

A Conductometric Urea Biosensor by Direct Immobilization of Urease on Pt Electrode

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ABSTRACT: Urease was immobilized on platinum electrode both by chemical binding and electropolymerization. The conductometric urea biosensor thus prepared showed a detection limit of 4.9×10^{-5} M and linear dynamic range from 4.9×10^{-5} to 5.8×10^{-3} M for urea concentration when the enzyme is covalently immobilized on Pt electrodes. Conductometric transducers respond to the changes in ionic strength thereby leading to uncontrolled inaccuracies. Such interferences were effectively suppressed by here the use of differential sensor pairs. It was shown that measurements in diluted fluids are possible with the use of a reference sensor having no immobilized enzyme. Specifically, the sensor constructed by covalent binding provided more reproducible responses than that prepared by electropolymerization. Namely the former preserved 80% of its initial activity over a period of 25 days. In order to provide more reproducibility, a method was developed for regeneration of the sensor surface so that, the renewed sensor would responded to urea almost as new.

KEY WORDS: Urea determination, Conductometric biosensor, Urease immobilization, Electro-polymerization, Differential conductometric transducer, Urease covalent binding.

INTRODUCTION

Since the biocatalytic reaction of urease primarily involves a pH change due to the decomposition of urea to carbon dioxide and ammonia, most urea biosensors are based on potentiometric mode of detection [1-14]. These include a mathematical model to describe dynamic behavior of potentiometric biosensors [15]. Also, a few developments in urea sensors are focused on amperometric method [16-21] using immobilization of urease onto membranes or conducting polymers. Moreover some other methods such as miniature thermal biosensor

[22, 23], plant tissue-based chemiluminescence [24] and the sensor based on a manometric assay of the carbon dioxide generated by the enzymatic hydrolysis of milk urea [25] have been developed for determination of urea.

The next group of urea biosensors developed are based on conductometric method [26-31]. The unique characteristic of the enzyme-catalyzed reactions studied by conductometric method is the change in solution resistance (reciprocal of conductance) during the biocatalytic process. Conductometric sensors are simply

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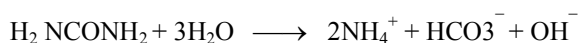
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constructed and from technological point of view are suitable for miniaturization and mass production. In addition, they do not need a reference electrode and unaffected by color or turbidity.

Lawrence and Moores [32, 33] have reported five categories of enzymes that, separately or in combination, allow the application of conductometric methods to enzymatic reactions. These enzymes were including a) amidases for generation of ionic groups b) dehydrogenases and decarboxylases leading to separation of unlike charges c) estrases for protein migration d) kinases that cause a change in the degree of association of ions and e) phosphatases and sulfatases that result in change in the size of charge-carrying groups. Among the processes mentioned, the urease-urea reaction in which the initially uncharged substrate is hydrolyzed to yield four charge-bearing species has attracted more attention:



The previous work on using the conductometric method for determination of urea was done in solution [34]. Thereafter, efforts have been made to use the conductometric method as a transducer for developing the urease based biosensors.

We report herein the design and characterization of a new detection cell for urea determination in which urease is immobilized both by chemical binding and electropolymerization on platinum electrode. More sensitivity was achieved using direct attachment of enzyme to the electrode surface. Interferences caused by fluctuations in the ionic strength and consequent conductivity changes were effectively suppressed by differential sensor pairs.

EXPERIMENTALS

Materials and methods

Urease from jack beans, E.C.: 3.5.1.5, bovine serum albumin (BSA), glutamate dehydrogenase from bovine liver (GLDH), E.C.: 1.4.1.3, adenosine diphosphate (ADP), and nicotinamide adenine dinucleotide (NADH), were purchased from Sigma Chemical Company (St. Louis, MO, USA). N-[3-(trimethoxysilyl) propyl] ethylamine and glutaraldehyde were purchased from Aldrich Chemical Company (Milwaukee, WI,

USA). Pyrrole, folin-ciocalteu's phenol reagent, copper (II) sulfate pentahydrate were prepared from the Merk Chemical (Darmstat, Germany). All reagents were commercially available and employed without further purification. Solutions of enzyme were prepared with doubly distilled water. Phosphate buffer, 1 mM, pH 7.6, was used in all measurements, unless stated otherwise.

Pyrrole electropolymerization carried out using a 263A Potentiostat-Galvanostat from EG&G (USA). All experiments were carried out at 25°C using a laboratory made isolated chamber equipped with an electronic temperature controller to achieve a constant cell temperature. Conductometric experiments were carried out in 1 mM phosphate buffer at pH 7.6. Spectroscopic measurements were carried out using a spectrophotometer UV-160, Shimadzu (Tokyo). Conductometric measurements were carried out using a laboratory designed and made differential conductometer.

Protein quantitation and enzyme assay

For protein quantitation the Lowry method [35] was used. For this purpose 0.1 ml of 2N NaOH was added to 0.1 ml of the sample or standard solution. The solution was hydrolyzed at 100°C for 10 min in a heating block or a boiling water bath. The hydrolyzate was cooled to room temperature and 1 ml of freshly mixed complex-forming reagent (a solution of sodium carbonate 2%, copper sulfate 1% and sodium potassium tartrate 2% in distilled water) was added. The solution was let stand at room temperature for 10 min. Then 0.1 ml of the Folin reagent, using a vortex mixer, was added and let the mixture stand at room temperature for 30-60 min. The absorbance was read at 750 nm, if the protein concentration was below 500 µg/ml, or at 550 nm if the protein concentration was between 100 and 2000 µg/ml. A standard curve of absorbance as a function of initial protein concentration was plotted and used to determine the unknown protein concentrations.

The assay of urease was carried out according to Worthington manual [36]. For this purpose, a solution of the enzyme (1 mg/ml) in 0.1 M phosphate buffer pH 7.6 was prepared. Immediately prior to use, solution was diluted further in buffer to obtain a rate of 0.02-0.04 ΔA/min. The spectrophotometer was adjusted to 340 nm and 25°C. In two cuvettes (sample and reference) the following reagents were added: phosphate buffer 0.1 M,

pH 7.6, 2.4 ml; adenosine-5'-diphosphate (ADP) 0.023 M in phosphate buffer, 0.1 ml; NADH 0.0072 M, 0.1 ml; α -ketoglutarate 0.026 M, 0.1 ml; urea 1.8 M, 0.1 ml and GLDH, 500 units/ml, 0.1 ml. The mixtures were incubated at 25°C for 5-10 minutes to achieve thermal equilibration. Upon obtaining a zero change in absorbance, 0.1 ml enzyme solution was added. The decrease of adsorption at 340 nm was recorded. The units of enzyme were calculated based on the following equation:

$$\text{Units/mg} = \frac{\Delta A_{340} / \text{min}}{6.22 \times \text{mg.enzyme} / \text{ml.reaction mixture}}$$

Enzyme Immobilization

The enzyme was immobilized on the surface of the conductometric transducers (2×2×40 mm Pt electrodes) by two methods: cross-linking in an albumin-glutaraldehyde film (method A) and entrapment by pyrrole electropolymerization (method B).

Method A required three steps to bind the enzyme covalently to the electrode surface. At first the sensitive parts of platinum electrodes were polished with aluminum oxide powders 0.5 and 0.03 μm , respectively. The polished electrodes were washed thoroughly with water, absolute ethanol and distilled water under ultrasound for 5 min, respectively. Electrodes were then, dipped in boiling 2M sodium hydroxide solution for 2 hrs and washed thoroughly with distilled water.

In the second step, the clean electrodes were anodized for 1.5 hrs at 2 Volt vs Ag/AgCl in 0.1 M sulfuric acid solution. The anodized electrodes were removed from the cell, washed thoroughly with distilled water, dried and refluxed for 1hr in an anhydrous 10% (V/V) solution of N- [(three methoxy silyl propyl) ethylene diamine] in toluene. The resulting silanized electrodes were rinsed thoroughly with toluene and ethanol.

In the third step, a 20 μl aliquot of 1.2 mg/ml solution of BSA and 30 μl of 1 mg/ml solution of urease (both in phosphate buffer 0.1 M, PH 7.6) was mixed and spread on the alkyl-amino-bound platinum electrode. Then 10 μl of 0.02% (W/V) glutaraldehyde was added to the surface of platinum electrode. The solutions were mixed rapidly with a thin glass rod. The film was allowed to form at room temperature for 30 min. The electrodes were washed with phosphate buffer and stored in buffer at 4°C.

Method B also required three steps to entrap the enzyme in polymer membrane. The electrodes that were polished using the procedure mentioned in method A were rinsed thoroughly with distilled water and dipped in boiling nitric acid 0.1 N for 10 min. Thereafter, they were washed with distilled water several times and dried at room temperature.

In the second step, 30 μl of freshly distilled pyrrole was dissolved in 0.9 ml phosphate buffer 0.1 M, PH 7.6. To this solution 100 μl of KCl 0.1 M was added. Then, one mg of urease was added to the pyrrole solution.

In the third step, the electropolymerization and entrapment of the enzyme were performed galvanostatically by applying a current density of 0.4 mA/cm² to the working electrode for 5 min. A three-electrode voltammetric cell was used for electropolymerization. A platinum electrode (2×2×40 mm) was used as the working electrode, while a Pt wire and an Ag/AgCl were employed as auxiliary and reference electrodes, respectively.

For both methods, after placing the electrodes in the cell the response current base lines were established, using a known volume of fresh buffer, thereafter substance additions began.

Experimental set-up and measurements

The design of the conductometric transducer used in this work is shown in Fig. 1. A low frequency wave-form (200 Hz) was generated by the sinusoidal generator. The output signal after preamplification was conducted to the biosensor.

Characteristics of the biosensors were measured at differential signal between the pairs of electrodes covered with the immobilized enzyme (used as working cell) and those without enzyme (used as blank). Measurements were conducted in a cell shown in Fig. 1.

Two identical pairs of platinum rods were used as electrodes. The dimensions of each of them were 2×2×40 mm. Two pairs of electrodes were spaced 10 mm apart. The sensing surface, which was in contact with the test solution, consists of four parallel electrodes of 20 mm length. The electrodes were fixed in places and covered with a flexible silicone rubber followed by a rigid sheet as support. Then they were kept tightly using two clips on both sides

of the cell. In each pairs the electrodes spaced one mm apart.

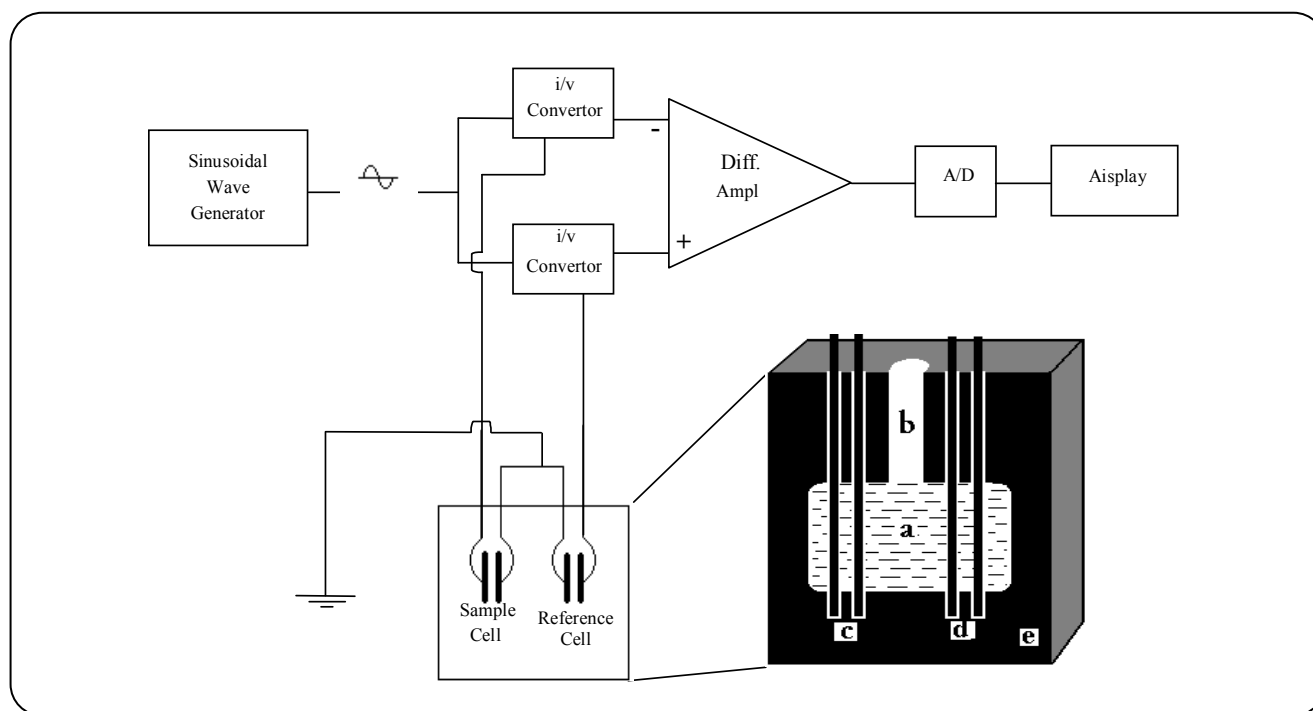


Fig. 1: Schematic representation of the overall conductometric cell layout and block diagram of the set-up used. The cell body (e) is made from Plexiglas. Two identical pairs of platinum rods with (pair c) and without (pair d) urease were used as sample and reference cells, respectively. In each cell the electrodes spaced 1mm apart. The dimensions of each of them were $2 \times 2 \times 40$ mm. Two pairs of electrodes were spaced 10 mm apart. The sensing surface, which was in contact with test solution (a), consists of four parallel electrodes of 20 mm length. The test solution compartment (a) is filled through inlet (b). The electrodes were fixed in places and covered with a flexible silicone rubber followed by a rigid sheet as support (not shown in the figure). Then they kept tightly using two clips on both sides of the cell

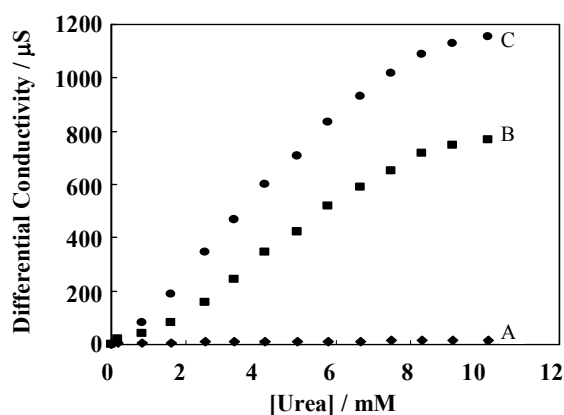


Fig. 2: Conductometric changes versus urea concentrations. Line A represents the conductometric change in the absence of urease as blank. Lines B and C indicate the conductivity responses in the same condition but in the presence of urease immobilized based on methods B and A, respectively. Urea samples were made in 0.1 mM phosphate buffer solution pH 7.6 and the measurements were carried out at 25 °C. For details see the text.

The design was chosen for simplicity and ease of renewing the electrode surface. Although improved analytical characteristics may be achieved with smaller cell spacing with continuous monitoring.

RESULTS AND DISCUSSION

Characterization of urea biosensors

The conductometric sensors for urea determination were constructed by using enzyme immobilization methods A and B. Initial blank measurements of transducer responses to urea solution, were made in 0.1

mM phosphate buffer solution. As the urea concentration was increased from zero to 10 mM (by addition from stock solutions made in the same buffers), almost no conductivity change was observed (Fig. 2, line A). When the electrode-pairs containing immobilized urease were replaced with bare electrodes, the conductivity increased by increasing the concentration of urea in buffer solution.

Table 1: Parameters for native and immobilized urease

Parameters Urease form	K_m (mM)	V_{max} (M/min)	Total enzyme used for immobilization (IU) ^a	Enzyme activity (IU) ^b	Recovery of activity (yield)(%)
Native (free) enzyme solution	4.2	15.0×10^{-3}	—	—	—
Immobilized, covalent binding (method A)	4.6	13.5×10^{-3}	0.39	0.085	21.7
Immobilized, lectropolymerized (method B)	4.5	13.0×10^{-3}	0.400	0.100	25.0

a) Mean values of three measurements based on Lowry's method. For details see the text.

b) For assaying the immobilized urease on Pt surface, the sensitive part of the sensor (2 cm length) was immersed in assay solution for one min. Then the absorbance was measured at 340 nm. The figures are mean values of five independent measurements based on the procedure reported by Worthington [36].

In Fig. 2, curves B and C indicate the conductivity response in the presence of urease, immobilized based on methods A and B, respectively.

Kinetic parameters of immobilized enzyme

Comparison of the kinetic parameters of immobilized enzyme with those for native enzyme solution revealed that both K_m and V_{max} values have changed after immobilization. The K_m of native enzyme in solution, changed from 4.2 mM, into 4.6 mM and 4.5 mM through immobilization by methods A and B, respectively. Moreover, the V_{max} value of enzyme solution, 15.0×10^{-3} $\mu\text{M}/\text{min}$, shifted to 13.5×10^{-3} $\mu\text{M}/\text{min}$ and 13.0×10^{-3} $\mu\text{M}/\text{min}$ when the protein immobilized through methods A and B, respectively. The difference in the kinetic parameters usually attributed to the effect of microenvironment and the immobilization process itself. The diffusional restriction is the other most dominant effect influencing the kinetics of the immobilized enzyme [37]. The restriction of substrate diffusion through the immobilized enzyme layer strictly affects the reaction rate. From the data reported in table 1, it is obvious that the diffusional constraint caused by covalent binding

(method A) is more severe than that caused by electropolymerization (method B). In other word, it seems the covalent binding of urease on electrode surface leads to its tighter bonding owing to multiple attachments. This probably produced a relatively higher stiffness and in turn a diffusional constraint.

Efficiency of enzyme immobilization

Estimation of the amount of bound urease is important for ascertaining the efficiency of immobilization methods, since it is well known that all the protein that is immobilized is not active. For this purpose the following steps were carried out. In the first step, after each immobilization procedure, the sensor was rigorously washed. Then, the amount of protein recovered in the washing solution was determined using the Lowry's method [35]. Quantification of the bound urease was performed by measurement of the difference between the amount of protein put into the immobilization mixture and that recovered after washing.

The remaining activity of immobilized urease on electrode surface was assayed using the procedure prescribed in Worthington manual [36]. The sensitive part of the sensor (2 cm length) was immersed in the assay solution for one min. The absorbance was then

measured at 340 nm. The yield of immobilized enzyme for both methods were characterized and compared. This consideration revealed that the recovery of enzyme activity in method B is 3.2% higher than that of method A. This indicated that cross-linking of the enzyme probably made more restriction for urease relative to electropolymerization.

pH profiles

The pH profiles of the immobilized enzymes and that of enzyme solution were studied using both optical method (Fig. 3, up) and conductometry (Fig. 3, down). For this purpose phosphate buffer solutions in the pH

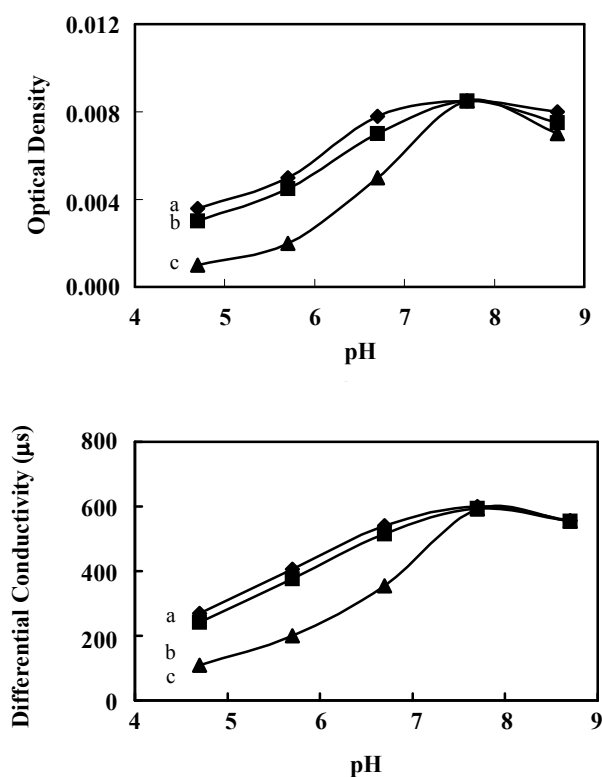


Fig. 3: Comparison of pH profiles for native and immobilized enzymes. Curves a, b and c represent the pH profiles of the enzyme immobilized by methods A and B and that for enzyme solution, respectively. For pH change, phosphate buffer solutions with a pH range from 4.8 to 9 were used. The pH profiles of the immobilized enzymes and that of enzyme solution were studied using both optical method (top) and conductometry (down).

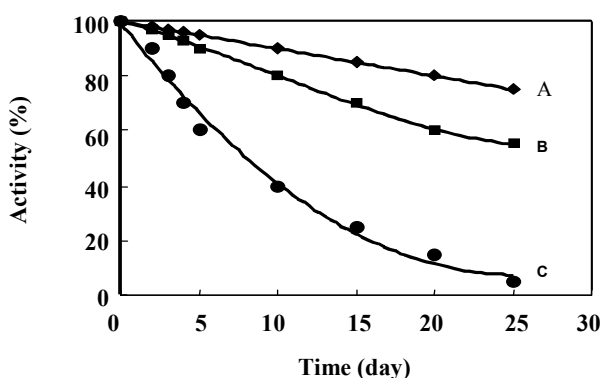


Fig. 4: Stability of urea biosensors over time. The urea samples were prepared in phosphate buffer 0.1 mM, pH 7.6. The percent activity of urease immobilized by methods A (curve A) and B (curve B) were compared with that of urease enzyme solution (curve C). For details of enzyme immobilization methods see the text.

and c indicate the pH change for the immobilized urease based on the methods A and B and that for free urease in solution, respectively. These results show that the optimum pH for the enzyme immobilized by methods A and B and that for enzyme in solution were about 7.6. It can be seen that the pH profiles for both immobilized methods are less affected by the pH change compared with the free enzyme. Moreover, it is obvious that the immobilization processes did not affect the optimum pH.

Storage stability

The storage stability of urea sensors were investigated over 25 days by measuring the response to urea solution up to 10 mM concentration in phosphate buffer, pH 7.6. The percent activity of urease immobilized by methods A and B were compared with that of the free urease in buffer solution. The data depicted in Fig. 4, show that the free enzyme in buffer solution deteriorated with time rapidly, while, the immobilized enzymes showed a reasonable stability. Specially, the sensor constructed by method A provided a more reproducible response than that prepared by method B. So that, over a period of 25 days, following immobilization method A, 80% of the initial activity was preserved.

Sensor surface regeneration

As described above, after a period of time the activity of biosensor decreased by 80% for method A. This implied the necessity of re-calibration of sensor due to gradual deterioration of immobilized enzyme. This also suggests that the surface of biosensor must be regenerated

for reproducible results. Accordingly, to renew the modified electrodes surface, the cell designed in such a way that removal of electrode pairs from the cell could be done simply. For this purpose, the enzyme-covered platinum electrode was dipped in the boiling nitric acid 0.1 N for 10 min. It was then, washed with distilled water frequently. At this stage, the immobilization method A was followed from step two. The electrodes, which were subjected to regeneration process satisfactorily showed reproducible results (data not shown).

Calibration curve

Fig. 5 represents a calibration curve resulting from five frequent measurements. The points depicted in this figure are the mean values of five independent

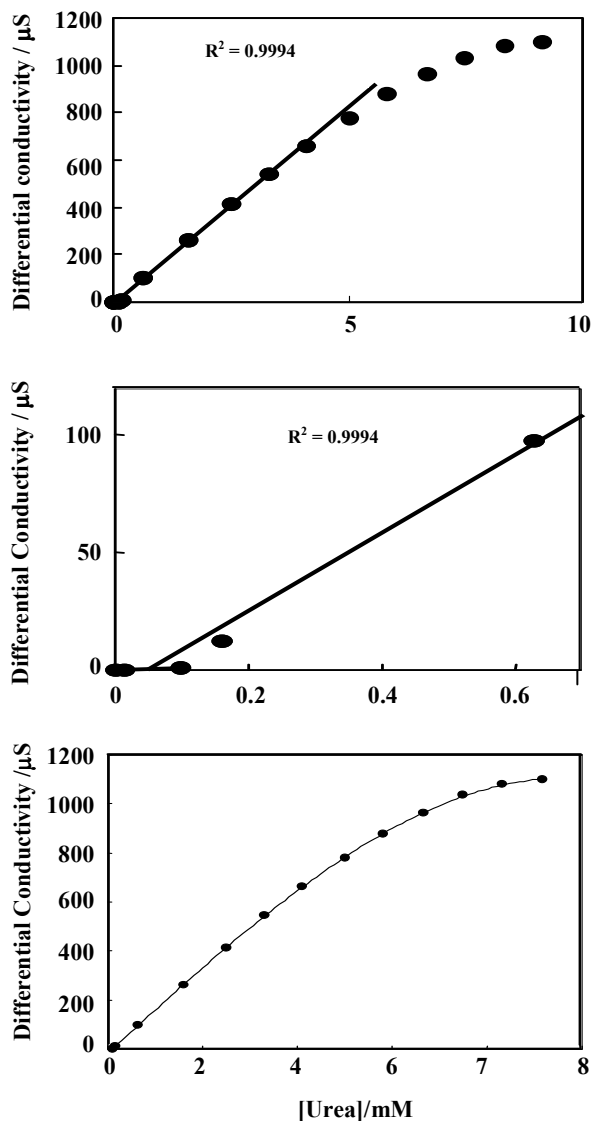


Fig. 5: Calibration curve for the urea conductometric biosensor. In the bottom, the whole calibration curve, in the top, the upper limit of dynamic range and in the middle the detection limit of linear range is observed. Urease was immobilized covalently based on method A. The urea samples were prepared in phosphate buffer 0.1 mM, pH 7.6. The points depicted in the figure are the mean values of five independent measurements at different concentrations of urea solution. The measurements were carried out at 25 °C. For a concentration within the linear range of conductivity response, the standard deviation was 1.58.

measurements at different concentrations of urea. For a concentration within the linear range of conductivity response, the standard deviation was 1.58, which indicated that the responses of the sensor were reliable. We were able to measure the concentration of urea in the range from 4.9×10^{-5} to 5.8×10^{-3} M. This indicates that the present sensor could be adaptable for clinical use from the viewpoint of its sensitivity and reliability. The detection limit, according to the IUPAC [38, 39], may be taken as the concentration corresponding to the point of intersection of the two extrapolated lines as shown in Fig. 5, middle. In Fig. 5, top, the upper limit of the linear range has been shown.

CONCLUSIONS

Since conductometric transducers suffer from the interferences due to ionic strength changes, it seems that they do not appear practical for in-vivo applications. It has been shown however that measurements in dilute fluids are possible with the use of a reference sensor having no immobilized enzyme. Another advantage of the sensor reported here is its stability. Specially, following the immobilization method A, 80% of the activity is preserved over a period of 25 days and the sensor provided more reproducible responses than that prepared by immobilization method B. More reproducible responses were achieved by re-calibration of the sensor after frequent operation or a period of storage. Regeneration of the immobilized enzyme layer on the sensor surface is preferred because the renewed sensor responds to urea almost the same as new one. From the viewpoint of sensitivity and reliability of the urea

biosensor developed it seems suitable for clinical applications.

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