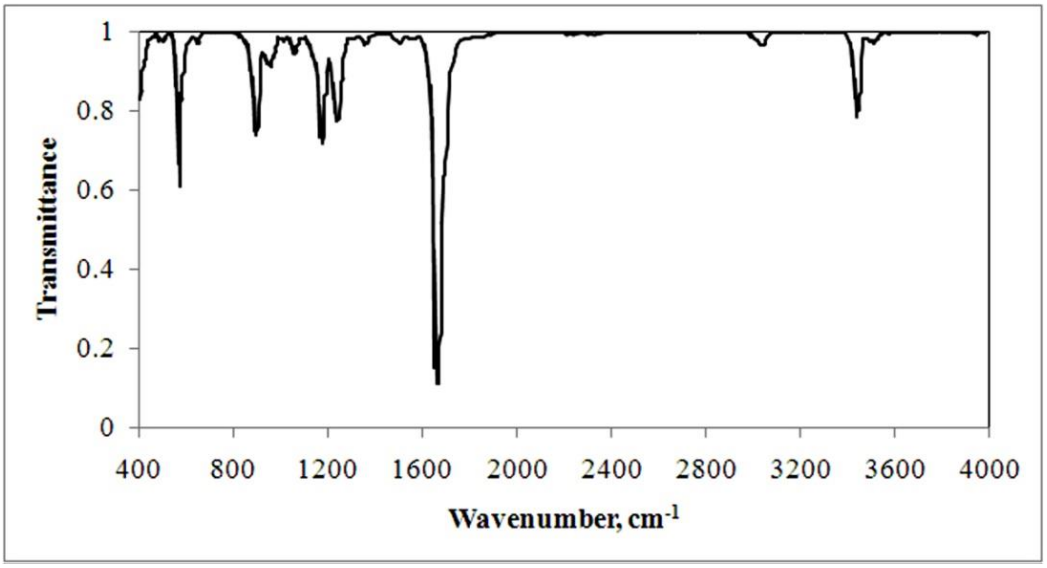

Figure 6. FTIR for the biomimic

Figure 7 shows the response due to oxidation of uric acid in presence and absence of the biomimic. It also shows the responses due to oxidation of uric acid in the range of 1-10 mg/dL by pure uricase enzyme. It is evident from Figure 7 that the response in absence of biomimic was much less compared to that in presence of biomimic. Thus the biomimic had a significant role in measuring the current response corresponding to the uric acid concentration. With increase in concentration of uric acid the response increased. Thus the biomimic catalyzed the oxidation. Figure 7 also showed that the responses due to biomimic and pure enzyme were similar. The biomimic also gave a linear response in the 0-10mg/dL concentration range of the uric acid.

The biomimic was much more stable compared to the pure enzyme. It was necessary to store the enzyme sensor at subzero temperature. However, the biomimic sensor was stable even at room temperature. After 90 days of storage at room temperature the biomimic sensor did not show significant variation in responses. Moreover the cost of the biomimic (INR 7.78 per electrode) was much less compared to the cost of the pure enzyme (INR 51.7 per electrode) (Table 2).

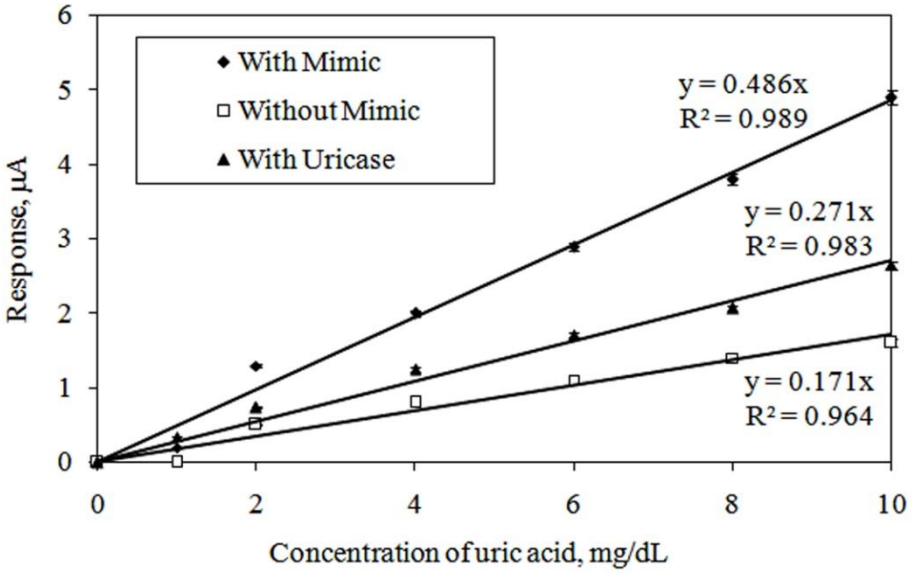

Figure 7. Responses for the sensors using biomimic and uricase enzyme.

Table 2. Price comparison for biomimic and uricase enzyme

Detector	Quantity immobilized per electrode	Price (INR)
Uricase	0.1 U	51.7
Biomimic	1 mg	7.78

IV. CONCLUSIONS

A stable sensor for uric acid was developed using a biomimic of uricase. The sensor gave a response comparable to that of the pure enzyme sensor. The sensor made of the synthetically prepared receptor biomimic was stable at room temperature. The cost involvement for making such a sensor was also negligible. Moreover the mimic could be produced using very simple laboratory facilities without much sample handling. The methodology may also be adapted for developing sensors of similar nature.

V. FUTURE WORKS

This work showed the potential of using phthalimide as a functional biomimic of uricase enzyme. Pure uric acid samples were used to study the role of biomimic as detector element in the electrochemical sensor. Research will be done to study the effect of interfering compounds. The final goal of this work is to measure uric acid in blood samples.

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