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Urease immobilized potentiometric biosensor for determination of urea

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A simple potentiometric biosensor for the determination of urea is reported in this work. The biosensor has been constructed by immobilization of urease at tungsten electrode using polyvinyl alcohol matrix followed by the sealing process with plasticised polyvinyl chloride. The sensing device gives fast and sensitive response to urea. A linear calibration curve in the concentration range of 0.01–50 mM urea is obtained with slope 42.7 mV per decade urea concentration (r^2 = 0.993), and detection a limit of 0.01 mM urea.

Keywords: Electroanalytical chemistry, Biosensors, Potentiometric biosensors, Enzyme electrode, Tungsten electrode, Immobilized enzyme, urea

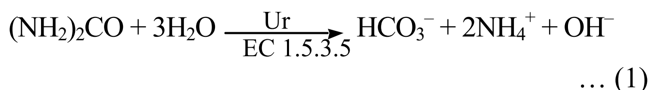
Urea is commonly found in blood and other body fluids that is formed from the breakdown of protein during tissue metabolism. It is known that the concentration of urea in the body fluid is a key indicator for some diseases such as kidney problem, heart coronary diseases, stroke, excessive protein intake or protein catabolism, malnutrition, pregnancy, shock and stress[1-2]. Therefore, a fast, selective, sensitive, reliable and accurate method for the determination of urea in clinical samples is needed for fast diagnosis of these diseases. The determination of urea in clinic samples is frequently carried out using UV-vis spectrophotometric procedures where the presence of color compounds in the samples interfere with the detection method and result in low measurement accuracy[3]. Therefore, it is important to develop a very sensitive and selective method for urea determination in clinic samples[4].

Various analytical methods have been reported for the determination of urea, including reflectometry[2,5-6], colorimetry[7-9], nuclear magnetic resonance[10], LC–MS[11], field effect transistor[12-13], acoustic wave[14], and electrochemical methods[15-17]. Most of the methods involve complicated procedures for sample pre-treatment, and are time consuming. Some require high cost instrumentation, need for skilled analyst, and high running operation cost that make these methods difficult for use in routine analysis in the clinical laboratory. Another problem is the presence of susceptible interfering agents in real samples resulting in poor accuracy. The development of electrochemical biosensor for urea with incorporation of urease can improve the selectivity of sensing device[18]. Urea biosensor with immobilisation of urease on amperometric determination system has also been reported[19].

The development of potentiometric biosensor is very attractive because of the cheap and simple instrumentation required, usually a glass pH-meter. Despite its responsiveness to potential changes, the glass electrode has limitations as a sensing device in a potentiometric biosensor because the electrode is thin, fragile and difficult to miniaturise. Various metal electrodes that are responsive to changes in pH include antimony, tungsten, iridium and polyaniline (PAn) semiconductors[20] and vanadium oxide[21]. Tungsten electrodes have been used as transducers in potentiometric biosensors for malate[22] and cholesterol[23]. Attempt has been made to enhance the selectivity of urea biosensor by coating the tungsten electrode with polymer membrane film[24]. Choosing the right matrix polymer is very crucial to immobilise the electrode as well as to protect the electrode from interference and enzyme fouling[22]. The design of potentiometric biosensors with various detection system including spectrometry, potentiometry, conductometry, coulometry, amperometry and inductometry has been reported[25-27]. Different types of matrices have been used in the construction of potentiometric biosensor such as chitosan[28], polyvinyl alcohol[29], fullerene[26], and thiophene copolymer[27].

The compatibility of tungsten electrode is extended for the determination of urea as it could act as a substrate electrode for enzyme immobilisation and retain its ability to sense changes in pH. The aim of this study is to demonstrate the potential use of PVA as an enzyme immobilization matrix for the construction of potentiometric biosensors using urease. The urea biosensor was constructed by immobilisation of urease (Ur) onto a tungsten (W)

electrode by using PVA matrix. Urea is catalytically converted to ammonium ion and bicarbonate as illustrated in reaction (1).



$$(NH_2)_2CO + 3H_2O \xrightarrow[\text{EC 1.5.3.5}]{\text{Ur}} HCO_3^- + 2NH_4^+ + OH^- \quad \dots (1)$$

The increase in potential can be detected at the underlying tungsten electrode on the enzymatic catalytic hydrolysis of urea. The potential change is related to the concentration of urea. Enzyme systems require to be buffered at pH 6 in order to achieve maximum efficiency. The buffer is also important to suppress the pH change produced in the enzymatic reaction. In this work, the use of a buffer is not immediately compatible with the measurement of change in pH, but the use of buffer solutions of suitably low buffer capacity allows what is in essence a local titration of the buffer[20].

Experimental

Urease (EC 3.5.1.5) from *Canavalia ensiformis* (jack bean) Type IX (75,265 units/g solid), urea, N-(hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), tris(hydroxymethyl)aminomethane (Tris base), tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl), were obtained from Sigma Chem. Co. Reagent grade K_2HPO_4, KH_2PO_4, KCl, and HCl were purchased from Aldrich Chem Co. Polyvinyl alcohol (PVA), poly(vinyl chloride) (PVC: low molecular weight type), 2-nitrophenyl octyl ether (NPOE) as membrane plasticizer, potassium tetrakis (*p*-chlorophenyl) borate (KTpClPB) as anionic additive, 2-chloromethylquinoline, and tetrahydrofuran (THF) were purchased from Aldrich Chem Co. All reagents were used without purification and Milli-Q grade reagent water was used for all solutions.

Potentiometric measurements were made with a Keithley 177-Microvolt Digital Multi Meter (Keithley Instrument, USA) connecting with Powerlab 2/20 Analog Digital instrument for data acquisition. A home-made tungsten wire electrode, made from a tungsten wire (1 mm dia., Aldrich Chem Co) was used as a working electrode in combination with Ag/AgCl reference electrode containing 3 M KCl internal solution from Bioanalytical System (BAS, USA) were used in all electrochemical experiments.

The enzyme biosensor was prepared as follows: The potentiometric biosensor was constructed by immobilisation of urease following the procedure outlined previously[24]. Immobilisation of enzyme onto tungsten (W) electrodes was performed starting from preparation of enzyme solution and dip-coating immobilisation procedures. Enzyme solution was prepared by dissolving 2.044 mg urease and 1.5 mg PVA in 1 mL MQ-water and mixed until a clear and homogenous solution was obtained (designated as PVA solution). The PVC solution was prepared from the mixture composition of PVC, plasticizer NPOE, with added the anionic additive for polymeric membrane KTpClPB in THF solution (PVC solution) following the procedures reported previously[30].

For the preparation of the tungsten electrode, W-wire (6 cm) was soaked in 1 M HCl for 1 min and rinsed with water and dried. Immobilisation of enzyme onto the W electrode was performed by dip-coating of W-wire (1 cm) in PVA solution containing enzyme and allowed to dry at room temperature to give the enzyme electrode (W/Ur-PVA). Variation in the thickness of membrane PVA was obtained via successive dip-coating in a PVA solution after achieving dry membrane film in the first coating. The procedures were then completed by coupling the entrapped enzyme in the polymer with PVC solution to obtain the modified enzyme electrode (W/Ur-PVA/PVC) and allowed to dry overnight. It is important to note that coating procedures in PVC solution has to be performed very fast to avoid enzyme denaturation in THF solvent. The enzyme electrode W/Ur-PVA/PVC was then rinsed with phosphate buffer (pH 6.0), and stored dry overnight in the refrigerator (4 ^oC). The electrode was conditioned for 5 min in Tris buffer (pH 6.0) before use, and was generally stored dry in the refrigerator (4 °C) when not in use.

Various buffer solutions, viz., acetate buffer, phosphate buffer, Tris buffer, HEPES buffer, and MOBS buffer, used in the experiments were prepared from buffer components and the pH was adjusted by dropwise addition of standard solution (Supplementary data, Table S1). These buffer solutions contained 0.05 M KCl supporting electrolyte prepared by dissolving the buffer components in deionised water and the pH adjusted using 0.1 M standard solution under a pH-meter, followed by the dilution to the volume. The concentrations of the buffer solution were defined from the weight of the buffer components. Buffer solution of phosphate was prepared by dissolving of KH_2PO4 and K_2HPO4 in deionised water and the pH was adjusted with addition of NaOH or HCl. Similarly, the acetate

buffer was prepared from sodium acetate and the pH was adjusted by using 0.1 M acetic acid. Buffer solutions of Tris base was prepared by dissolving tris(hydroxymethyl)aminomethane and the pH was adjusted with HCl, Tris-HCl was prepared from tris(hydroxymethyl)aminomethane hydrochloride and the pH of the buffer was adjusted by using 0.1 M NaOH. The same procedure was followed to prepare MOBS buffer by dissolving 4-(N-morpholino)-butanesulfonic acid in deionised water and the pH adjusted with HCl.

Potentiometric determination of urea was conducted using W/Ur-PVA/PVC versus Ag/AgCl in 0.01 M Tris buffer solution (pH 6.0). After a steady background potential was obtained, urea standard solution (from a stock solution of 1 M urea) was added to 10 mL Tris buffer solution followed by stirring for 5 s. The enzymatic reaction was monitored continuously on a voltmeter until a steady state potential was reached via the catalytically converted ammonium ion and bicarbonate produced at the electrode surface. The potential change produced is related to the concentration of the urea in the solution. The potential measurement on a modified W/PVA/PVC electrode was also carried out as for the enzyme electrode to determine the range of the pH change (pH 4.0 - 11) which was due to the bacidity of urea directly rather than as a consequence of the enzyme reaction.

Optimisation was carried out to obtain the best potential signal for urea biosensor by using urea standard solution. The best condition for one parameter was kept to optimise another experimental parameter[23]. Optimisation was conducted for investigating the effect of buffer solution at various type of buffer solutions (acetate, phosphate, and Tris buffer at different pH (5.0–7.5) and at concentration range (0.1–100 mM) to obtain the best solution for urea biosensor. Another parameter to be optimised was the effect of thickness of membrane films prepared by dip-coating procedures 1 to 5 times.

Results and discussion

Before constructing the enzyme electrode, the response of PVA modified electrodes in the absence of enzyme (W/PVA/PVC) was investigated in 0.01 M buffer solution at wide range pH as showed in Fig. 1. It is observed that the slope at a bare tungsten electrode was 55.8 mV pH^{-1}. On increasing the thickness of the matrix PVA, coated 1 to 5 times (Fig. 1), the sensitivity of potential response

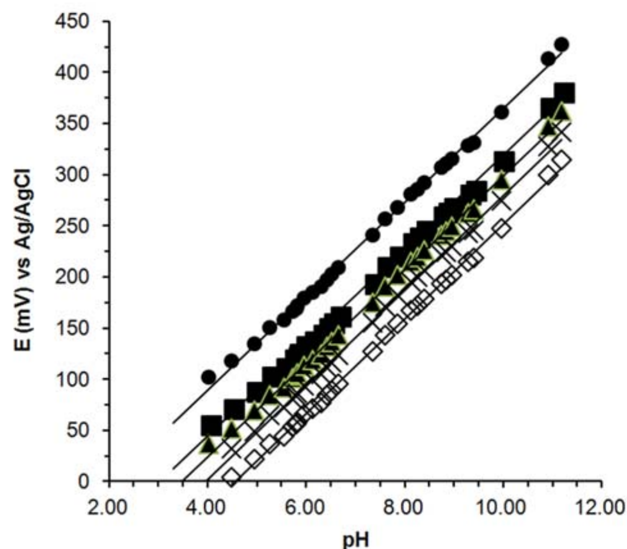


Fig. 1 — Response sensitivity of tungsten electrode (W) and modified coated PVA electrode (W/PVA/PVC) to pH changes in various pH buffer solutions (pH 4.0–11.2). [The buffer solutions were acetate (pH 4-5), Tris-HCl (pH 5.0 - 6.5) and Tris-base (pH 7.0 - 11.2), each at 0.01 M. The pH was adjusted using HCl or NaOH. (●) bare tungsten electrode, and, modified W/PVA/PVC (■) coated 1 time, (▲) coated 2 times, (x) coated 3 times, and (□) coated 5 times. Measurements are relative to Ag/AgCl reference electrode].

decreased. However, the slope was constant at the electrode prepared with PVA films of different thickness. The results show that tungsten electrode responds to pH change that is suitable for use as detection system for catalytic conversion of urea. It is important to use buffer solutions at low buffer capacity to allow what is in essence a local titration of the buffer[22]. It is observed that W electrode can act as solid-state material for the construction of urea biosensors. However, there is need to immobilize urease on to the electrode by using polymer matrix and maintaining the environmental conditions to keep enzyme activity constant. Thus, the PVA matrix was chosen since it dissolves in water along with the enzyme, and the membrane film containing enzyme can be immobilised on the electrode surface. The immobilised enzyme is then sealed with insoluble plasticized PVA to keep the activity of immobilized urease and to protect from enzyme leaching.

Response of the enzyme electrode W/Ur-PVA/PVC to urea has been evaluated. Typical responses of an enzyme electrode to increasing concentrations of urea are shown in Fig. 2(a). The time from injection of sample until reaching a steady state potential was about 6 min. In the absence of the

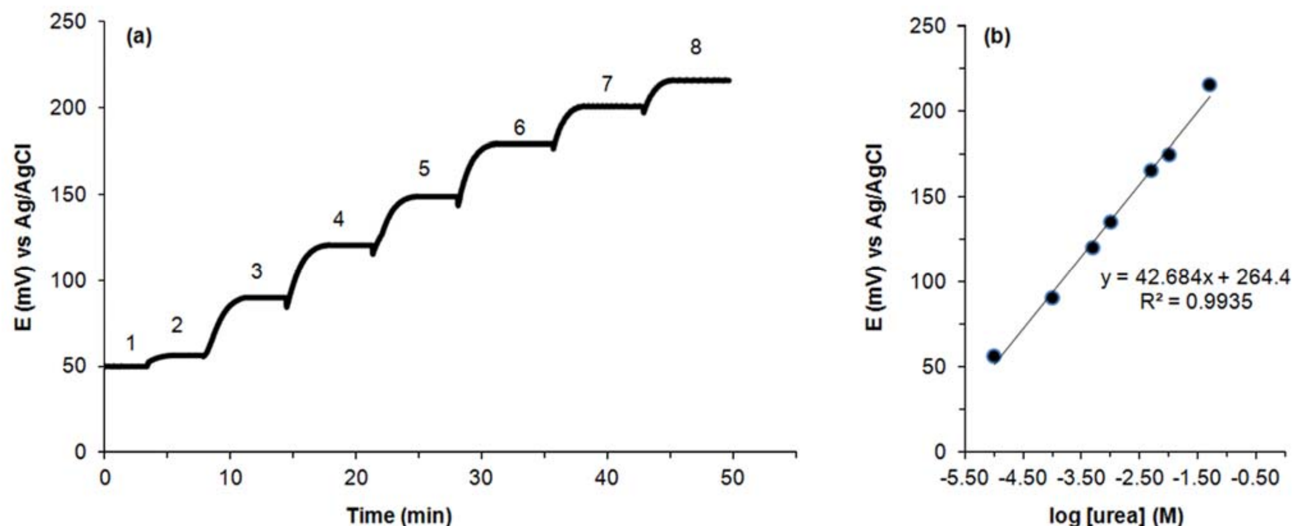


Fig. 2 — (a) Typical steady state potential-time curve for successive injection of (1) 0.0 mM, (2) 0.01 mM, (3) 0.1 mM, (4) 0.5 mM, (5) 1.0 mM, (6) 5.0 mM, (7) 10.0 mM and (8) 50 mM urea in 0.01 M Tris buffer solution. [pH 6.0]. (b) Calibration curve for urea standard solution. [The enzyme electrode was constructed by coating (1 time) the tungsten wire electrode with PVA containing urease].

enzyme (for W/PVA/PVC electrode), there was no potential response observed to an injection of high concentration of urea (50 mM). The calibration curve for urea standard solution is presented in Fig. 2(b). The linearity range of the plot lies in the concentration range of 0.01–50 mM urea, with a slope of 42.7 mV decade^{-1}. The smallest concentration detected was 0.01 mM urea. The linear range is greater than that reported previously for potentiometric biosensor for urea[25].

The effect of the thickness of PVA film used as matrix polymer to immobilise enzyme urease has been investigated. Different enzyme electrodes were prepared with between one to five times dip-soaked in a mixture solution of PVA-urease. Variation in the thickness of membrane matrix, i. e., variation in the amount of immobilized enzyme on the electrode surface, was obtained by increasing the soaking time. The calibration curve for enzyme electrode constructed at varying number of coatings of PVA film is presented in Fig. 3. The increase in number of coatings during preparation of the electrode resulted in decrease in the sensitivity of the urea biosensor, although the linearity concentration range of detection system remained the same for enzyme electrode prepared with film coated 1-3 times (Table 1), while the electrode with very thick membrane film (coated 5 times) has the lowest linearity concentration range. This variation in response with the amount of enzyme-modified polymer deposited is not consistent with theoretical

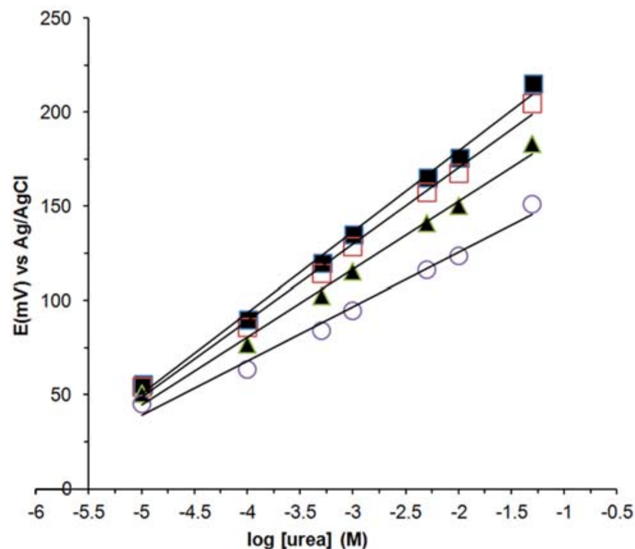


Fig. 3 — Calibration plots for urea biosensors as a function of the number of coatings for PVA films used as a matrix polymer to immobilise urease on the surface of tungsten electrode. [(●) coated 1 time, (□) coated 2 times, (▲) coated 3 times, and (○) coated 5 times. Measurements are relative to Ag/AgCl reference electrode].

models for biosensors where the catalytic activity of the enzyme membrane increases with enzyme loading[31]. The time response to reach steady baseline was also longer at the enzyme electrode on increasing the thickness of membrane PVA. The response time was expected to be shorter on increasing the units of enzyme. However, this was not the case in the present study, probably because the activity of immobilzed enzyme in the electrode was sufficient for catalytic

Table 1 — Performance of PVA enzyme electrodes constructed at various dip-coating times[a]

Dip-coating (times)	Response time (min)[b]	Linearity range (mM)	Sensitivity (slope, mV decade^{-1})
1	6	0.1 - 50	42.89
2	7	0.1 - 50 mM	40.74
3	9	0.1 - 50 mM	36.46
5	13	0.5 - 50 mM	30.02

[a]The enzyme electrode was prepared with variation of dip-coating in PVA-urease solution followed by sealing with plasticised PVC.
[b]Response time calculated from injection of 0.1m Murea until a steady baseline was obtained.

conversion of urea. The longer response time at the thicker film is probably affected by the thickness of the membrane on the electrode surface. Therefore, the enzyme electrode prepared by one coating was chosen as optimum for the urea biosensor. The biosensor constructed under these conditions gave a sensitive and fast responses to urea (see Table 1).

It is known that potentiometric measurement is affected by various parameters such as the concentration of the buffer, pH of the buffer, and the loading of the enzyme in electrode surface[25]. Since the pH is a critical parameter for enzymatic activity and stability of biosensors the effect of pH buffer was examined for urea biosensor in Tris buffer over the range of pH 4.0–9.0. The best response of the enzyme electrode to urea was obtained at pH 6.0. The biosensor was also been tested at the same pH range with different buffer solutions such as phosphate, HEPES and MOBS. It was found that Tris buffer (pH 6.0) gave the most sensitive response to urea.

It has been demonstrated the PVA matrix is compatible for incorporating the urease in the electrode surface. The use of PVA in potentiometric biosensor is mainly to find an alternative for immobilization techniques for urease in which it is compatible with maintaining bioactivity of enzyme to urea, and dip-coating technique is used to control the thickness of the biorecognition layer in the electrode surface. Since the PVA is water suluble, it is neccessary to sealed the PVA membrane with PVC. The plastisized PVC, due to rough, porous and hydrophobic nature, can protect the enzyme on the electrode surface. The incorporation of 2-nitrophenyl octyl ether (NPOE) as membrane plasticizer, potassium tetrakis (*p*-chlorophenyl) borate (KTpClPB) as anionic additive, 2-chloromethylquinoline in PVC film makes porous and elastic membrane in the construction of biosensor.

In the present work, preliminary study for the preparation of urea biosensor with immobilisation of urease in PVA matrix has been successfully conducted to obtain a fast biosensor for urea assay. The potentiometric urea biosensor is very attractive because the instrumentation is relatively inexpensive. The tungsten wire electrode used as a sensing device act as solid-state electrode and can be applied for the construction of potentiometric biosensors for urea. The PVA serves as a compatible matrix to immobilise urease on the electrode surface. The detection linearity lies in the range concentration of 0.01–50 mM urea, with slope of 42.7 mV decade^{-1} urea, and detection limit of 0.01 mM urea. The developed potentiometric biosensor is simple and allows further modification for commercial purposes. The best conditions for clinical application of the present biosensor are under investigation.

Supplementary data

Supplementary data associated with this article are available in the electronic form at http://www.niscair.res.in/jinfo/ijca/IJCA_57A(02)175-180_SupplData.pdf.

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