

Development of potentiometric urea biosensor based on urease immobilized in PVA–PAA composite matrix for estimation of blood urea nitrogen (BUN)

Sandeep Kumar Jha ^{a,1}, Anita Topkar ^b, Stanislaus F. D'Souza ^{a,*}

^a Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai 400085, India

^b Electronics Division Bhabha Atomic Research Centre, Mumbai 400085, India

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Abstract

A urea biosensor was developed using the urease entrapped in polyvinyl alcohol (PVA) and polyacrylamide (PAA) composite polymer membrane. The membrane was prepared on the cheesecloth support by gamma-irradiation induced free radical polymerization. The performance of the biosensor was monitored using a flow-through cell, where the membrane was kept in conjugation with the ammonia selective electrode and urea was added as substrate in phosphate buffer medium. The ammonia produced as a result of enzymatic reaction was monitored potentiometrically. The potential of the system was amplified using an electronic circuit incorporating operational amplifiers. Automated data acquisition was carried by connecting the output to a 12-bit analog to digital converter card. The sensor working range was 1–1000 mM urea with a response time of 120 s. The enzyme membranes could be reused 8 times with more than 90% accuracy. The biosensor was tested for blood urea nitrogen (BUN) estimation in clinical serum samples. The biosensor showed good correlation with commercial Infinity™ BUN reagent method using a clinical chemistry autoanalyzer. The membranes could be preserved in phosphate buffer containing dithiothreitol, β-mercaptoethanol and glycerol for a period of two months without significant loss of enzyme activity.

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1. Introduction

Determination of blood urea nitrogen is an important routine test widely used in clinical laboratories, as elevated urea level in blood sera deciphers kidney disease, stone in urinary tract or even bladder tumour. Whereas, its decreased level indicates severe liver malfunction. The normal range of urea in human serum is between 1.7 and 8.3 mM and level increases up to 100 mM under patho-physiological conditions [1]. Although colorimetric and spectrometric methods are most commonly used [2,3], simple and fast method for determination of urea is in demand.

More recently, biosensors have emerged as a promising technology, especially for applications requiring rapid and continuous monitoring. Biosensors are being applied to a wide variety of analytical problems such as in medicine [4–6], environment [7–9], food and process industries [10,11], security and defence [12]. Although urea biosensors emphasizing on better sensitivity [13–20] or higher response range [21] are reported, not much effort has been made in resolving the drawbacks of enzyme instability, difficulty in storage and handling, and fragility of the immobilization matrix.

Some of these drawbacks were obviated in our recent studies, wherein we developed a mechanically stable conjugate membrane of polyvinyl alcohol (PVA) and polyacrylamide (PAA) prepared on cheesecloth support by gamma-irradiation [22]. PVA provided flexibility to the membrane and cryo-polymerization generated porosity in the structure. The mixed properties of the individual polymers and use of cheesecloth as the impregnating support provided sufficient tensile strength to the membrane to

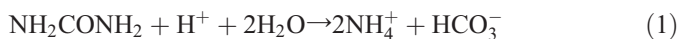
* Corresponding author.

E-mail address: sfidsouza@barc.gov.in (S.F. D'Souza).

¹ Present address: Department of Nanoscience and Engineering, Myongji University, San 38-2 Namdong, Cheoin-Gu, Yongin, Gyeonggi-do, 449-728, South Korea.

withstand rough treatments such as repeated washings. The membranes entrapped sufficient amount of the enzyme, however a slow leakage of the enzyme was seen during reuse. Cross-linking the enzyme with 0.2–0.4% glutaraldehyde in cold minimized the leaching.

We used this membrane in our present study to develop a potentiometric biosensor for urea. The sensor was based on the enzymatic reaction of urea hydrolysis catalysed by urease, which is described in Eq. (1).



The electronic circuit was designed for automated measurements and the biosensor was developed as standalone equipment to suite clinical needs.

2. Materials and methods

2.1. Materials

Polyvinyl alcohol (PVA) of degree of polymerization [1700–1800] and degree of hydrolysis [98–99 mol%] was procured from Loba Chemie, India. Urease (E.C. 3.5.1.5.) powder from Jack Bean was purchased from Sigma–Aldrich (catalogue no. U1500). Infinity™ Urea (Nitrogen) (Powder) reagent and ammonia ion selective electrode (ISE) (model no. 9512 BNWP) were from Thermo Scientific Inc. All other chemicals were of analytical reagent grade. A 12-bit analog to digital converter card (ADC) (model PCL 207) was procured from Dynalog India Ltd. Other electronic components were purchased from the local market.

2.2. Preparation of enzyme membrane

The enzyme membranes were prepared as per the procedure described earlier [22]. Polymerization mixture contained PVA (5%) (w/v), acrylamide (10%) (w/v) and bis-acrylamide (3%) (w/v), dissolved in 10 mM sodium phosphate buffer of pH 7.4 by heating under boiling water bath for 10 min, followed by cooling down to room temperature. Urease (2.5 g) (w/v), dithiothreitol (DTT) (1 mM), β -mercaptoethanol (β -ME) (1 mM) and 125 μ l

glycerol were mixed with 50 ml of this viscous slurry and homogenized by stirring with a glass rod. Ten 100 cm² cheesecloth pieces were soaked in the slurry and frozen in toluene-dry ice bath (–78 °C). These were then exposed to 1.2 kGy of Co⁶⁰ γ -rays in a gamma-cell (model: 220, Atomic Energy of Canada Ltd, Ottawa, Canada) at a dose rate of 10 Gy/min using air as the gas phase. After irradiation, membranes were soaked in phosphate buffer (pH 7.4) containing 0.2% glutaraldehyde (v/v) for 20 min and were finally washed with phosphate buffer. The membranes were partially dried at room temperature and stored at 4 °C.

2.3. Analytical methods

Urease activity in the membrane was determined as previously reported [22]. Membranes of size 5 cm² (2 cm \times 2.5 cm) were soaked in phosphate buffer containing 50 mM urea for 1 min. 20 μ l aliquot of the sample was removed and the ammonia liberated was estimated colorimetrically by the phenol-hypochloride method [23].

Urea was estimated by Infinity™ blood urea nitrogen (BUN) reagent method [24]. Appropriate amount of serum sample was mixed with 300–1000 μ l of reagent and analyzed by a chemistry autoanalyzer (model-express plus, Ciba–Corning Ltd.). The urea concentration was proportional to the absorbance change at 340 nm over a fixed time interval.

2.4. Reaction vessel and electronic circuit designs

The reaction vessel and electronic circuit used for biosensor studies were developed in our laboratory. The schematic diagram of the vessel is shown in Fig. 1. An ammonia ion selective electrode (ISE) was placed in a measuring cylinder with provision of buffer flow through tubes. Samples were injected into the vessel through a syringe. A buffer tank was placed at a height to maintain the flow of buffer to the vessel by siphon action. The flow was controlled with the help of stopper cocks. The working volume of the vessel was 10 ml.

An electronic circuit was designed to acquire sensor signal. This comprised of operational amplifiers (op-amp) to amplify the output potential of the ammonia selective electrode. Due to

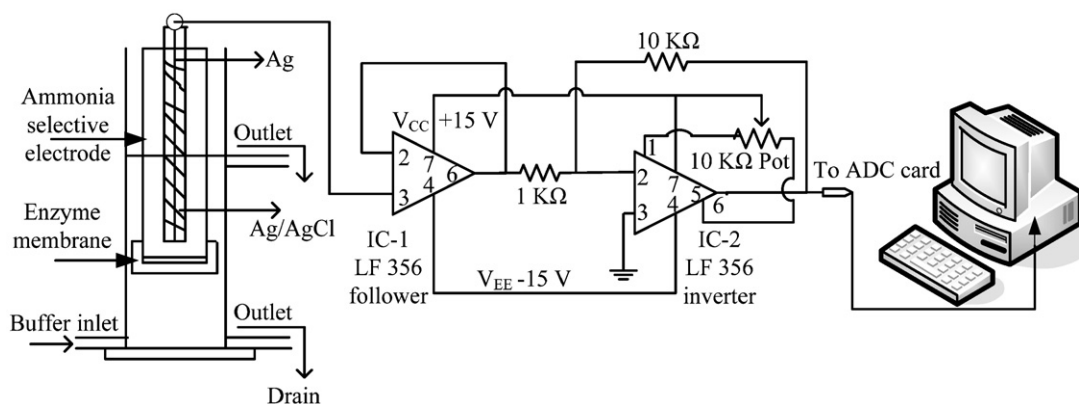


Fig. 1. Schematic of flow-through reaction vessel and circuit diagram of electronic assembly used for urea biosensor.

high impedance characteristics of the electrode, a high input impedance, low offset voltage op-amp (LF 356) was used as voltage follower at the first stage (IC-1, Fig. 1). The potential developed due to the formation of ammonia was amplified ten-folds further with additional op-amp inverter (IC-2, Fig. 1). The circuit diagram of the instrument is also included in Fig. 1.

2.5. Data acquisition and analysis

Data acquisition was automated by connecting the amplified output of the instrument to a 12-bit ADC card integrated with a computer. The ADC card had a resolution of 2.44 mV for a full-scale voltage of ± 5 V. A programme was written using C++ language to acquire the ADC card output data. The programme also provided step by step guidance to users through its interface. The electronic noise in the circuit was minimized using two approaches, first by isolating the circuit in an aluminium casing and further by software based real-time averaging of input signal. The ADC card read instrumental analog output through a fixed channel at a sampling rate of 10 K with respect to analog ground. Mean of the potential values per second was recorded in the computer for further analysis. Post acquisition analysis including data plot, curve fitting and statistical analysis were performed using software Origin.

2.6. Sensor measurements

The enzyme membranes were allowed to equilibrate to room temperature before measurements. The membrane (2×2.5 cm²) was tied to the tip of the electrode with the help of an O-ring and then placed in the cylinder. The baseline potential of the sensor was achieved with flow of buffer for about 15 min (at flow rate of 20 ml/min). Subsequently, 0.5 ml of sample was injected in the vessel through buffer inlet to initiate enzyme–substrate reaction. As the reaction was over, the liquid inside the vessel was drained off and membrane was washed by flow of buffer. The process was repeated with different urea concentrations (1–1000 mM) to obtain standard curve. The experiments were performed at least in triplicates and enzyme-free membranes were used as control. The sensor was subsequently tested on synthetic urea samples (1–1000 mM) and urea concentrations calculated from calibration equation of biosensor were compared with the actual amounts used. Possible interference on sensor response from other species in the blood such as ascorbic acid was examined by recording the biosensor response with synthetic samples containing 1–10 mM of ascorbic acid and fixed urea concentration (10 mM).

For ascertaining reusability, a single enzyme membrane was used recurrently for estimation of urea in a span of 3–4 h by washing in between measurements. Aliquots (0.5 ml) of 100 mM urea were injected in the vessel each time and urea concentration was calculated from the calibration equation for 120 s response time. The sensor was also tested on clinical samples derived from different patients. Serum or whole blood samples was analyzed for blood urea nitrogen (BUN) using biosensor and subsequently with Infinity™ BUN reagent method in a chemistry autoanalyzer.

2.7. Shelf-life of the membranes

To determine optimum storage condition, the membranes were either kept moistened with buffer or completely submerged in it. In one set, the buffer contained 1 mM each of DTT and β -ME and also 0.25% glycerol, whereas, no additives were used in second set. These membranes were stored at either 4 °C or room temperature (25 °C). The enzyme activities in these membranes were estimated at regular interval for a period of two months.

3. Results

The enzyme membranes prepared under ideal conditions and stored for one day were used for all sensor measurements. These contained 1.2 U/cm² enzyme activity and about 1.12 mg/cm² protein content. The average thickness of these membranes was 0.89 mm (SD 0.08, $n=10$).

3.1. Calibration of the biosensor

Biosensor response was obtained upon injection of urea sample in the vessel containing stabilized membrane. The potential of the system, as recorded over time, showed an increase in magnitude and saturated to a level particular to urea concentration. Baseline corrected sensor responses for entire urea concentration range and as also in buffer are shown in Fig. 2. The sensor response was rearranged to depict urea concentration (C , mM) with respect to potential change from baseline (δV , volt). The magnitude of δV showed linear correlation for the entire sensor range at 30 s response time, whereas, it was non-linear for other durations and saturated beyond 120 s (Fig. 3). Hence, 120 s was chosen as sensor response time. At this point, sensor response had reached over 90% of its maximum for 100–1000 mM urea and 75% for lower concentrations. The equation obtained upon curve fitting of sensor response at 120 s represented rectangular hyperbola between 1–1000 mM urea. The calibration equation was

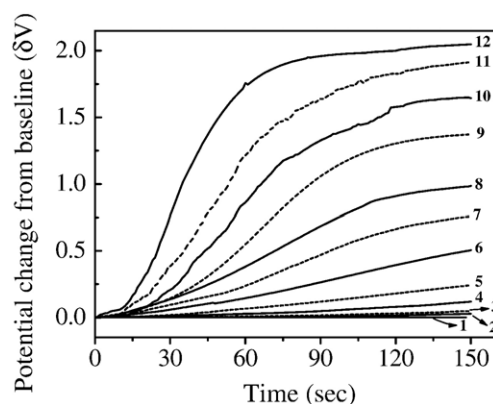


Fig. 2. Baseline corrected response curves of the biosensor with: (1) phosphate buffer pH 7.4; (2) 1 mM, (3) 5 mM, (4) 10 mM, (5) 20 mM, (6) 50 mM, (7) 100 mM, (8) 150 mM, (9) 300 mM, (10) 500 mM, (11) 800 mM and (12) 1000 mM urea.

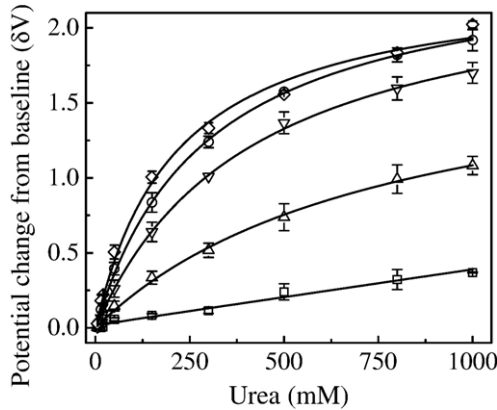


Fig. 3. Calibration curve for urea at (□) 30 s, (△) 60 s, (▽) 90 s, (○) 120 s and (◇) 150 s response time of the biosensor.

simplified (Eq. (2)) and coefficients in the expression were fed into software for calculating urea concentrations.

$$c = \frac{293.53 \times \delta V}{(2.48 - \delta V)} \quad (2)$$

The sensor response in plain buffer had a noise of 2.5 mV (Fig. 2). Therefore, lower detection limit and resolution of the sensor were deduced using Eq. (2) as 1 mM and 0.3 mM urea respectively.

3.2. Sensor selectivity, reusability and sensitivity

Ammonia ISE was used as transducer to make sensor specific towards ammonia. There was negligible interference on sensor response by ascorbic acid, as tested with synthetic samples containing 1–10 mM of ascorbic acid and fixed urea concentration (10 mM). The enzyme-free control membrane also showed negligible response. Urease membranes could be reused several times with proper washing in a span of 3–4 h and sensor response was more than 90% reproducible till 8 reuses (Fig. 4).

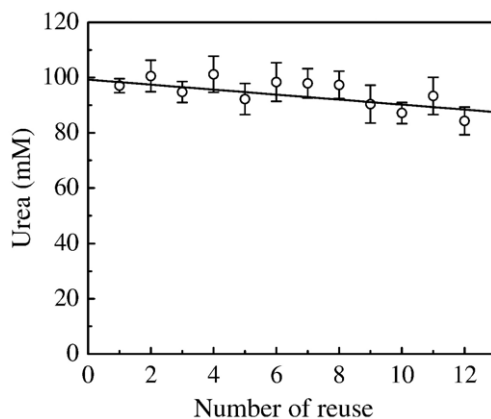


Fig. 4. Reusability of enzyme membranes: Synthetic urea samples (100 mM) were analyzed repeatedly using single membrane in a span of 3–4 h at room temperature. Biosensor response was reproducible to more than 90% till 8 reuses of membrane.

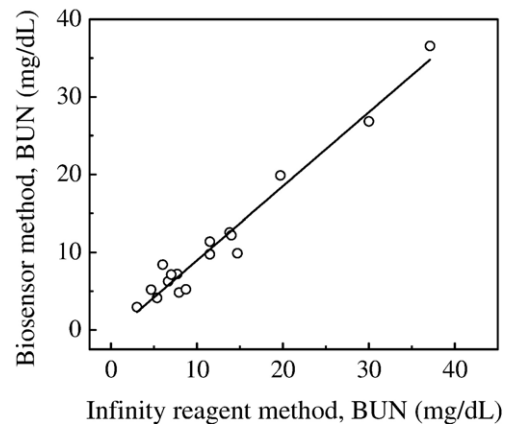


Fig. 5. Correlation between measured values of BUN in clinical serum samples analyzed by biosensor and Infinity™ reagent methods.

The biosensor was tested on synthetic urea samples and concentrations calculated from calibration equation were compared with the actual amounts used. Statistical analysis of this comparison yielded regression coefficient and slope of 0.997 and 0.944 respectively for the samples tested within sensor working range. The corresponding values in lower concentration range (1–15 mM) were 0.983 and 1.042 respectively. The sensor sensitivity, defined as ratio of δV and C , was calculated from biosensor calibration equation and were found as 8.3–7.4 V/M urea (for 1–40 mM range), 4.0–7.4 V/M (40–550 mM) and 2–3 V/M of urea for rest of the working range.

3.3. Biosensor testing on clinical blood samples

The biosensor was tested on clinical serum samples and BUN values calculated from calibration equation was compared with that obtained using commercial Infinity™ BUN reagent method (Fig. 5). The linear curve fit of comparison yielded an equation, $y = 0.952 \times x - 0.549$ with regression coefficient of 0.962.

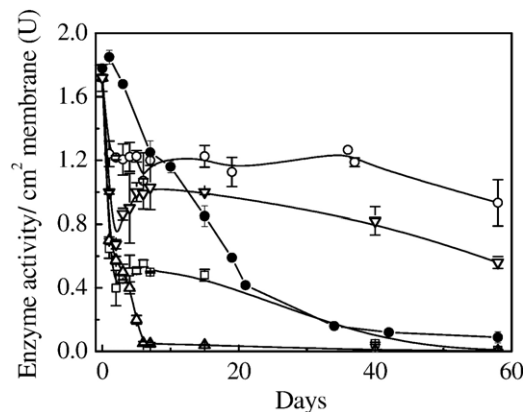


Fig. 6. Stability of urease membrane at (□) 25 °C and (○) 4 °C when stored moistened with buffer containing DTT, β-ME and glycerol. Stability at (△) 25 °C and (▽) 4 °C when submerged in buffer containing DTT, β-ME and glycerol. Stability at 4 °C (●) when stored moistened with buffer devoid of DTT, β-ME and glycerol.

3.4. Shelf-life of the enzyme membranes

Urease activity in stored membranes was estimated till two months. The enzyme activity was observed as 1.8 U/cm² on the first day, which decreased and got stabilized from second day onwards to 1.2 U/cm² when kept moistened at 4 °C (Fig. 6). Storage at room temperature led to significant loss of enzyme activity over time. Loss of enzyme activity was also seen when membranes were kept submerged in buffer. Upon dry storage, membranes dehydrated and became brittle. The enzyme activity in the membrane prepared without additives reduced continuously with time even when stored under optimum conditions (Fig. 6).

4. Discussion

Potentiometric biosensor, that often uses large sized commercial ISEs as transducer require enzyme membranes to be tied directly at the sensing tip. The membrane also should be prepared under mild conditions for better retention of enzyme activity. We used PVA–PAA conjugate membranes as matrix prepared using γ -irradiation to provide gentle condition for enzyme immobilization. Use of cheesecloth as additional support layer during preparation of enzyme membranes proved useful in improving the tensile strength and reusability of the membranes. Cross-linking of enzyme in the matrix using glutaraldehyde was necessary to maintain integrity of the membrane as well to minimize enzyme leaching during use. Use of flow-through reaction vessel for sensor measurements helped in making the membranes reusable through quick and gentle washing between analysis.

Urease activity in stored membranes, when observed for long-term shelf-life, decreased and got stabilized from second day of preparation when kept moistened at 4 °C (Fig. 6). The reason for loss of enzyme activity on first day of preparation was not clear. Storage at room temperature led to significant loss of enzyme activity over time. Loss of enzyme activity was also seen when membranes were kept submerged in buffer at room temperature. This was obvious for the reason that urease is sensitive towards temperature [25]. Dry storage was not possible for the reason that PAA–PVA gels upon complete dehydration yield a hard solid in low humidity environment as also mentioned earlier [26]. Therefore, the optimum storage condition was to moisten and preserve the enzyme membranes at 4 °C. Addition of β -ME and DTT to the polymerization mixture was useful in retention of enzyme activity, whereas, glycerol helped in delayed drying of moistened membranes. The enzyme activity in the membrane prepared without these additives reduced continuously with time even when stored under optimum conditions (Fig. 6).

Selectivity and specificity of the biosensor remains cause of concerns in potentiometric systems, primarily because various ions and radicals from sample can impart a charge transfer across the electrode. Therefore, pure enzyme was used as biological recognition element to avoid unselective reactions. An ammonia selective electrode was used in the equipment to solve the problem of specificity, as it would only detect

ammonia species formed by the action of urease on urea. It was also observed that there was no influence of common interferrant like ascorbic acid on sensor response.

Use of relatively inexpensive and simple electronic circuit in combination with ADC card based automated data acquisition and software controlled signal conditioning enhanced the sensitivity of the equipment, which otherwise lacks in commercial pH meters. The gain of operational amplifier used in the circuit was set to tenfold of input signal to avoid amplification of noise. Data visualization, real-time data averaging and interpretation of potential change into substrate concentration were simplified using the program written in C++.

The response time of a biosensor is often set to a time where sensor response reaches more than 90% of maximum value for any analyte concentration. This definition holds true only for linear range of calibration. Therefore, response time of biosensor was calculated on the basis of saturating trend of response (Fig. 3), which was 120 s in this case. The calibration equation for urea obtained for this response time, represented rectangular hyperbola which was fed in the software to calculate and display urea concentration after the response time. Software based interpretation of the results minimized the need for manual calculations, therefore non-linearity of calibration curve or manual interpretation of response time did not affect sensor accuracy.

The biosensor showed best sensitivity in clinically important low concentration range (1–40 mM urea) and blood samples could be analyzed without pre-treatments. The benefit of having a wide detection range of sensor (1–1000 mM urea) was the possibility to analyze samples with high urea concentrations, such as, adulterated milk or from effluent treatment plants. The sensor also had a good correlation with commercial Infinity™ BUN reagent method for determination of urea. Hence, the urea biosensor developed was suitable for clinical analysis and can also find use with non-clinical samples.

5. Simplified description of the method and its applications

Urease was immobilized within mechanically stable conjugate membrane of PVA–PAA prepared on cheesecloth support by γ -irradiation followed by cross-linking with glutaraldehyde. The enzyme could be stabilized within the membrane using DTT, β -ME and glycerol as additives. A potentiometric urea biosensor was developed using this membrane in conjugation with ammonia selective electrode and placed within a flow-through reaction vessel designed for this purpose. The potential output from the electrode increased with liberation of ammonia by enzymatic reaction of urease with urea. The signal was amplified and sensed using an electronic circuit developed with LF-356 based op-amps. The standalone equipment was interfaced with a computer using an ADC card and software developed in C++. The biosensor was calibrated against synthetic urea samples and tested for its detection range, sensitivity, reusability and shelf-life. It showed a wider response range for urea and was reusable and stable for over two months. The whole blood or serum samples were analyzed with the biosensor and the clinical data was correlated

with existing commercial method using urea autoanalyzer. The urea biosensor was suitable for BUN analysis and may also find uses in environmental as well as milk urea nitrogen (MUN) analysis because of its broad detection range, reusability and longer shelf-life.

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