The Development of A Urea Biosensor Based on BSA Embedded Surface Modified PANI/2AP composite Film

Suresh More[1],Kiran Paithankar[2], Vikas Gade[3], Sunil Mirgane[4*]

[1,2]*Jaihind Polytechnic, Kuran, Savitribai Phule University, Pune, India*

[3]*Department of Physics, Shri Anand College Pathardi, Savitribai Phule University, Pune, India*

[4]*Department of Chemistry, JES Art, Science and Commerce College Jalna, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, India*

ABSTRACT

A Potentiometric biosensor based on bovine serum albumin (BSA) embedded surface modified PANI/2AP has been developed for the detection of urea in water sample. The enzyme, urease (Urs), was covalently linked to free amino groups present over the BSA embedded modified surface of the conducting PANI/2AP composite film electrochemically deposited onto an indium–tin-oxide (ITO) film. The biosensor has been characterized by UV–visible, infrared spectroscopy and SEM. Potentiometric response of the enzyme electrode (Urs/BSA- PANI/2AP /ITO) were measured as a function of urea concentration in phosphate buffer. It has been found that the electrode responds to low urea concentration with wider range of detection. The electrode showed a linear response range of current 0-30µA for 1-100mM solution of urea in PBS at pH 7.0. The response time is about 50-70 s reaching to a 90% steady-state potential value. These results indicate an efficient covalent linkage of enzyme to free amino groups of the BSA molecules over the surface of PANI/2AP composite film, which leads to high enzyme loading, an increased lifetime stability of the electrode and an improved wide range of detection of low urea concentration in aqueous medium.

Keyword: - *Urea, Polymer, Biosensor, Enzyme, BSA, Peptide linkage, PANI 2AP.*

1. INTRODUCTION-

Biosensor is a device that uses specific biochemical reactions mediated by remote enzymes, immune systems tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals (Mc Naught and Wilkinson 1997). Beginning of biosensors may be dated to 1962, when Clark known as the father of the biosensor concept published an experiment in which glucose oxidize (GOX) was entrapped at a Clark oxygen electrode using dialysis membrane (Clark Jr. and Lyons 1962).

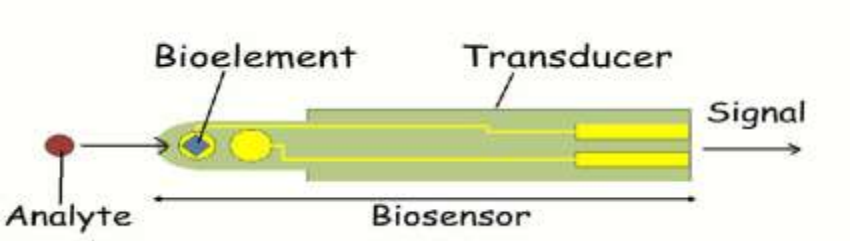

Fig.1 Biosensor Model

The most successful commercially available biosensors are those for measuring glucose in blood samples representing about 90 % of the global biosensor market utilize glucose oxidase or glucose dehydrogenase Monošík et al. 2012). Variety of enzymes were used for biosensor construction, for example oxidoreductase enzymes were used for lactate (Huang et al. 2009 Huang et al. 2008, Katrlík et al.1999, Pereira et 2007) Many factors have influence on the performance of enzyme based biosensors, such an enzyme deposition ,the use of a suitable pH, temperature and in some cases a cofactor can help to maintain the abilities of the enzyme. Another factor that can have an effect on the electrode performance is the type of immobilization method used to retain the enzyme as well as the thickness of the enzyme layer on the sensor. Polymer films are used in biosensors to help electron transfer from biocomponant to the electrode surface, for the incorporation of mediators and in the immobilization of enzymes. It proves that the polymeric material can give better stability and remove interference. Therefore it is advisable to use polymer in all biosensors. However the choice of polymeric material and method by which the films are being synthesized is very crucial because different polymeric materials have different blocking capacities for the analytes. Therefore, different expertise from different such as Physics, Electronic, Chemistry, Biology are expected to come together to do some innovative research in this newly emerging interdisciplinary research area i.e. biosensors. The normal level of urea in serum is 8–20 mg/dL. High level of urea in blood causes chronic or acute renal failure, urinary tract obstruction, dehydration, shock, burns and gastrointestinal bleeding, whereas a substantial low level of urea concentration causes hepatic failure, nephritic syndrome, and cachexia[1]. Conducting polymers have become the materials of choice for recent technological advances in biotechnology and have been extensively reviewed by various researchers [2–4]. These matrices are used as supports for biomolecules, resulting in biosensors that have enhanced speed, sensitivity and versatility in diagnostics to measure desired analytes. These devices are finding ever increasing use in clinical diagnostics. Conducting polymers are conjugated polymers that can be synthesized by chemical methods as well as electrochemical methods, providing easy modulation of various properties (e.g., film thickness, conductivity, and functionalization, use of various supporting electrolytes and the ability of serving as an electrochemical transducer it self). Additional merits are that enzyme molecules can be entrapped during electro-polymerization in one step and also that the polymer film uniformly covers the surface of substrate electrodes of any shape or size [5,6].Various conducting polymers, like polyaniline (PANi), polypyrrole and polythiophene, have been used for the fabrication of biosensors. Among them, polypyrrole is one of the most extensively used conducting polymers in the fabrication of urea biosensors [7–8]. The versatility of this polymer is determined by (i) itsbiocompatibility, (ii) capability to transduce energy arising from the interaction of analytes and analyte recognizing sites into electrical signals that are easily monitored, (iii) capability to protect electrodes from interfering material, and (iv) easy ways for electrochemical deposition on the surface of any type of electrode. Among other conducting polymers, polyaniline is often used as the immobilizing substrate for biomolecules and as an efficient electrocatalyst. However, the necessity to detect bioanalytes in neutral pH ranges leads to electro inactivity of the deposited film, discouraging the use of polyaniline and polythiophene as biosensing materials for the detection of urea.Adeloju et al. have fabricated amperometric, flow injection, urea biosensors using electrochemically entrapped urease in polypyrrole[9–11].

The influence of flow rate and various other analytical parameters (e.g., applied potential and polymerization time) have been studied. Under optimized conditions, the amperometric response was linear between 3 and 15 mg/l of urea. The sensitivity and detection range were further improved by increasing the enzyme loading and utilizing pulsed amperometric detection [12]. Using pulsed amperometric flow injection sensors based on polypyrrole, detection limits of 60 mg/l and linearity in the 100–450 mg/l range has been observed. However, a shelf-life of only 2 weeks could be achieved using this

technique. This sensor suffers from a limitation of quantitative urea estimation in biological samples, as it needs pretreatment with anion exchange separators for the removal of interferants and needs further improvement to minimize the interferant effect [12].Efforts have been made to improve the sensitivity of the polypyrrolebased urea sensor by using composite films with polyion complexes. Komaba et al. have reported a potentiometric urea sensor based on nucleophilic electrolytes and urease, indicating improved urea response with a slope of 31.8 mV per decade [8]. However, it has been found that only a small number of urease molecules could be immobilized using the electropolymerization process. To overcome this problem, the polyanion complexes, polyacrylic acids, have been used to enhance the degree of immobilization and the response was found to increase to 53 mV per decade [14]. It has been demonstrated that the sensitivity can be furtherincreased by using polyion complexes (i.e., polycation polystyrene sulphonate and polyanionpolyacrylic acid) and, using a polyion complex, the sensitivity was found to be enhanced to 110 mV per decade. This high sensitivity has been attributed to effective immobilization of the large amount of urease by electropolymerization of the electrode with a precoatedpolyion complex [15]. However, the experiments on shelf life and sensor reproducibility have not been reported.To minimize enzyme desorption from the desired immobilization material and to obtain increased enzyme electrode stability, various approaches and immobilization methods have been used. A urea biosensor achieving the electrochemical entrapment of urease in a polypyrrole matrix, including a stability of around 2 months, has been reported [12]. This biosensor, however, cannot be reused. A functionalized copolymer of pyrrole and N-3aminopropylpyrrole[9]has been used for the fabricationof thin film urea biosensors.

In this system, urease was covalently immobilized onto the copolymer matrix. The sensor acted via covalent binding with free –NH2 groups of the polymer. This sensor exhibited improved characteristics, such as 2-month stability at 4–6 ◦ C and urea detection found in the range of 0-30μA for 1-100mM solution of urea in PBS pH7.0, Sensor stability of this system has been attributed to a porous morphology, owing to the large, inserted PTS dopant. This results in high enzyme loading and covalent binding with copolymers having free –NH2groups. A polypyrrole based urea biosensor has been reported that provides advantages via eliminating the need for an optical indicator, dye and pH sensitive reagent. Polypyrrole itself acts as a pH sensitive indicator in the near-IR range, with the change in urea concentration at the linear range of 0.06–1 M urea [7].Polyaniline, among other conducting polymers, is known to have a broad range of tunable properties, desirable electrochemical activity, chemical stability, structural flexibility and, above all, two redox couples in a convenient potential range. A pH urea sensor has been reported in both aqueous and non aqueous media via electropolymerization of polyaniline in dry 0.5 M acetonitrile. This sensor retains its reproducibility for more than 9 months. However, the sensor has not yet been used for clinical samples [16].

A copolymer of polyanilinepoly(n-butylmethacrylate) (Pn-PBMA) homogeneous composite films has been obtained using poly(vinyl methyl ether) (PVME) and poly(vinyl ethyl ether) (PVEE) as dispersants. This copolymer has been characterized for its morphological, electrical and mechanical properties [17]. These composites have been used for the detection ofH2O2 , NH3 and toward the fabrication of urea and uric acid biosensors. PolyanilineNafion composites have been used to develop an potentometric urea biosensor that is based on polyaniline–perfluorosulfonated composite electrodes. This system uses a flow injection analysis system, has been fabricated and is reported to have a detection limit of 0.5 M as well as a response time of 40 s [18]. Polyaniline–Nafion-based composite films have been immobilized with urease via electrochemical and casting methods for the fabrication of amperometric urea biosensors [9]. Urease immobilization on these composite films has been achieved by electrochemical immobilization and the casting method. It was found that the sensitivity of composite electrodes immobilized with the casting method is greater than that of the electrochemical immobilization method. The sensitivity and detecting limit of this urea sensor were found to be 0.7 A (mg dl−1)−1 cm2 and 6 mg dl−1 , respectively, when urease was immobilized by a glutaraldehyde (GA) cross-linker; and 5.27 (mg dl−1)−1 cm^2 and 0.3 mg/dl, when immobilized by a Nafion network [19].

However, no studies have been carried out for the stability of urease and the estimation of urea in clinical samples. For practical applications of urea biosensors, problems like reproducibility, long term stability and clinical sample measurements (e.g., in urine and serum) remain the major thrust. Although, conducting polymer based urea sensors have a lot of advantages (e.g., stability, tunable properties, flexibility and biocompatibility) they cannot work properly in high ionic strength samples. Furthermore, the response of these sensors is highly dependent on the buffer capacity of the sample, resulting in a narrow dynamic range and low sensitivity. It may be noted that the use of additional perm-selective membranes on top of the enzymatic membrane could substantially reduce the dependence of

sensor response on buffer concentration and significantly extend its dynamic range. For example, the use of Nafion and polyvinylene phenylene membrane on top of enzymatic layers has been reported to substantially improve the dynamic range of urea biosensors and reduce the dependence of buffer concentrations [20].Current developments in conducting polymer based biosensors show that electrochemical affinity sensors, based on molecularly imprinted conducting polymers, have a great potential for direct electrochemical sensing. This is because they enable deliberate control of the molecular structure at the electrode surface, resulting in the additional advantage of higher affinity to the target analyte. Therefore, nanostructured conducting polymers and tailored, molecularly imprinted conducting polymers are likely to be the best choice to obtain enhanced stability and reproducibility in urea biosensors.

2. EXPERIMENTAL

2.1. Materials and methods

Urease (EC 3.5.1.5, 104 Units/mg) and N-(3-dimethylaminopropyl)-N-ethyl carbodiimide hydrochloride (EDC) were obtained from Sigma–Aldrich. Bovine serum albumin (BSA) was purchased from Merck chemicals .N-Hydroxysuccinimide , Aniline and 2 amino pyridene monomer were obtained fromSpectrochem. p-Toluenesulphonicacid was procured from Spectrochem . Urea and other chemicals were of analytical grade and used without further purification.

2.2. Preparation of BSA embedded surface modified conducting PANI/2AP films

The BSA embedded surface modified conducting PANI/2AP films (0.25 cm × 0.25 cm) were electrochemically prepared on ITO glass plates from an aqueous medium containing Aniline (0.1 M),2 amino pyridine (0.1 M), p-toluene sulphonic acid (0.1 M) and BSA (10-5 M), at a fixed voltage of 1 V versus Ag/AgCl. The polymer films were prepared at injecting at potential 0.4 mV

2.3. Fabrication of enzyme electrode (Urs/BSA-PANI/2AP /ITO)

The enzyme, urease, was covalently attached to the exposed free amino groups (from BSA) at the surface of the PANI/2AP film through carbodimide coupling reaction. The electrode BSA/PANI/2AP /ITO was immersed in a phosphate buffer solution (0.1 M, pH 7.0) containing 0.05 M EDC, 0.03 M N-hydroxysuccinimide and 10 mg/mL Urs for 1hr. The enzyme electrode was rinsed with buffer solution (pH 7) to remove excess unbound enzyme. All experiments were carried out at about 27 ◦ C. The enzyme electrode was stored under dry conditions at 10 ◦ C in a refrigerator when not in use.

2.4. Apparatus

The FT-IR spectrum was recorded using Shimadzu FT-IR-8400 series, using KBr pellets in the region 350–4000 cm_1 .The scanning electron micrograph was recorded, This facility provided by Dept. of Physics, University of Pune, India. The JEOL JSM-7500 Fis an ultra-high resolution field emission scanning electron microscope (FE-SEM) equipped with a high brightness conical FE gun and a low aberration conical objective lens. The improved overall stability of the JSM-7500 F enables you to readily observe your specimen at magnifications up to 1,000,000 x s with the guaranteed resolution of 1 nm. The energy filter (r-filter) makes it possible to observe the fine surface morphology of nanostructures. UV-visible spectroscopy- Analytic Jena specord 210 plus Wavelength (200 nm-800 nm) was used to study the oxidation state of synthesized polymer film. This facility was extended by DIAT, Pune.

Electrochemical polymerization and galvanostatic measurements were done on A computer controlled Potentiostat/Galvanostat, indigenously designed and fabricated in the Materials Research Laboratory, Department of physics, Shri Anand College, Pathardi, Dist. Ahmednagar. (MS) India was employed for the electrochemical synthesis of polymer film by using potentiometric (Galvanostatic) method. Galvanostatic measurements were carried out in a conventional three-electrode cell configuration consisting of a working electrode (Urs/BSA- PANI/2AP /ITO), Ag/AgCl reference electrode and Graphite as a counter electrode. A stirring bar and magnetic stirrer provided convective transport. All measurements were performed at about 25 ◦ C in buffer solution (pH 7.0).

3. RESULTS AND DISCUSSION

3.1. Characterization of enzyme electrode (Urs/BSA-PANI/2AP /ITO)

The scheme given in Fig.2 shows the fabrication of enzyme electrode (BSA/PANI/2AP /ITO), where in the free amino groups present at the surface of the BSA modified PANI/2AP film have been utilized for the covalent attachment of enzyme, urease, through peptide linkage with a carboxylic acid group, using the linkage reagents EDC and NHS [33,34]. The enzyme electrode (Urs/BSA-PANI/2AP /ITO) was characterized by FTIR spectroscopy it shows FTIR spectra of PANI/2AP, BSA-PANI/2AP. FTIR spectra of PANI/2AP and PANI/2AP/BSA samplesareshowninFig2.

In a spectrum the band observed at 3650 to 3433 cm-1 is due to N-H stretching. The polymer shows the absorption bands at 2923.62, 2923.25, 2923.21 and 2825.55 cm-1are due to asymmetric C-H stretching and symmetric C-H stretching vibrations. The absorption peaks observed at 1654.43, 1637.67 and1637.68 cm-1 we reattributed to C=C stretching in aromatic nuclei. The bands obtained at1600-1500 cm-1 corresponds to C-H stretching in aromatic compounds. Absorption bands at 1476.12, 1489.67, 1490.77 and 1491.15 cm-1evidencedto C=N stretching in aromatic compounds. The polymer shows absorption bands at 1300-1200 cm-1which confirms the C-N stretching of primary aromatic amines.

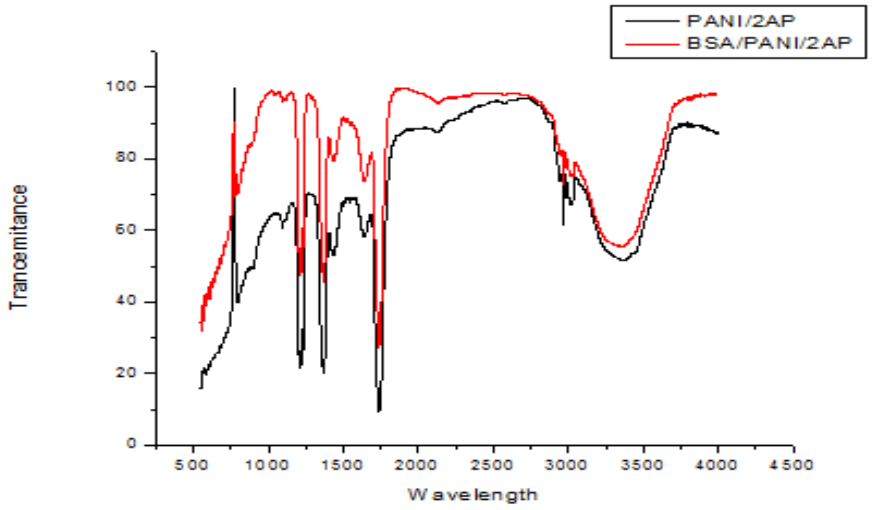

Fig.2. FTIR of BSA-PANI/2AP /ITO)

3.2 SEM micrographs of (A) PANI/ITO; (B) BSA-PANI/ITO; (C) Urs/BSA-PANI/ITO

The physical morphology of the native PANI/2AP and BSA embedded surface modified BSA-PANI/2AP /ITO film with and without enzyme (Urs) immobilization were characterized by scanning electron microscopy. A typical SEM picture of PANI/2AP/ITO (Fig. 3A) displays a three dimensional porous open structure with uniform PANI/2AP monomers granules. However, the SEM picture of BSA embedded PANI/2AP film exhibits a large numbers of aggregated globular particles along with the PANI/2AP monomers granules (Fig. 3B), which indicates the presence of globular protein (BSA) over the surface of the PANI/2AP film. When the enzyme was covalently immobilized over the surface of the BSA-PANI/2AP /ITO matrix, the porous structure was disappeared and instead bright rectangular shape particles have been seen on the surface of the polymer matrix (Fig. 3C) at the magnification of 20,000, which may be assigned to enzyme (Urs) molecules.

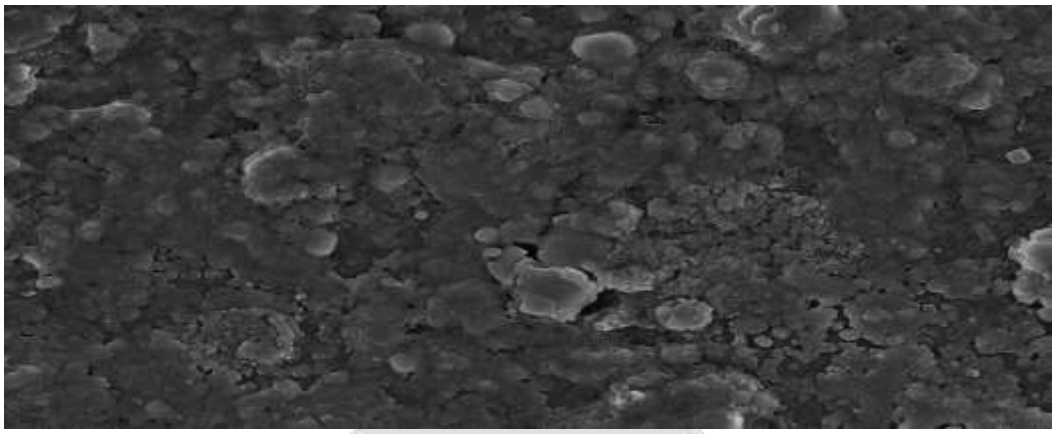

Fig.3A SEM OF PANI/2AP

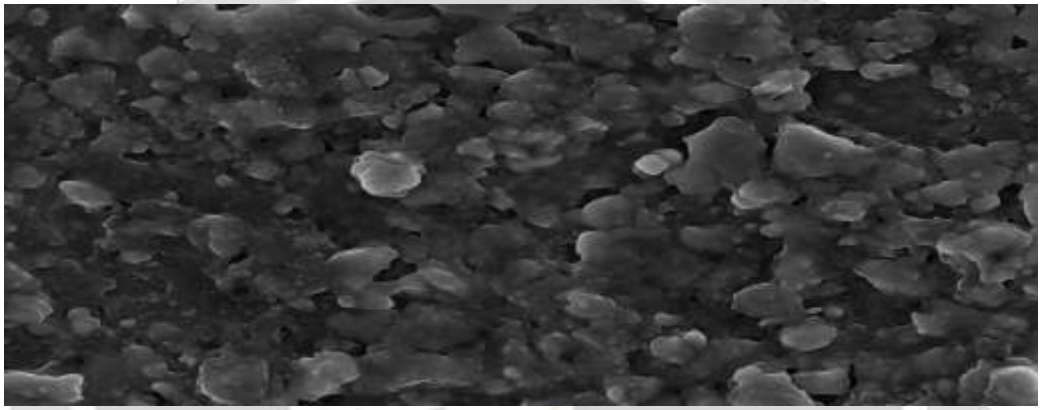

Fig.3B SEM OFPANI/2AP/BSA

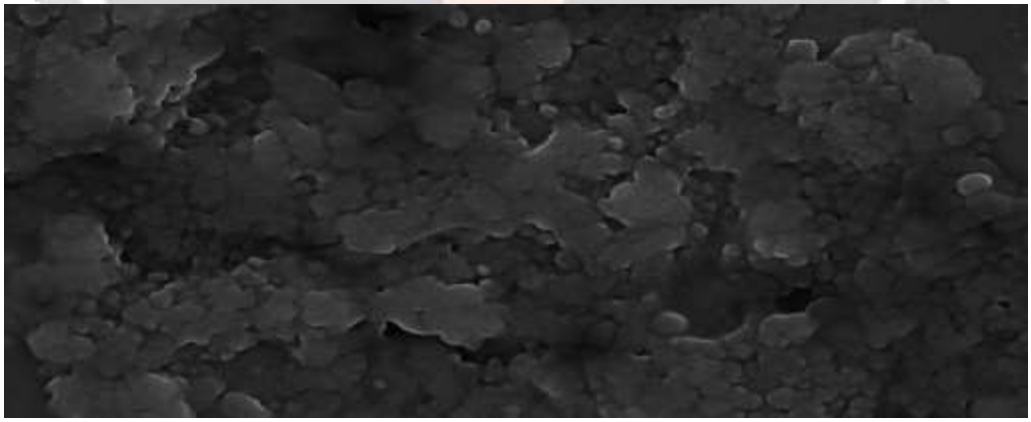

Fig.3C SEM OF PANI/2AP/BSA/URS

Fig. 3.SEM micrographs of (A) PANI/ITO; (B) BSA-PANI/ITO; (C) Urs/BSA-PANI/ITO

3.3 Potentiometric response characteristics AND Sensor measurements

The enzyme membranes were allowed to equilibrate to room temperature before measurements. The baseline potential of the sensor was achieved with flow of buffer for about 15 min (at flow rate of 20 ml/min). Subsequently, 50 ml urea sample having different concentration (1-100 mM) was taken simultaneously in the different beaker and the Urs/BSA- PANI/2AP /ITO electrode deep in it to initiate enzyme–substrate reaction.

As the reaction was over, the urea sample was drained off and membrane was washed by flow of buffer. The process was repeated with different urea concentrations (1– 100 mM) to obtain standard curve. The experiments were performed at repeatedly and enzyme-free membranes were used as control. The sensor was subsequently tested on synthetic urea samples (1–100 mM) and urea concentrations calculated from calibration equation of biosensor were compared with the actual amounts used. Urea concentration was calculated from the calibration equation for 50-70 s response time.

In the present case, assuming that the enzyme is uniformly distributed throughout the film, there action takes place predominantly on the surface of the film in the lower concentration. However, the reaction on the surface of the film and the diffusion occurring simultaneously at higher concentrations delays the response time. With increasing concentrations of glucose, the response current also increased and finally reached to steady state value. The current –time relationship when the potential of the enzyme electrode was set at 1V is as shown in Fig.4. It was observed that there response current of theenzymeelectrodeeasilyreachestosteadystate.Therelationshipbetweenresponsecurrentandurea concentration with phosphate buffer,at7.0pH, is shown in Fig.4.It was found that, current increases with increasing urea concentration in the range1–100mM.For ascertaining reusability, a single enzyme membrane was used recurrently for estimation of urea in a span of 2-3 h by washing in between measurements.

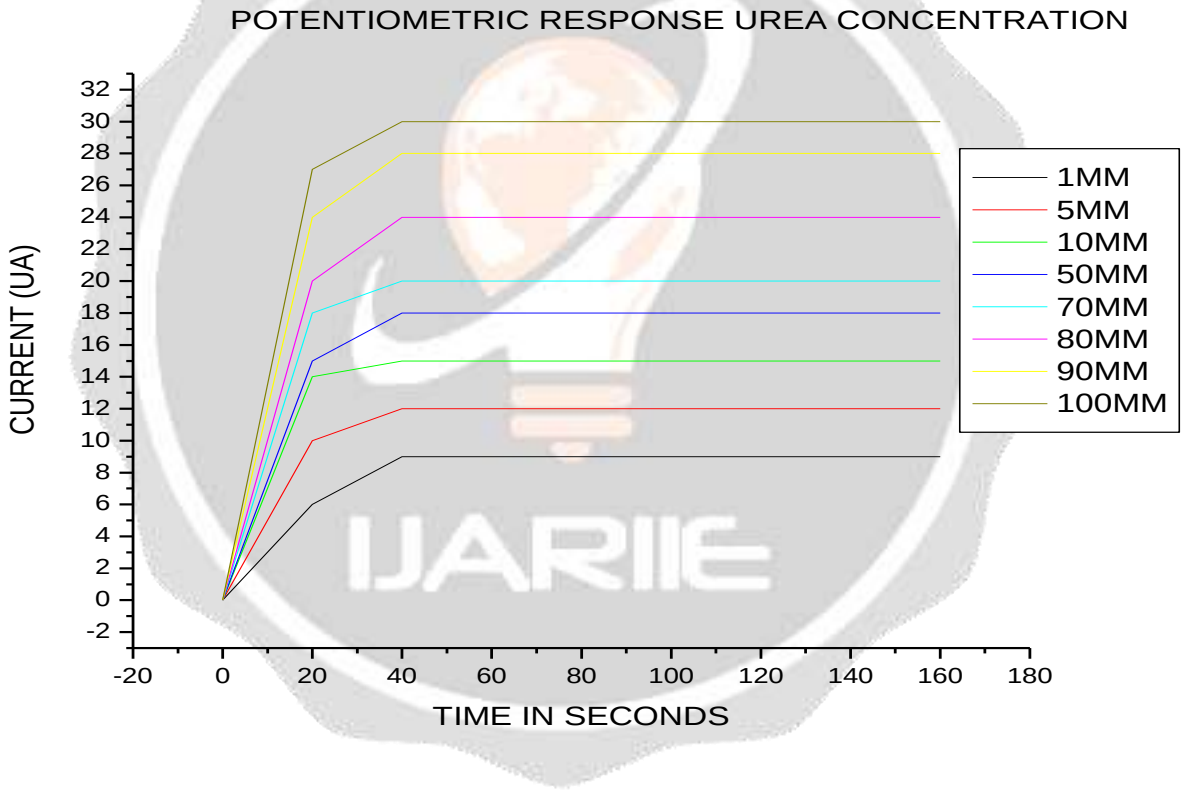

Fig.4 POTENTIOMETRIC RESPONSE UREA CONCENTRATION

4. CONCLUSIONS

This study has demonstrated the advantages of developing a bovine serum albumin (BSA) embedded surface modified conducting PANI/2AP film as an immobilizing matrix for the entrapment of enzyme, urease. The enzyme urease, efficiently binds with the free NH2 groups provided by the BSA molecules at the surface of the PANI/2AP film through carbodiimide-coupling reaction. This has lead the surface modified PANI/2AP film to have increased loading of enzyme, urease, which results in long term stability and a wide linear response range of detection, at much lower urea concentration in aqueous solution in comparison to the reported surface unmodified PANI/2AP biosensor. The low cost and simple method of fabrication of surface modified immobilizing material also makes this biosensor more advantageous than recently reported biosensors.

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