Chapter – 3

Characterization of

Urea Biosensors
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CHARACTERIZATION OF UREA BIOSENSORS

3.1 MEASUREMENTS

The fabricated urea biosensor is now ready to be used for measurements. The setup consists of a working electrode and the other reference electrode and measuring unit with high impedance. The potentiometric biosensors combine the selectivity of enzymatic methods of analysis with the simplicity of ion selective electrode measurements. The result is a device that can be used to determine the concentration of a given compound in solution quickly. This method requires a minimum sample preparation for measurements.

The investigations were carried out in two ways:

➢ In one a conventional Ag/AgCl reference electrode with double junction (inner and outer filling solution supplied with the same) from ORION (Model 90-02) was employed.

➢ The other measurements were carried out with disposable reference electrodes fabricated in the Laboratory.

The screen-printed silver electrodes were used as working electrode in both the ways of urea determination. All measurements were made at room temperature with the aid of the pH/ISE/mV, ORION make, model: 920A meter. The block diagram for potentiometric measuring setup is shown in figure 3.1.
Figure 3.1 Block Diagram of Potentiometric Setup
Before the measurements, urea biosensors were equilibrated in the sample buffer until a stable potential was obtained and then immersed together with double junction reference electrode in stirred electrochemical cell with 30 ml of 7 pH buffer. The measurements were carried out in batch process and the potential change was then observed at steady state value. Between trials the electrode was rinsed with double distilled water and then immersed in the sample buffer. After setup was ready, the fabricated urea biosensors were studied for their characterization.

The analytical principle of the biosensor is based on the hydrolysis of urea in presence of an enzyme urease. The reaction results in the formation of ammonium ions.

\[
(NH_2)CO + 2H_2O + H^+ \rightarrow 2NH_4^+ + HCO_3^-
\]

The ammonium ions are sensed in urea biosensors and the change in potential determines the logarithmic concentration of urea.

To begin with, first, the standard ammonium working electrode and reference double junction electrodes were dipped into the buffer solution. The standard NH₄Cl solution was prepared to check the functioning of reference electrodes. The NH₄Cl was added step wise in known amount and observed potential change proportional to the concentration is shown in the figure 3.2.
Figure 3.2 Calibration curve for NH$_4^+$ conventional electrodes.
3.2 PARAMETERS OF UREA BIOSENSORS: PART – I

3.2.1 RESPONSE CURVE

The fabricated urea biosensor was studied with respect to the reference electrode. The response of a sensor is shown in figure 3.3.

To check whether the sensor can detect urea, standard urea solution of different molar concentration was added to the buffer. The electrode responded rapidly and in a stable manner to the changes in the urea concentration with a response time of less than 3 minutes.

The response time of a biosensor is normally the time taken for it to reach steady state when there is no further variation in the signal. Response time depends upon analyte, co-substance and product transport through different membrane; their thickness and permeability are critical. They also depend upon the activity of the molecular recognition system; the higher this activity, the smaller is the response time.

3.2.2 CALIBRATION

Once an electrode responds correctly to an analyte, it needs to be calibrated by varying the analyte concentration. Calibration refers to the process of applying several signals to a biosensor device, and measuring the output signal, so that input-output relation of that biosensor can be derived with certain accuracy.
Figure - 3.3 Response curve for Urea Biosensors.
For the calibration of fabricated urea biosensor the same reference electrode was used. The calibration curve obtained by plotting, the potential difference across the two electrodes against the logarithm of the concentration of urea is shown in figure 3.4. The calibration is perhaps the most important part because all the features depend on the goodness of calibration of the single biosensor. This calibration curve is utilized to find the concentration of unknown urea solution.

The curve flattens out on both sides of the linear zone with potential change. The upper plateau corresponds to high concentrations of urea, which saturate the active sites of the enzyme. The lower plateau corresponds to the detection limit of the transducer. The only useful part of the curve is the linear zone where a variation in substrate concentration gives a variation in the potential at the transducer.

To check the validity of the calibration curve, the concentration of few samples of known strengths within the range of detection limits, but not used for constructing the calibration curve, was estimated using present biosensor.

The following table 3.1 shows comparison of the concentrations of urea in solution obtained using potentiometric biosensor with known concentrations of urea. The concentrations of urea have also been obtained with UV-VIS-NIR spectroscopic method by measuring absorbance at 425 nm.
Figure - 3.4 Calibration curve of urea biosensor.
Table 3.1 Validation of the calibration curve of urea biosensors.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Known Urea Concentrations (mM)</th>
<th>Measured with Biosensors (mM)</th>
<th>Measured with UV-VIS method (mM)</th>
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<tr>
<td>1</td>
<td>1</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2.1</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
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</tr>
<tr>
<td>4</td>
<td>6</td>
<td>6.2</td>
<td>6.8</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>7.9</td>
<td>8.2</td>
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</table>
3.2.3 REPRODUCIBILITY

The biosensors were fabricated in batch process. Hence it was necessary to study the response of urea biosensors to see the behaviour of sensors. From the batch of six sensors the potential change of four sensors is shown in figure 3.5.

The linearity of the potential with the concentration was observed for these four sensors. A linear regression was carried out for the potential and concentration of urea for all these four biosensors. For each sensor the results are reproducible. The slope of the curve in this zone corresponds to the sensitivity of the biosensor because it expresses a variation in the signal obtained as a function of the analyte concentration. The average slope obtained is 54.6±1 mV/decade and the straight line is the best-fit line passing through the experimental data points, the regression coefficient was around 0.997. Similar results have been obtained with biosensors fabricated in other lot.

3.2.4 SHELF LIFE

The storage stability of the biosensors was investigated over a period of one week. During the week, slope was determined once a day. When not in use the biosensor was stored at 5°C in a buffer. The performance of biosensors was observed as a function of time.
Figure 3.5 Linear curve of four biosensors from a batch of six biosensors.

- $y(S1) = 54.766x + 208.48$
- $R^2 = 0.997$
- $y(S2) = 55.806x + 211.09$
- $y(S3) = 55.833x + 211.33$
- $y(S4) = 54.825x + 207.57$
The lifetime of the biosensor was in the range of 3-4 days, during these period a slight decrease in electrode response was observed. This lifetime is sufficient enough for disposable biosensors. The response of biosensor was measured for number of samples. A gradual decrease in potential was observed after average 30 measurements of the sample, implies the activity of enzyme but after the limit its activity started diminishing. A short-term drift for few minutes is represented as an error bar of data points in figure 3.6.

3.2.5 EFFECT OF pH

The major problem for pH sensitive electrodes is that the sensor response is strongly dependent on the buffer capacity of the sample. The pH change produced in the course of enzyme catalyzed reaction is suppressed by the buffer used which leads to a narrow dynamic range and a loss in sensor sensitivity. The urea sensitive pH electrodes are not so stable as the others are. The effect of different pH buffer concentrations on the urea biosensor response is shown in figure 3.7. The figure shows negative slope with increase in pH of the buffer solution.

The exhaustive study was carried out by Ru Qin Yu et. al [1] with number of organic compounds as possible hydrogen ion carriers, amines and other compounds capable of protonation to produce a pH sensitive response. The excellent carriers such as methyl dioctadecylamine (MDODA), dioctyl octadecylamine (DOODA),
Figure - 3.6 Response of biosensors with number of measurements.
Figure - 3.7 Response of biosensor to different pH buffer.
didecyloctadecylamine (DDODA), N,N-di(2-ethylhexyl)-octadecylamine (DEHODA), (4-nitrobenzyl)dioctylamine (NBDOA) and some nicotinic acid ethers including octadecyl ether (NAODE) and hexadecyl ether (NAHDE) were synthesized. By using quantum chemistry calculations, the relationship between the structure of ionophore and electrode potential response characteristics was reported. In search for carriers with improved potentiometric characteristics they discovered that in the carrier molecule different functional groups are responsible for the potentiometric response to the solution pH in different pH regions. In order to obtain a carrier which would show Nernstian response covering very wide pH range, one can introduce into the same carrier molecule a functional group capable to create pH response in the acidic region and simultaneously another functional group, which is responsible to the alkaline region [2]. Hence, ethylenediaminetetraacetic acid (EDTA) was used as one of the component in the buffer solution of pH 7.

3.2.6 SELECTIVITY

The selectivity is probably the most important characteristic of ion-selective electrodes. It has become accepted that the liquid membrane ISE response can be satisfactorily described by considering the phase boundary potential at the sample-membrane interface [3]. Considering that the phase boundary potential is the main potential-determining factor in polymer membrane based ion-selective electrode, it can be concluded that a Nernstian electrode slope is expected if the
discriminated ion of interest fully displaces the primary ion from the interfacial layer of the membrane.

Selectivity depends upon the choice both of the biological receptor and the transducer. Most enzymes, except alcohol or amino-acid oxidase, are very specific. In contrast, bacteria, yeast or tissue cultures are very non-specific. Owing to the high specificity of the urease enzyme for its substrate the selectivity coefficient for urea biosensor would be practically zero.

3.2.7 LOWEST LIMIT OF LINEAR RANGE
The lowest limit of linear range can be determined by extending the straight line through linear points where it crosses the x-axis. In calibration curve, i.e. figure 3.4 indicates the lowest limit of linear range of the biosensor as $6.3 \times 10^{-5}$ mol/l for the urea biosensors.

3.3 PARAMETERS OF UREA BIOSENSORS: PART II
3.3.1 FABRICATED REFERENCE ELECTRODES

The measurements were carried out with the aid of fabricated reference electrodes against the fabricated working electrodes. The results are plotted in figure 3.8, reveals that the sensor has sufficient linear range. The biosensors responded rapidly in the stable manner to changes in the urea concentration. The slope in the linear range of
Figure - 3.8 Calibration curve of fabricated urea biosensor.
these biosensors were between 20.6 and 28.4 mV/decade (average 24 mV/decade) and the biosensors responded linearly to changes in the urea concentration between $10^{-4}$ and $3 \times 10^{-2}$ mol/l. The lifetime of these biosensors was in the range of 2-3 days and was stored at 4°C when not in use.

The slope obtained from the plot of potential against log of (Urea C), figure 3.9 using fabricated urea biosensors is found to be less when compared with the slope obtained employing conventional reference electrode figure 3.5. The exact reasons for these are not known at this time. Probably thorough investigations might suggest the factors responsible for the lowering of slope.

The curve shows that the biosensors have a good linear least square fit with correlation coefficient of 0.998. The following table depicts the statistical data for one particular biosensor.

### 3.4 FACTORS AFFECTING THE PERFORMANCE

#### 3.4.1 IMMobilIZATION

The way in which the enzyme layer is formed on the surface of the transducer is of paramount importance in the construction of biosensor. Immobilized enzymes have normally more stability and higher activity. Among the methods for immobilization, entrapment of urease enzyme within a gel of polymers is best suited for urea
Table 3.2 Statistical data analysis of fabricated urea biosensor.

<table>
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<th>Regression Statistics</th>
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<tr>
<td>Multiple R</td>
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<td></td>
</tr>
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<td>R Square</td>
<td>0.99808837</td>
<td></td>
<td></td>
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<tr>
<td>Adjusted R Square</td>
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<tr>
<td>Standard Error</td>
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<td></td>
</tr>
<tr>
<td>Observations</td>
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<table>
<thead>
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<th>MS</th>
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<td>2088.455</td>
<td>1.37E-06</td>
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<td>Residual</td>
<td>4</td>
<td>6.785714286</td>
<td>1.696429</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
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<td>3549.7</td>
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</table>

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<th>P-value</th>
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<td>1.793080047</td>
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<td>45.69961</td>
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<table>
<thead>
<tr>
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<th>Upper 95%</th>
<th>Lower 95.0%</th>
<th>Upper 95.0%</th>
</tr>
</thead>
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<td>33.07874</td>
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<tr>
<td>26.72825</td>
<td>30.18604</td>
<td>26.72824733</td>
<td>30.18603839</td>
</tr>
</tbody>
</table>
\[ y = 28.457x + 38.057 \]
\[ R^2 = 0.9981 \]

Figure - 3.9 Linear curve for urea biosensors with fabricated reference electrodes.
biosensor. It enhances the sensitivity and reproducibility of biosensors. Entrapping urease enzyme for the target molecule of urea activates the Ag electrode. Exposure of this activated electrode to the target species results in the change in potential of this electrode.

The pH is another important factor, since most enzymes have a pH range of maximum activity and on either side of domain activity false off rapidly. The pH optimum of an immobilized enzyme may not be the same as the soluble enzyme. In fact the pH profile alters depending the carrier used. Bound to a negatively charged matrix, the pH optimum shifts to higher pH, while on positively charged support, the converse is true [5]. Therefore, the pH of the polymer matrix used in the present investigations lies between 6 to 6.5.

3.4.2 ENZYME KINETICS

Virtually, an enzyme catalyzes every biochemical reaction. Enzymes are extraordinary effective catalysts, commonly enhancing reaction rates by a factor of $10^5$ to $10^{17}$. To be active, some enzymes require a chemical cofactor, which can be loosely or tightly bound.

Kinetics is an important method for the study of enzyme mechanisms. Most enzymes have some kinetic properties in common. As the concentration of substrate is increased in hyperbolic fashion to approach a characteristic maximum rate $V_{\text{max}}$ at which essentially, the entire enzyme is in the form of ES complex. The substrate
concentration giving one-half $V_{\text{max}}$ is the Michaelis Constant $K_m$, which is characteristic for each enzyme acting on a given substrate.

The Michaelis–Menten Equation:

$$V_0 = \frac{(V_{\text{max}} [S])}{(K_m + [S])}$$

relates the initial velocity of an enzymatic reaction to the substrate concentration and $V_{\text{max}}$ through the constant $K_m$ and $V_{\text{max}}$ can be measured: they have different meanings for different enzymes. The limiting rate of an enzyme, catalyzed reaction at saturation is described by the constant $K_{\text{cat}}$. The ratio $K_{\text{cat}}/K_m$ provides a good measure of catalytic efficiency. Activity of the enzyme, thickness of the layer and permeability of the enzyme layer influences the response time of biosensor.

C. Eggenstein and K. Cammann et. al. [4] studied efficiency of biosensors using different urease concentrations (between 1 and 10 mg/ml in the polymer solution). The best potentiometric response was obtained with an enzyme loading of 5 mg/ml, various levels of cation exchanger in the casting solution were also evaluated. A maximum urease response was obtained using 1.4 % polymer solution.

Figure 3.10 shows the calibration graph of the biosensors for the first, second, third and fourth day. The figure reveals that in the linear range the slope was 53.9 mV/decade. The slope gradually starts decreasing with the passage of days.
Figure - 3.10 Calibration curve of biosensor on 1st, 2nd, 3rd and 4th day after its fabrication
Enzyme loading can change the slope of response. The slope increases with increase in enzyme loading until a level is reached when no further change is possible.

3.4.3. EFFECT OF POLYMER MEMBRANE

Different polymeric materials like PVC, polyvinylpyrrolidone, polyestersulphonic acid, poly(carbamoylsulfonate) and photo-crosslinkable materials etc. can be used as gel matrix for entrapping the enzymes[6]. In urea biosensor the enzyme urease, which catalyze the reaction of hydrolysis of urea to ammonia and carbonate, was immobilized with such type of polymeric materials. Poly(carbamoylsulfonate) (PCS) and Polyethyleneimine (PEI) polymers have been employed in this investigation to immobilize urease. Best results concerning adhesion capability, slope, linear range and lifetime were obtained with PCS mixed with PEI as gel material. This material not only adhered excellently to the PVC membrane, but also showed minimal diffusion resistance to the substrates. Experimental analysis confirms the same behaviour of polymer layers in fabricating disposable urea biosensors.

The Nernst response of a PVC membrane ion selective electrode is referred to the standard ion transfer potential which is determined by the standard Gibbs energy of ion transfer from the aqueous phase to the membrane phase. It is interesting that the presence of PVC does not have a significant effect on ion transfer process. It has been
reported [7] that upon contact with aqueous solutions the PVC plasticized membrane absorb water that is distributed non-homogeneously over the membrane phase reaching the maximum concentration at the interface.

3.4.4 TEMPERATURE

Urea biosensors use enzymatic reactions and so an increase in temperature will also increase the catalytic activity and hence the rate of reaction. Figure 3.11 shows the response of double matrix membrane as a function of temperature. From the figure, it seems that response of a biosensor is practically unaffected by variations in temperature, provided that the range of temperature remains between 10°C and 42°C. Above 42°C, the reduction in the urease enzyme activity corresponds to denaturation of the enzyme and below 10°C, the apparent activity of an enzyme lay diminishing causing a drop in the biosensors response. The present work has been carried out at around 30°C, i.e. 12°C below the maximal working temperature, which limits the denaturation thereby prolonging the lifetime of the biosensor.
Figure 3.11 Temperature dependence curve of urea biosensors.
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