

Reagentless Porous Silica Based Electrochemical Urea Biosensor

Tanu Bhardwaj^{1*}, Prabhash Mishra², S. S. Islam² and Vinita kumari¹

¹*Bioelectronics and Instrumentation Program, Department of Biochemistry, Faculty of Science, Jamia Hamdard*

²*Nanosensor Research Laboratory, Dept. of Applied Sciences and Humanities, Faculty of Engineering and Technology, Jamia Millia Islamia*

Abstract

Conventional urea biosensors strongly require large well-known amount of urea sample with reagents under analysis. Due to which a troublesome task of collecting high amount of sample and expensive reagents originates. In this paper, we have reported a reagentless disposable impedimetric urea biosensor. For this objective, urease enzyme was immobilized into the matrix of porous silica sol-gel. Meanwhile, silica sol-gel quality was improved by supplementation of polyethylene glycol. For the impedimetric study, gold screen printed electrodes were fabricated onto the surface of alumina substrate. Optimization of the parameters like urease concentration, urea concentration, pH and temperature were done to find best suitable conditions for the biosensor. Characterization of the resulting biosensor was done by Scanning Electron Microscopy (SEM). Immobilization and biosensor's response was studied by electrochemical impedimetric method. It is found that the resulting biosensor had response time of only 6-7 seconds. It requires only 2-3 drops of sample. The linear response corresponds to 0.001mM to 70mM. As the impedimetric method of urea biosensing is simple, cheap and efficient, it can find practical utilization.

1. Introduction

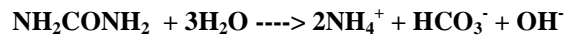
There is a massive demand of an efficient urea biosensor for various applications as alteration in its level may affect human body as well as different industries like milk, cosmetic, food etc [1-3]. In medical domain, selection procedure for a biosensor includes many parameters. One of them is necessary amount of sample required for the analysis of patient. Hence, technology is running forward to create

reagentless biosensor's that may not hamper diagnosis of serious patients only due to persistence of necessity of large amount of samples. Conventional urea biosensors need more reagent and sample solution for analysis [3-5]. In this research paper, we have developed a urea biosensor that needs only 2-3 drops of sample. For this intention, sol-gel immobilization technique was employed for encapsulation of enzyme.

For development of an enzymatic biosensor, immobilization is very important step. Various enzyme immobilization techniques like adsorption, covalent bonding, cross-linking, sol-gel encapsulation etc. are reported [6-7]. The sol-gel chemistry is highly versatile in nature. This results in the production of sol-gel with controlled structure, composition, morphology and porosity and can be utilized for development of variety of sensors. Silica sol-gel immobilization technique is reported to have unique feature of adjustable pore size depending upon the size of biomolecule and negligible reduction in enzyme activity after encapsulation [8-13]. Therefore, this has become an area of research interest as biomolecules retain their biological functionality, specificity and reactivity in the solid state environment of sol-gel after entrapment [14]. Hence the sol-gel-based system can be a potential host matrix for chemical and biochemical sensing [15]. The advantage of sol-gel based sensor is that it offers larger surface for immobilization of the enzyme, hence increasing the efficiency of the sensor. Further enzyme is protected inside the sol matrix, resulting in higher stability of enzyme [11 and 16]. To enhance the stability of sol-gel polyethylene glycol was used as additive[17].

Here we report the studies related to the development of urea biosensor by encapsulation of enzyme urease into the matrices of silica sol-gel. In human body, urea is the end product of protein degradation. The normal

level of urea in serum is 8–20 mg/dL. Our urea biosensor utilizes the simple technique of impedance measurement for urea analysis. The complete urea hydrolysis by enzyme urease results a large change in pH due to presence of resulting product ammonium ion which further results in change in impedance of sol gel matrix. Thus the variation of impedance can be correlated to the urea concentration [3-4].



2. Experimental

2.1 Material

Urease and urea were purchased from Sigma-Aldrich. Remaining chemicals were of analytical grade and purchased from Merck. All aqueous solutions were prepared by doubly distilled and deionized water.

2.2 Preparation of sol-gel matrix

For preparation of silica sol-gel, tetramethylorthosilicate (TMOS) was used as precursor and methanol was used as solvent. To enhance the stability of sol-gel polyethylene glycol was used as additive. Silica sol-gel was developed by mixing an optimized composition of 4ml tetramethylorthosilicate (TMOS), 4ml methanol and 6ml polyethylene glycol in a beaker. It was then stirred by magnetic stirrer at 60°C for 15 minutes. The pH of the solution was maintained at 7 by addition of ammonium hydroxide. This was followed by addition of 10ml of phosphate buffer solution (PBS) and the solution was stirred for 1 hour by magnetic stirrer. The sol-gel solution was kept at 4°C for 1 day. This gel was exploited as a support for the interaction between the urease and urea.

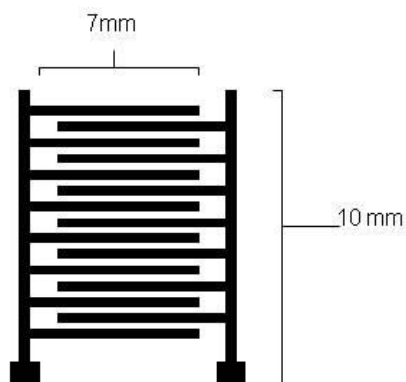


Figure 1. Interdigitated Screen printed electrodes

2.3 Fabrication of Electrodes

The interdigitated electrodes were used for the impedimetric urea biosensing. Eight pairs of gold interdigitated electrodes were fabricated (Figure 1) using screen printing technique on an alumina substrate. Then, the printed gold electrodes were cured at 500 °C for 15 minutes.

2.4 Immobilization of Urease and fabrication of biosensor

Urease solution was prepared by adding 3mg of Urease into 525 µL solution of deionized water and glycerol (1:1 solution). 500µl of this Urease enzyme solution was mixed with 100µl of silica sol-gel stock solution and this mixture was pipetted out to evenly distribute onto the interdigitated electrode sensing area of the biosensor. This biosensor was stored at 40°C for 1 day before use.

As urease reaction rate is influenced by various parameters like urease concentration, urea concentration, pH and temperature, so, these parameters were optimized to get best suitable conditions for the biosensor.

The surface morphology of the resulting sol-gel was studied using the scanning electron microscope (Nova Nano SEM450) before and after immobilization of urease.

The biosensor's impedimetric response was studied by Keithley 4200-SCS semiconducting system in frequency range 1KHz-1MHz at 5mV.

3. Optimization of Urease Enzyme Kinetics

For maximum response out of an enzyme based biosensor, we must know the conditions at which the specificity and sensitivity of an enzyme is highest. Specificity and sensitivity of any enzymatic reaction rate relies upon various parameters like enzyme concentration, substrate concentration, pH and temperature. In order to optimize enzyme concentration, urea solution (1M) was prepared in 40ml of 0.01M phosphate buffer ($\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$) and subjected to different enzyme concentration. The pH of the solution was monitored for 1 hour. A reaction rate curve was plotted against enzyme concentration (Figure 2). It was found from the graph that 3mg urease was the optimum enzyme concentration with maximum reaction rate and specificity. Beyond this concentration the reaction rate diminished. This may be due to non-competitive feedback inhibition and denaturing impact

due to the presence of ammonium ions on urease enzyme activity.

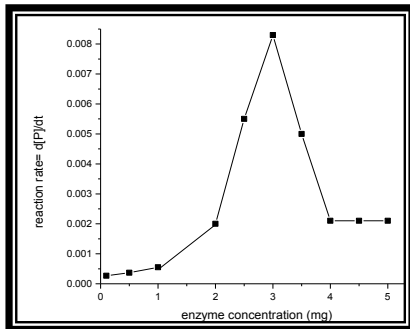


Figure 2. Reaction rate versus enzyme concentration

Similarly, to optimize substrate (urea) concentration, 3mg of urease was dissolved in 40 ml of 0.01 M Phosphate buffer and varying concentrations of urea was added into the solution. Again, pH changes were observed for one hour. From the observations, Michealis-Menton curve was obtained (Figure 3). It can be observed from the graph that 70mM was the optimum urea concentration as the saturation of active sites started from this level.

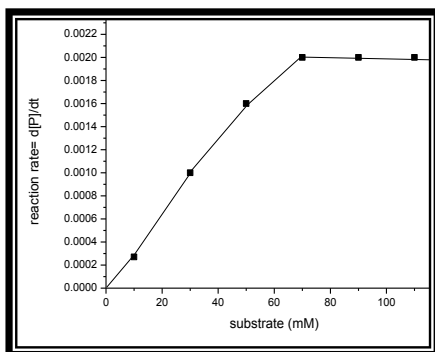


Figure 3. Reaction rate versus urea substrate

Effect of buffer pH was also studied in the same manner by preparing 70mM urea solution with 3mg urease enzyme in 40ml of 0.01 M phosphate buffer of varying pH values. In Figure 4, effect of buffer pH on enzymatic reaction rate is shown. The reaction rate was highest for neutral pH 7 and the activity of enzyme decreases at acidic and alkaline pH. Similarly, low or high temperature effects were studied on enzymatic reaction rate with 70mM urea solution and 3mg urease enzyme in phosphate buffer of pH 7. In Figure 5, reaction rate versus temperature curve was plotted which showed that the optimum temperature for urease is 30°C with highest reaction rate.

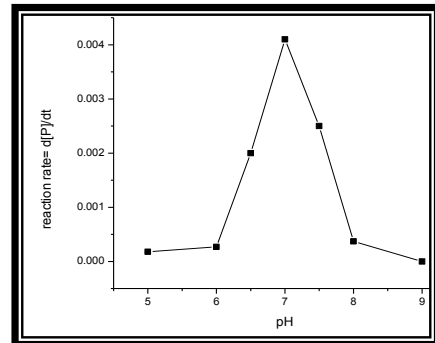


Figure 4. Reaction rate versus buffer pH

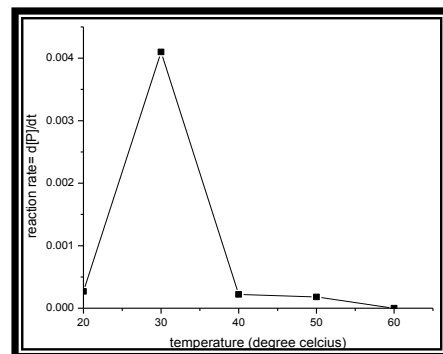


Figure 5. Reaction rate versus temperature

4. Result and Discussion

A crack free porous silica sol-gel was successfully developed for immobilization of urease. This was confirmed by scanning electron microscopy (SEM) image in Figure 6 which shows the presence of symmetrical network of spherical pores with an average diameter of around 1µm. However after encapsulation of urease enzyme, pores in sol-gel layer are not visible in SEM studies, Figure 7, as they are covered with the enzyme.

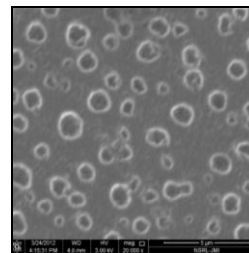


Figure 6. SEM Image of crack free silica sol-gel sample prepared after adding polyethylene glycol

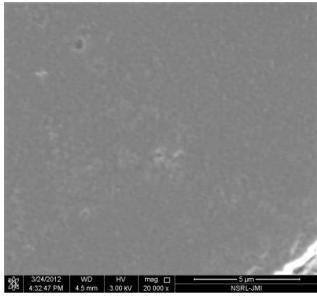


Figure 7. SEM image of sol gel sample with urease enzyme

From the impedimetric study, it is found that impedance of the sol gel decreased considerably after encapsulation of Urease as shown in Figure 8. This may be explained by the fact that when electrically charged enzyme is added into sol-gel layer of biosensor, the overall capacitance of the system raises as electrical permittivity of layer due to enzymes increases. Thereby, impedance, which is inversely proportional to capacitance, reduces. Furthermore, these enzymes increase conductivity that decreases resistance as well as impedance. Hence, gross impedance decreases. Similarly, the decrease in impedance after addition of urease is due to hydrolysis of urea in the presence of encapsulated urease and formation of charged ammonium ions on the surface of urea biosensor. It is also found that once 2-3 drops of urea solution was dropped onto the surface of urea biosensor impedance of system further decreased. It leads to formation of more of ions due to which conductivity increases and the impedance further decreases. Fig. 8 shows the response of the biosensor upon addition of 0.001mM of urea solution onto the biosensor's surface.

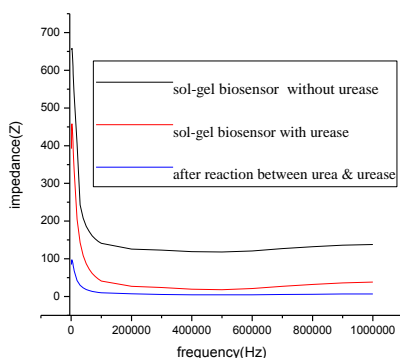


Figure 8. Impedance curve of sol-gel matrix in absence of urease, presence of urease and after urea & urease reaction

The operation of this urea biosensor is shown in the form of an equivalent circuit in Fig. 9 which includes double layer capacitance (C_{dl}), charge transfer resistance (R_{ct}), resistance of the electrolyte solution (R_s) and dielectric capacitance (C_{di}). Being frequency dependent, capacitance offers more impedance at lower frequencies ($<10\text{KHz}$) in contrast to resistance and the current goes through low impedance path R_{ct} , R_s and R_{ct} . This region is known as capacitive region. At frequencies higher than 10KHz, the impact of resistive impedance is high in comparison to capacitive. Meanwhile, the capacitive impedance decreases with increase in frequency. This region is called resistive region. So, the current flows through capacitance C_{dl} or C_{di} , R_s , C_{di} .

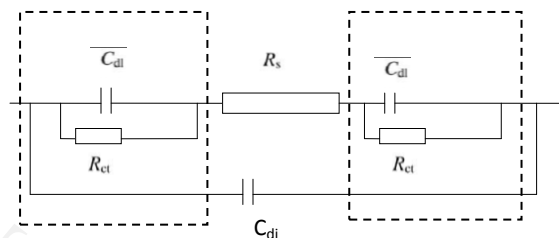


Figure 9. Equivalent circuit for the impedance measurement. (C_{dl} - double layer capacitance; R_{ct} - charge transfer resistance; R_s - resistance of the electrolyte solution; C_{di} - dielectric capacitance)

5. Conclusion

A reagentless disposable impedimetric biosensor was developed for detection urea. The biosensor shows response between 0.01mM to 70mM of urea. From the SEM and impedimetric studies, it is found that silica sol gel can be used to immobilize urease enzyme by trapping a huge amount of enzyme in the pores. Screen printed gold interdigitated electrode array is used and found to be successful in monitoring the impedance change of sol- gel matrix after applying only 2-3 drop of urea solution. Based on the equivalent circuit analysis, it is clear that the interdigitated electrode array has monitored the changes in the double layer capacitance, solution resistance, dielectric capacitance and charge transfer resistance of the electrode resulting from the generation of ions during catalysis of hydrolysis of urea. Thus, from these studies, our urea biosensor seems to be suitable for further investigations and testing for medical practice.

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