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Urea Biosensor based on Conducting Polymer Transducers

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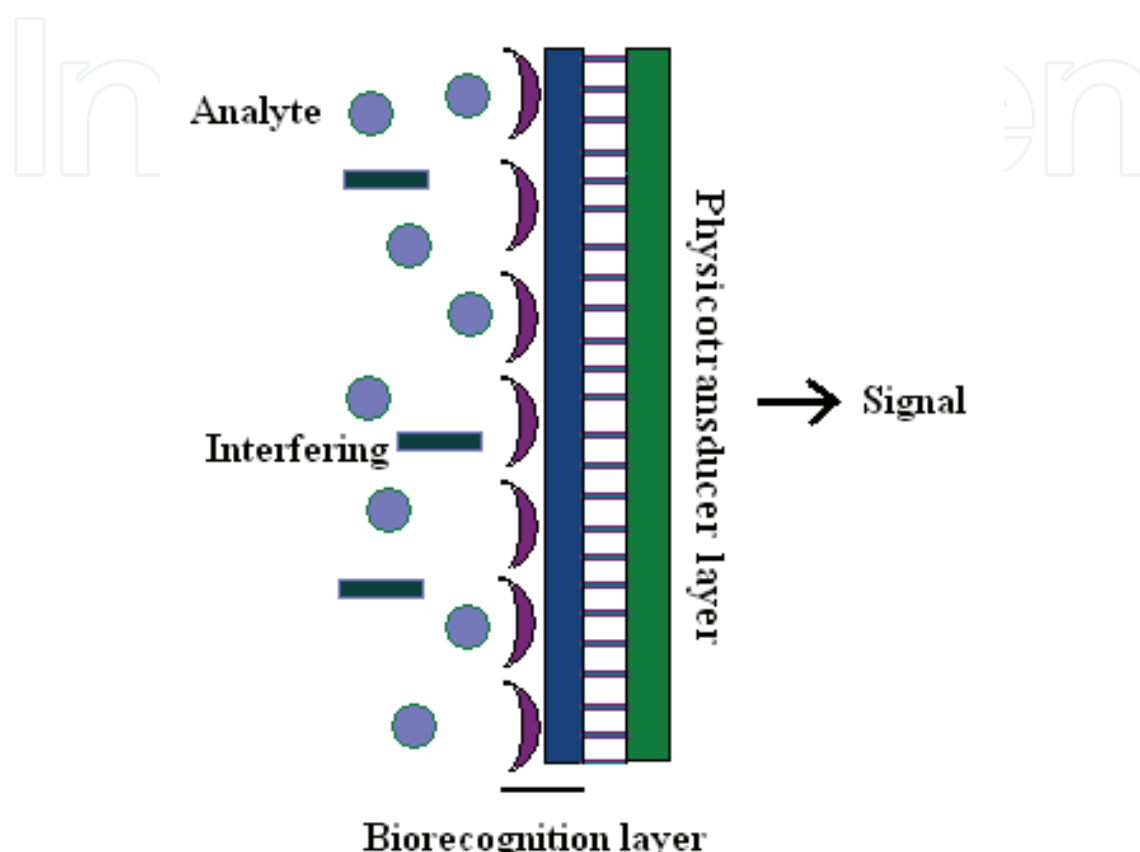*India*

1. Introduction:

Urea is one of the final products of protein metabolism. In clinical analysis and dairy industry urea is a very significant parameter as excess in blood serum from its permissible range causes dysfunction of the kidney. Hence, its analysis is significant and is carried out frequently in various laboratories [1, 2]. Urea is an omnipresent compound present in blood and various organic fluids. It passes directly into milk through diffusion. Therefore, milk is the second major biological sample for the study of urea concentration [3]. A periodic monitoring of urea in milk can be used to predict the state of animal's health and predict the protein requirement in its diet [4]. Besides milk, presence of urea in agricultural land as a pollutant due to excessive use fertilizers is also widely known. Various methods used for the determination of urea (without use of biocatalyst) are gas chromatography, calorimetric and flourimetric analysis etc [5]. However, these methods need sample pretreatment which stands as a major disadvantage in their versatility of applications. Alongside, these methods can not used for field monitoring. Therefore, devices developed based on biocatalyst "urease" to analyze urea also known as urea biosensors are of vital importance. The first urea biosensor was developed by Guilbault et al. [6] followed by many clinical and biochemical analysts. For the fabrication of the urea biosensor, urease is immobilized over a substrate. The immobilized urease there after catalyzes the urea conversion in to ammonium and bicarbonate ions based on enzyme substrate reaction. Many biosensors have been developed for the determination of urea in the biological samples namely spectrometry [7-9], potentiometry with application of pH sensitive electrode or an ion selective electrode or an ion sensitive field effective transistor [10-13], conductometry [14-16], coulometry [17], amperometry [18] and inductometry [19]. Amongst these methods, detection through electrochemical mode is highly adopted and versatile. This method involves the use of electrochemical urea biosensor. In the development of electrochemical urea biosensors, immobilization of urease over electrodes is the key parameter which decides the sensitivity and reproducibility of the sensor.

Urea biosensors are collectively formed by biorecognition layer - biocatalyst i.e. urease, a biotransducer – to read change in physical environment and convert into electronic signal. The complete working principle of the biotransducer component of urea biosensor is shown in Scheme 1. For making biotransducer and choice of substrate, conducting polymers are

most widely being researched. These materials have a key advantage in facilitating the conversion of physiochemical change into an electronic signal. A close harmony between the selective biorecognition system and physiochemical transducer also helps in overall performance of the biosensor.

Scheme 1. The biotransducer components of a urea biosensor composed of biorecognition layer having an intimate contact with transducer.

2. Various electrochemical techniques of urea sensing based on conducting polymers:

The electrode configuration of the conducting polymer based biosensor varies according to the sensing mechanism. A potentiometric and amperometric signals can be read through a single electrode [20]. Conductometric sensor devices, on the other hand, are most easily monitored by passing charge between two electrodes. Further low cost biosensors can be obtained using screen printed electrodes and reducing its size that is called miniaturized screen printed biosensors. Eggenstein et al [2] described urea sensitive biosensor electrode in the shape of test strips. Further, Trivedi et al [21] developed a miniaturized low cost screen printed disposable NH_4^+ ion sensitive electrode with double matrix membrane. Detection and estimation of urea in milk sample in this sensor is based on potentiometric changes occurring at the interface.

As mentioned before, in urea biosensors the enzyme immobilized to the electrode surface catalyzes the hydrolysis of the urea, in an overall reaction leading to the formation of ammonium, bicarbonate and hydroxide ions as shown below:

$$\text{Urea} + 3H_2O \xrightarrow{\quad \text{Urease} \quad} 2NH_4^+ + HCO_3^- + OH^-$$

The ionic products of the above reaction change the electronic properties of the biosensor electrode (modified with conducting polymers), which can be observed by various electrochemical techniques as follows:

2.1 Conductometric technique

There are many processes taking place in the vicinity of the polymer film which may lead to change in mobility and density of the carriers which thereby lead to a change in conductivity of these polymers. Conducting polymers are most suitable for conductimetric biosensor fabrication as their conductivity is sensitive to surrounding ions and their mobility. In case of conductimetric biosensor film conductivity can be calculated by four probe or two probe DC interrogation. In two probe interrogation, resistance of the polymer film is measured at a fixed potential with an increasing concentration of analyte. However, in case of four probe interrogations current is measured by sweeping the potential with the polymer fixed between a pair of interdigited microelectrode [22]. Current developed due to the potential drop is related to the film conductivity. The reason of potential drop is the interaction of analyte gaseous or ionic product cause the degree of doping of the polymer directly affects the conductivity. Sometimes analyte interactions cause a conformational transition of the polymer chain from "compact coil" to an "expanded coil" through the process of secondary doping, which might be accompanied by concomitant change in conductivity [23, 24]. Both types of interrogation have been applied for urea biosensors as the products of enzymatic reaction in both the methods are able to change the electronic property of the polymer.

These biosensors can be fabricated by putting enzyme layer over the polymeric transducer. Jha et. al. [25] used electropolymerized polyaniline-polystyrene (PANI-PSS) with brevibacterium ammoginase as a biocatalyst for urea biosensor. The immobilized cell works as a source of unpurified urease to develop conductometrc urea biosensor. The pH dependent change in resistivity of the polymer was also used as a basis for sensing. The response was linear over a range of 0-75mM urea with a sensitivity of 0.125 mM^{-1}. Ortega et. al. [26] reported a urea biosensor based on NH_3 gas interaction by two probe method with DBSA-doped polyaniline-poly(styrene-co-potassium acrylate) in a poly(n-butyl methacrylate) matrix. He also estimated urea concentration normal to pathological level.

2.2 Impedimetric technique:

Impedimetric transducer measures the effect of sinusoidal varying, nonperturbing voltage relative to a suitable reference electrode in term of impedance. The current originate allows the transfer function to real (Z") and imaginary (Z') component of impedance that changes as a function of the concentration of the analyte after each exposure. The impedimetric response can be measured either at one frequency (electrochemical impedance EI) or in a frequency range (electrochemical impedance spectroscopy EIS) [27]. The response might be direct or indirect depending on the interactions of the analyte with the recognition layer embedded in the conducting polymer or the analyte convert into some reaction product

after reacting with recognition layer and affects the carrier density (doping) or mobility (swelling) of the conducting polymer. Enzymatic biosensor based on conducting polymer comes in the category of the indirect and genosensors in the direct impedimetric biotransducer either by EI or EIS. There is still a debate whether impedimetric biosensor is truly or signal comes from the conformational change of the polymer. Ho et. al. [19] formed an impedimetric urea biosensor based on poly(methyl vinyl ether)/maleic anhydride which transformed rapidly in response to alkaline pH which was a link to enzyme reaction between urea and urease. The polymer was screen printed onto the interdigited screen printed carbon electrode and the electrode was overlaid with absorbent pad containing the associated enzyme. This type of electrode design was used for simple integrated and disposable sensor for clinical use.

2.3 Amperometric technique:

Amperometry is most commonly used technique for biosensors based on conducting polymers. Devices based on amperometry measure the change in current as a consequence of specific chemical reactions which take place at biotransducer electrode surface under non-equilibrium condition. The principal of the amperometry is based on the efficiency of the electron transfer between the biomolecule and underlying electrode surface in presence of electron mediator or conducting polymer. Moreover, amperometric biosensors are not only limited to the redox enzyme but also related to the biocatalyst reaction and interaction of the reaction product with conducting polymer to induce change in current [28]. Urea biosensor is the typical example of biocatalytic amperometric biosensor where ammonium ion which is a product of biocatalytic reaction interacts with polymer to induce a change in conductivity of the polymer. Adeloju et al [18] developed an amperometric urea biosensor based on flow injection analysis (FIA) to get linear responses between 3-15mg/l at the optimum condition of pH and potential of buffer and electrode respectively. The presence of interfering ions (KCl and KNO_3) at the concentration upto 0.01 M did not interfere with the estimation of the urea in blood and urine samples without any pretreatment. Rajesh et al [29] reported an amperometric urea biosensor with urease covalently immobilized with conducting copolymer poly (N-3 aminopropyl pyrrole-co-pyrrole) film. Amperometric response was measured as a function of concentration of urea, at a fixed bias voltage of 0.0 V vs. Ag/AgCl in a phosphate buffer (pH=7.0). The electrode gave a linear response range of 0.16-5.02 mM. W. J. Cho [30] described a new procedure for the urea determination through polyaniline-Nefion composite electrode which effectively sensed ammonium ion by amperometric technique. The sensor showed a detection limit as low as 0.5 µM (very less) with short response time. Nefion shortened the diffusion path and thus increased the sensitivity of the biosensor.
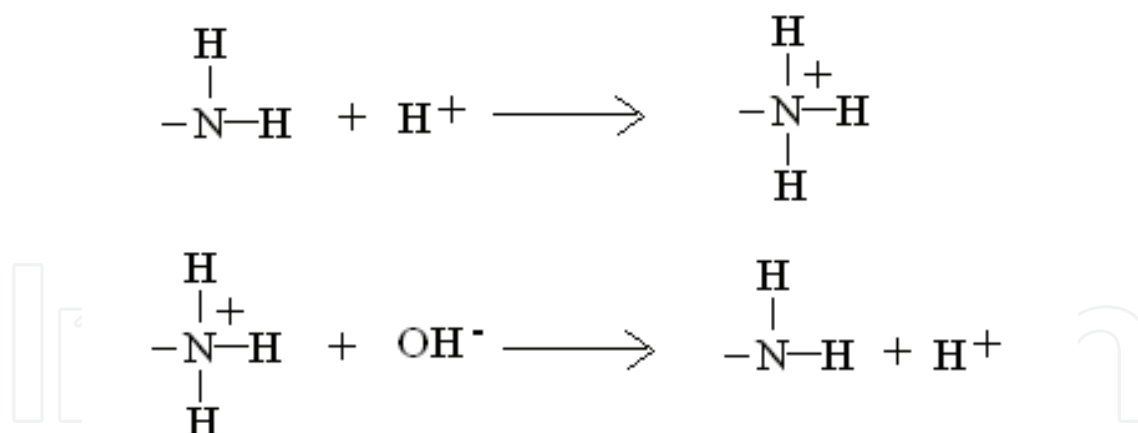
2.4 Potentiometric technique:

Electrochemical estimation of the urea is mainly carried out using potentiometric techniques because of a simpler mode of construction of the sensor electrode, detection and general availability of the instrumentation required for their utilization. On the other hand, for fully exploiting this technique, more durable and maintenance-free ion-selective electrodes (ISEs) which can be easily miniaturized are needed. Conducting polymers are widely studied for estimation of the urea by pH sensitive potentiometric technique. These polymers not only act as good transducers but also unique support to the immobilization of the enzyme due to

having capability for direct electron transfer between enzyme and polymer through the π electron (hydrophilic and hydrophobic interaction). In the following, we shall discuss about the critical parameters of solid state potentiometric urea biosensor based on conducting polymer pH transducer.

2.4.1 Conducting polymer based pH transducers:

pH sensor is widely known amongst all types of chemical sensors. A unique property possessed by conducting polymers is their low resistivity and conjugation. In addition, conducting polymers are known for better electronic communication through pi electrons. They also enhance ion to electron transduction performance of sensors and thereby increase the linear dynamic sensitivity, response time and stability of the sensor [31-40]. pH sensor is a type of chemical sensor extensively studied for the identification of the hydrogen ion concentration of the medium depending on the interaction and ion exchange between polymer and hydrogen ion in the bulk. Conducting polymer found in various oxidation states depends on pH and potential change affects conductivity, thus the change in the electronic parameter (change in electronic structure) measured by different type of electrochemical techniques for the identification of the pH. The mechanism of the potentiometric pH sensor based on conducting polymer is directly related to protonic interaction of the monomers. Amino group containing polymers have high affinity to the proton due to their behavior as protonation/deprotonation nitrogen i.e. Lewis base site [41]. The second mechanism based on the reaction of H^+ onto amino groups because of polarization creates local charge density excess at the electrode surface in comparison to the bulk concentration. The pH response mechanism of membrane may be represented by the following equation [42]:

$$-\overset{\overset{\displaystyle H}{|}}{N}-H \ + \ H^+ \ \longrightarrow \ -\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle H}{|}}{N}}\overset{+}{-}H$$

$$-\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle H}{|}}{N}}\overset{+}{-}H \ + \ OH^- \ \longrightarrow \ -\overset{\overset{\displaystyle H}{|}}{N}-H \ + \ H^+$$

The build up of charges on the inside of the conducting polymer membrane is proportional to the amount of hydrogen ions in the external solution as shown in figure 1.

The potential difference across the membrane in the potentiometric mode of detection is proportional to the logarithm of the ionic concentration in the external solution. Undissociated acidic groups (not participating in the doping) also participate in the H^+ sensitivity [43]. Various conducting polymers having some weak acidic groups over their backbone (self doped polymers) can explored for the pH sensor application, structures of some of them have been shown below in figure 2.
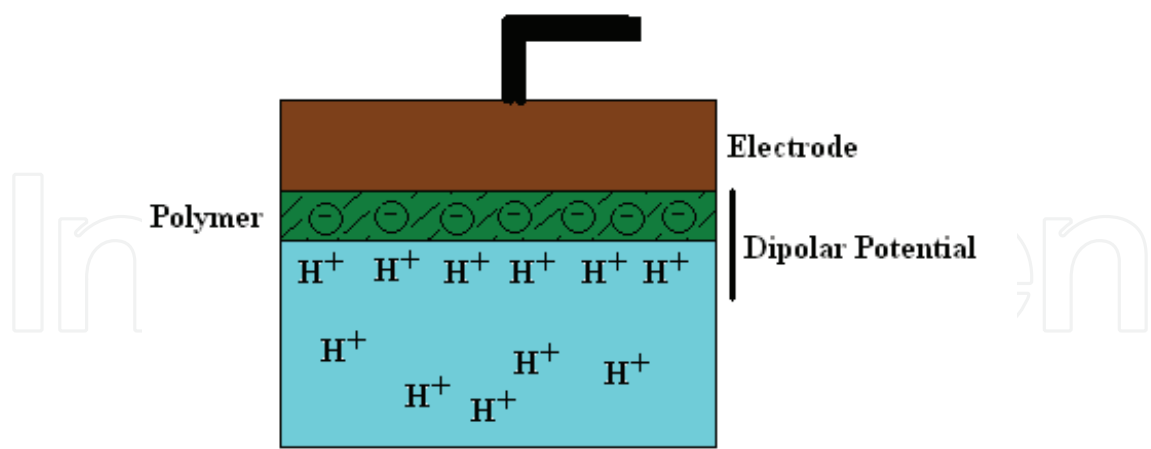
Fig. 1. Dipolar layer formation over the surface of conducting polymer modified electrode

Polyanthranilic acid

5-carboxy Polyindole

Poly(3'-propylsulfonate2,2':5',5'-terthienyl)
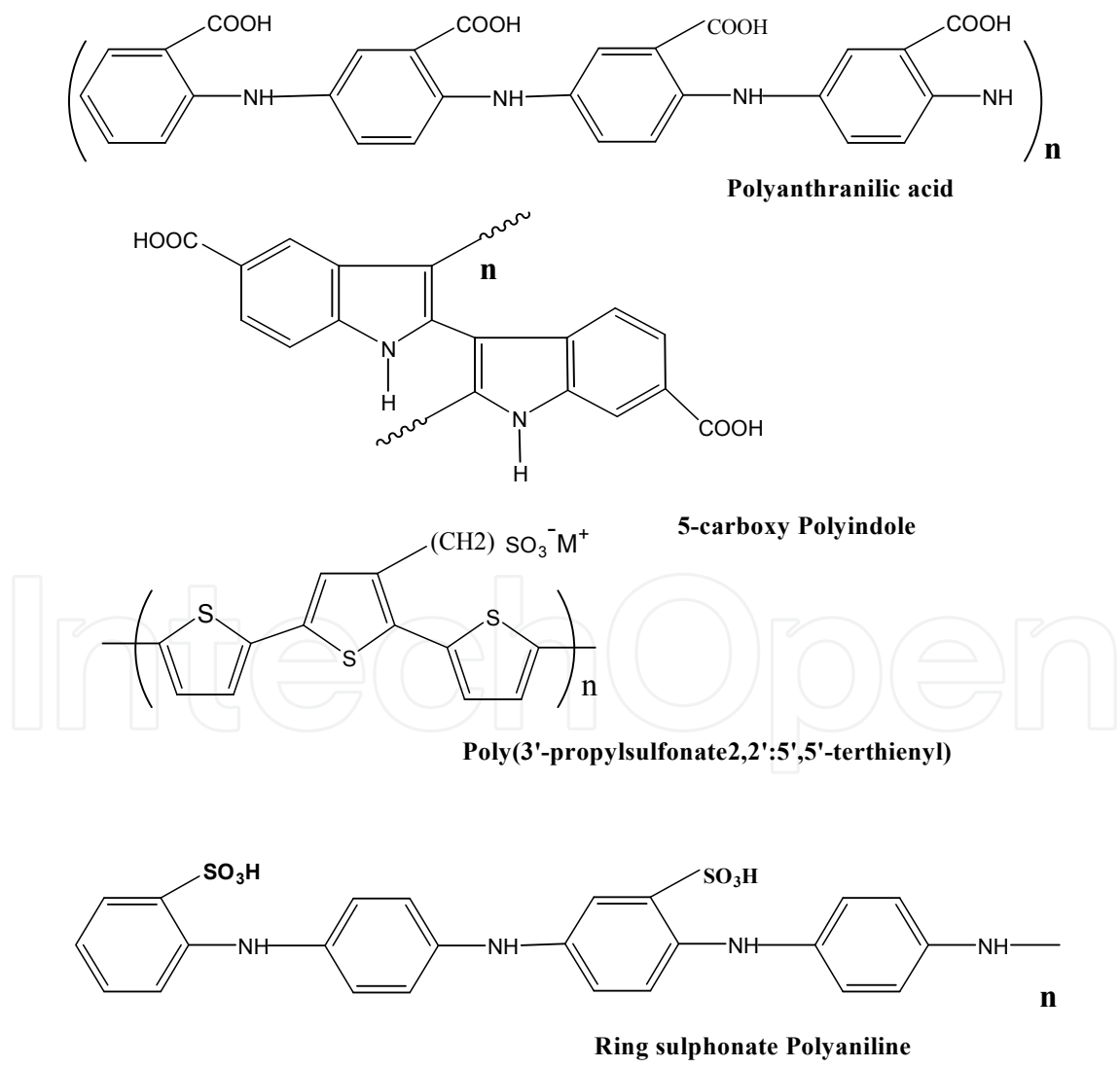
Ring sulphonate Polyaniline

Fig. 2. Electronic structure of self doped conducting polymers

Amongst the various conducting polymers, polyaniline has been found most suitable for pH sensing in aqueous medium. Pandey et. al. [44] found that hydrophobic ion exchanger used as a dopant for the polyaniline synthesis in acetonitrile facilitates pH estimation in the both aqueous and non-aqueous medium. Potentiometric chemical sensors relate open circuit potential to the concentration of analyte (with the generation of constant dipolar potential essentially required for baseline recovery after each addition of the electrolyte) by using an ion-selective or conducting polymer based electrode as a physical transducer.

These are selective, have large dynamic ranges and are nondestructive. They are frequently used for pH sensing. During the last decade, enormous efforts have been made on developments of potentiometric pH transducer based on polymer modified electrode. Charge over the polymer (oxidation state) is compensated by the counter i.e. dopants, thereby giving the ion exchanging opportunity to the polymer [45]. Thus the performance of the sensor is affected by oxidation state and structure of the polymers. Responses of various conducting polymers for pH are mentioned in table1.

Conducting Polymer	Dynamic Linear Range (M)	Slope (mV decade $^{-1}$)	Detection Limit (M)	Reference
Polyaniline (PANI)	pH 2-9	52.7	1×10^{-9}	T. Lindfors et. al. [48]
Polypyrrole	N.M	58	N.M	H. Kaden et. al. [49]
Polyaniline ultrathin films	pH 3-9	55-59	1.0×10^{-9}	C. H. Ge et. al. [50]
Polyaniline and poly (o-ethoxyaniline)	3 < pH < 9–10	~60 or 50	1.0×10^{-9}	C Slim et. al. [51]
Polypyrrole (PPy)	2-11	48	1.0×10^{-11}	B. Lakard et. al [42]
Polydiethylenetriamine (PPPD)	2-11	34	1.0×10^{-11}	B. Lakard et. al [42]
Polyethyleneimine (PEI)	2-11	46	1.0×10^{-10}	B. Lakard et. al [42]
Polypropyleneimine (PPI)	2-11	43	1.0×10^{-10}	B. Lakard et. al [42]
Orthomethoxyaniline and ortho-methylaniline	2-11	63.6	NM	C. A. Lindino et. al. [52]
Polyaniline and its substituted derivatives	2-9	62.9±0.9	1×10^{-9}	T. Lindfors et. al. [53]

Table 1. pH sensing parameter of a number of reported conducting polymers.

2.4.1.1 Effect of polymer composites on pH responses:

It is reported that conducting polymers having some more ion exchanging moieties viz functional group, dopant and other polymer modifier to form composites or blends not only affect total pH sensitivity but give stable immobilization matrix also. Different insulation polymer has been tried as a matrix for sensing membrane. PVC derivatives with carboxylate

groups (~1.8 %) got much more attention by the researchers. The reason is the COOH groups which principally remain undissociated. The weak acidic PVC or similar polymers are partly protonated upon contact with the aqueous solution and are being used to fabricate so-called ionophore-free H+-selective liquid membrane electrodes.

Prissanaroon-Ouajai et al. [46] developed pH sensor based on polypyrrole (PPy) with hydroquinone monosulphate and oxalate co-doping. Co-doped PPy showed the response slope of -54.67±7 mV/pH and a non-Nernstian slope of -35.0±7 mV/pH.

In our work for the first time we used 5-carboxy polyindole (5-carboxy PIn) and 5-carboxy PIn-Polyvinyl chloride carboxylated (PVC carboxylated) composite for the development of pH transducers. Incorporation of the functional group over polyindole and PVC (carboxylated) in polyindole matrix not only increases the sensitivity of the sensor but also provided stability to the sensing membrane. Besides, it also facilitated post synthetic processing. We measured the pH dependence of our electrodes by recording the open circuit potential in various buffer solutions ranging from pH 10 to 1 in 5ml buffer solution of 0.1mM using standard addition or batch method at the constant stirring of 100 rpm. Before each measurement, the sensor was immersed into pure water in order to ensure the same starting conditions (for batch method). Results are presented in figure 3 for 5-carboxy PIn and 5-carboxy PIn-carboxylated PVC composites based sensors. Both the electrodes demonstrated very good linearity. For 5-carboxy Pin and 5-carboxy PIn-Polyvinyl chloride carboxylated composite, we found a slope of 38.5 and 47.5 mV/pH respectively, which showed semi Nernstian behavior indicating that less than one proton per electron is involved in changing the stationary potential.
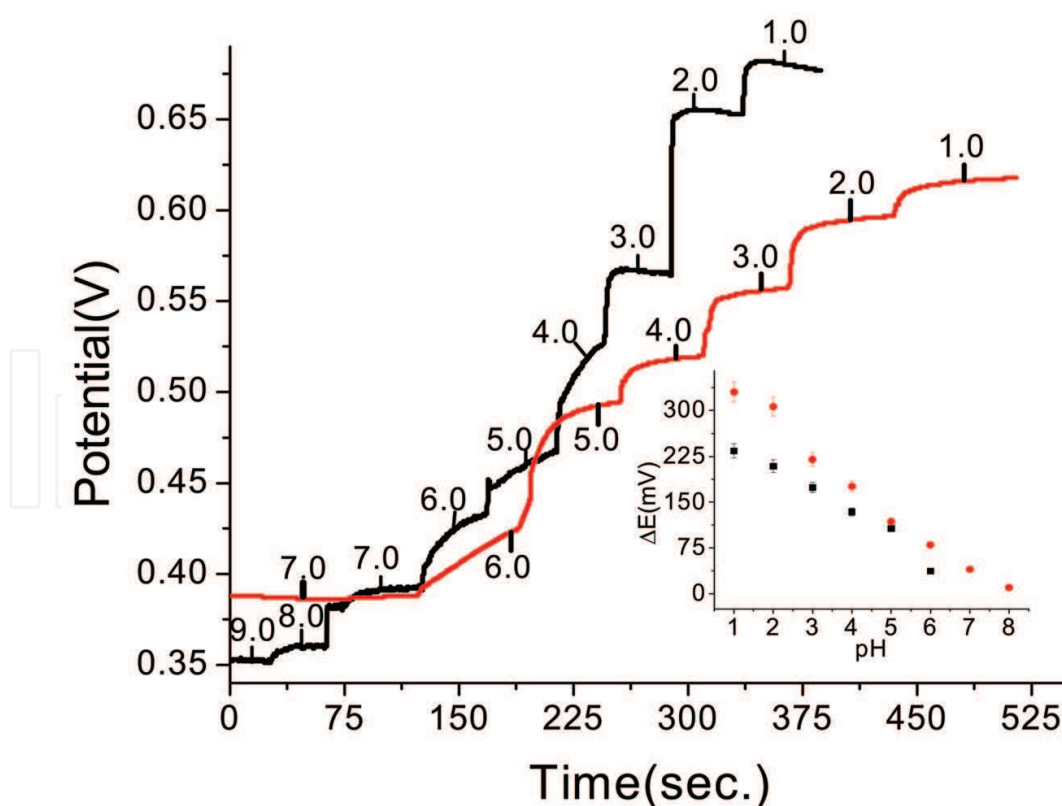

Fig. 3. Potentiometric pH sensing behavior of 5-carboxy PIn (Red) and 5-carboxy PIn-carboxylated PVC composite (Black)

2.4.1.2 Effect of Multiwall carbon nanotube (MWCNT)-Carboxylated on pH responses:

CNTs are well known as suitable materials for chemical sensing in which electron exchange is the basis of the mechanism. CNTs networks form an electrical contact to an outer device; whereas the sensitive coating acts as a transducer and converts physical phenomenon into electrical signals. Carbon is also known to give a pH response. In addition, it was found that thin carbon nanotube networks work as a conductive and transparent backbone structures for conducting polymer coating thereby increasing the electronic conductivity of the system. Use of carboxylated CNTs increases pH sensitivity with polymer not only due to the undissociated carboxylic group but also due to its reduced size which increases effective surface area. Kaempgen et. al [47] found that in polyaniline coated over CNT, pH response falls in the range of super Nernstian and linearity in response increases with increase in the amount of polyaniline. MWNTs helps in the total pH sensitivity of the electrode. Similarly our synthesized nanocomposite showed an increase in slope up to 69mV/pH for wide range of pH 10-1.0 as shown in figure 4.
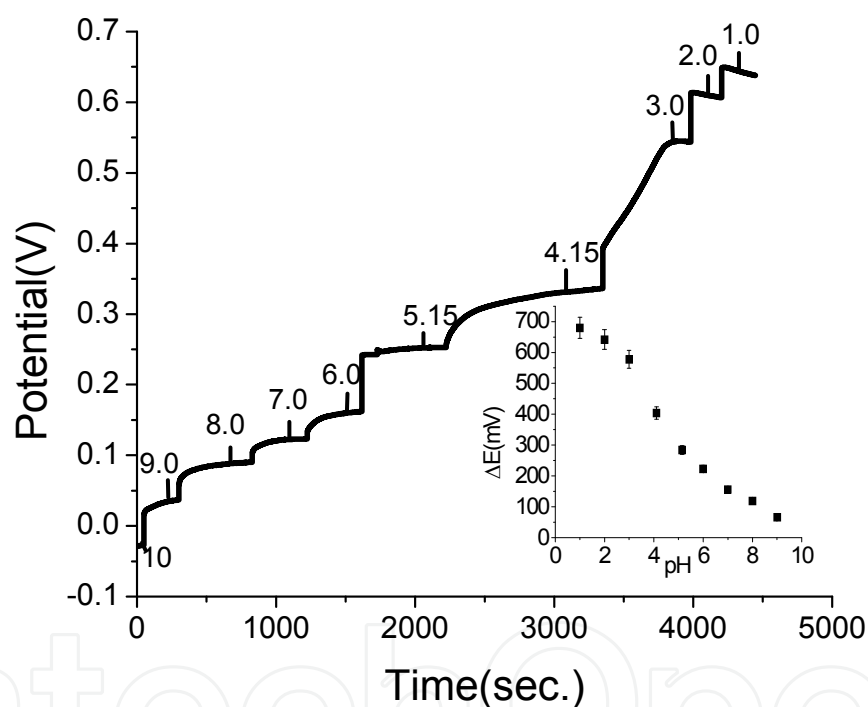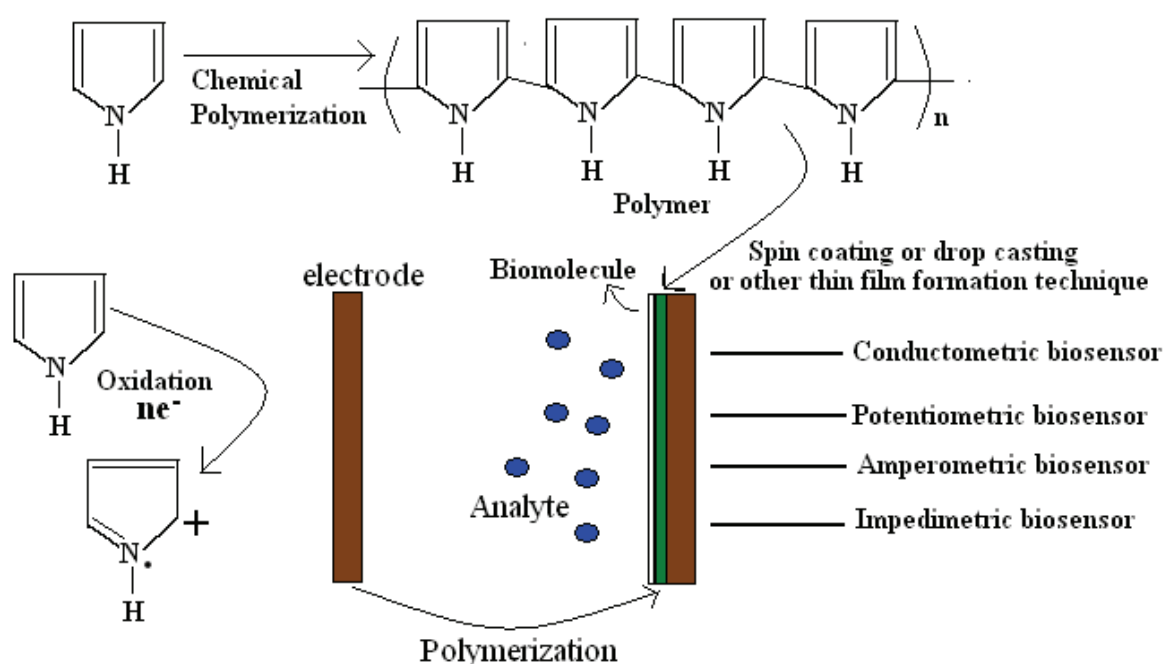

Fig. 4. Potentiometric pH sensing behavior of 5-carboxy PIn-carboxylated PVC-Carboxylated MWNTs nanocomposite.

2.4.2 Importance of conducting polymers for biosensors:

Biosensor is an electroanalytical device which transduces a physicochemical change in the presence of biological or biologically derived molecule such as enzymes, tissues, bacteria, yeast, antibodies/antigens, liposomes, organelles and DNA etc. Biosensors represent a new trend emerging in the diagnostic technology. Various types of matrices have been proposed for the immobilization of enzymes such as membranes, gels, carbon, graphite, silica, polymeric films etc. Conducting polymers are attractive materials for electron transfer over

the surface of the electrode as well for immobilization [54, 55]. The overall performance of electrochemical biosensor is characterized by metal substrate, biocatalyst immobilization and electronic and morphological properties of the conducting polymer. There are some advantages of using conducting polymers as transducing materials. Firstly, they are easy to synthesize through chemical and electrochemical polymerization techniques. Secondly, the ease of fabrication of sensors from these materials on substrate either by spin coating and/or drop casting from solution or directly by anodic electrochemical polymerization from an electrolyte solution [56, 57]. These polymers can be synthesized over the electrodes by electropolymerization also. The biosensor development by two different ways of polymer thin film formation is shown in Scheme 2.

Scheme 2. Schematic representation of conducting-polymer based biosensors fabrication technique by different methods of polymer film formation on solid and conducting substrate.

Biomolecules can be directly immobilized with the electropolymerization process to get these molecules in a specific arrangement or orientation. It is a widely adopted method for biosensor development. Beside, chemically synthesized polymers have their own advantages in post synthesis modification to desired mechanical and electrical properties. Moreover, these polymers can be molded by other materials which are more suitable for biomolecules immobilization which helps in enhancement of various properties. Conducting polymer is being researched as a material to enhance various analytical transduction properties of biosensors such as speed, sensitivity and versatility because of availability of various stable electronic states. Electrical (electronic state) and mechanical properties of conducting polymers can also be tailored by applying electrical stimuli thus affect the biochemical transduction performance. In the biosensor application conducting polymers can perform various roles viz. as a catalyst, a redox mediator, an ON/OFF switch,

ion sensitive membrane and as chemiresistor which shows change in the resistivity because of biochemical reaction. Due to biochemical reaction between the analyte and biomolecule various products are formed viz. proton, different ion and gases etc. These products related to a specific type of analyte which modulates the electronic and optical properties of the conducting polymer [58-61, 45], and thereby becomes a source of typical analytical signal which can be monitor at the electrode surface by various electrochemical techniques either in the change in impedance, change in open circuit potential (dipolar potential) change in current at fixed applied potential and conductivity etc. The sensitivity of the biosensor depends upon the electron relay between conducting polymer and solid electrode support over which polymer is fabricated. Here, we chemically synthesized 5-carboxy polyindole (5-carboxy PIn) by using two miscible solvents (water and ethanol) and prepared polymer used for the composite formation by solution casting method [62]. Carboxylated PVC and MWNTs used as a conventional and nanofiller (20 and 10wt. % respectively). Use of the filler not only modifies mechanical, morphological and electrical properties of the polymer but also helps to improve the chemical and biosensor performance of the polymers.
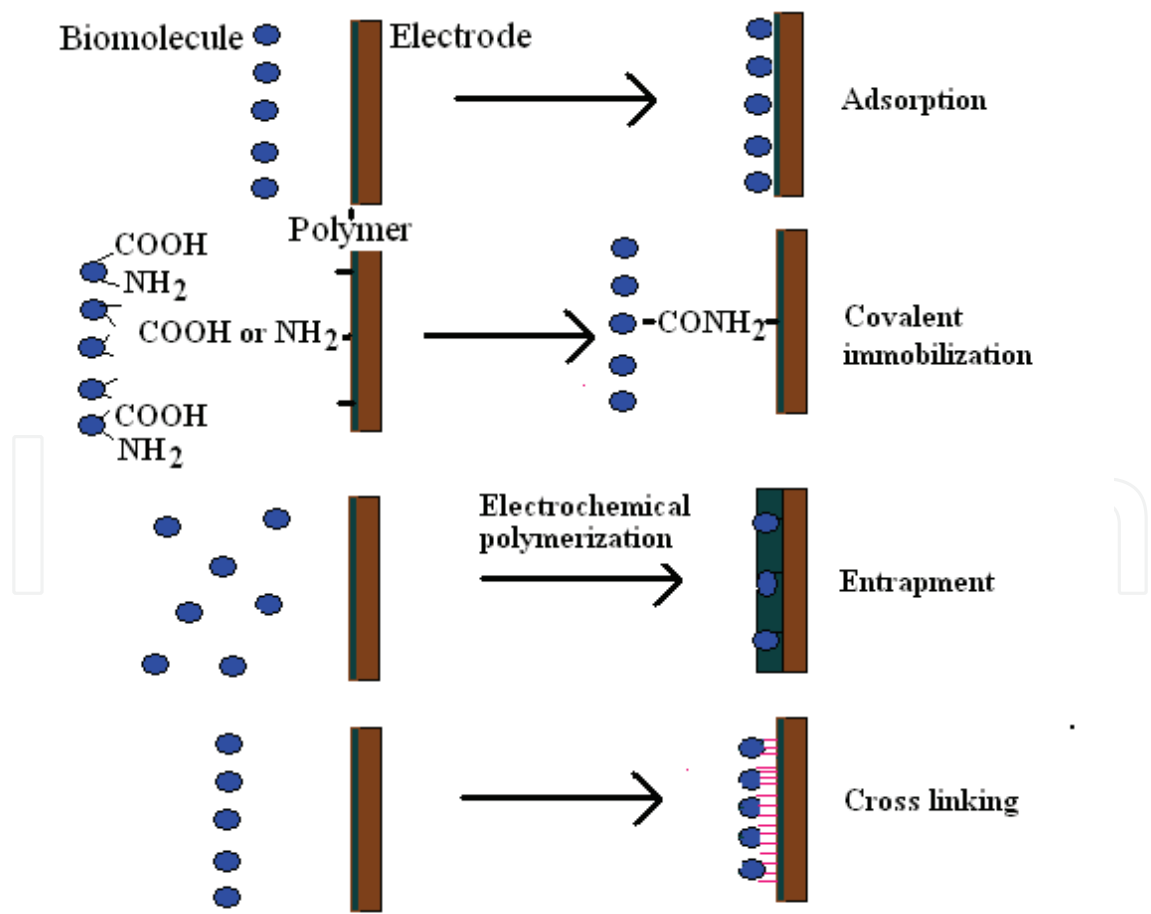
2.4.3 Immobilization of urease over the polymer membrane for urea biosensor:

Solid state biosensors are adopted more as compared to liquid state as the stability of the enzyme is very less in the liquid state. The immobilization of the biological component should be at the environmental conditions otherwise it results in decreased enzyme activity. Thus research is going on in the field of solid state biosensor based on polymer [63]. The activity of immobilized molecules depends upon various parameters such as surface area, porosity, hydrophilic character of immobilizing matrix, reaction conditions and the methodology chosen for immobilization [64, 65]. The popularity of using conducting polymers in biotransducer application is mainly due to various reasons such as availability of a spectrum of immobilization techniques for bioreceptor (even molecule through cell) location, each of which is compatible with maintaining bioactivity, faithful presentation of the bioreceptor to the analyte, and controlled thickness of the biorecognition layer. All these parameters are related to polymer suitable for immobilization due to rough, porous and hydrophilic nature. The incorporation of large size dopants, dodecylbenzene sulphonate (DBS) into polypyrrole film during electropolymerization makes PPy more porous [66, 67]. Moreover the incorporation of the nanoparticle in the conducting polymer not only increases the surface area and nanofabrication but also changes various properties viz morphological and electrical which help in the immobilization of the biomolecules and better performance of the biosensor by proper diffusion of the analyte specific product [68]. Enzyme over the immobilization matrix must be in the form of a thin layer with efficient loading. The immobilization of the suitable enzyme on the surface of the sensing electrode is a critical step in fabricating the biosensor of required choice. Additionally, the method used for enzyme immobilization should not affect the quality of the urea biosensor in terms of cost per use; it's easiness in carrying and its effectiveness in the transducer performance [69]. Immobilization of urease in conducting polymer can be achieved by several techniques.
These include physical adsorption, electrochemical entrapment, chemical cross linking and covalent coupling. These methods have their own advantages and disadvantage as mentioned in table2.
The steps involving in different type of immobilization method are shown in Scheme 3.

Way of immobilization	Advantage	Disadvantage	Reference
Physical adsorption	No structural change of biocatalyst and cheap, fast immobilization	Physical interaction easily braked by change in environment condition cause leaching	M. J. Syu et. al. [75]
Entrapment	One step biosensor fabrication, low cost	Unsymmetrical distribution of biocalatyst cause diffusion barrier	S. B. Adelojo et. al. [18]
Cross linking	Stable interaction, moderate cost	Side effect of chemical used for immobilization, it take time, difficult enzyme recovery	W. J. Cho [30]
Covalent bonding	Stable interaction with high enzyme loading	Side effect of chemical used for immobilization, it take time, difficult enzyme recovery	Rajesh et. al. [29]

Table 2. Different method of biomolecule immobilization

Scheme 2. Schematic representation of process in different immobilization methods.

Amongst all the immobilization methods covalent immobilization is the most advanced one. In this method biomolecule is incorporated in the conducting polymer matrix through complementary functional group chemistry. This way of immobilization confers strong interaction between polymer and biomolecule that can be achieved by amide or ester linkage in the presence of some coupling agent. Covalent immobilization not achieved in polymer only can be participate with other moieties (having functional group) present with conducting polymer after modification viz plasticizer or fillers. Covalent immobilization not only inhibits leaching of the enzyme but also provides thin coating of the enzyme which facilitates fast ionic movement thereby increasing sensitivity and long life stability of the biosensor. Rajesh et. al [29] immobilized urease in the copolymer poly-(N-3 aminopropylpyrrole-co-pyrrole) in the presence of 1-ethyl -3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) as coupling agent in aqueous medium. 95% baseline recovery in 40 second was thereafter observed. Thus it can be concluded that carbodiimide coupling reaction has a feature of strong covalent bonding with the enzyme with the matrix, which in turn is responsible for high enzyme loading at the surface of polymeric modified electrode.

We used 5-carboxy PIn, 5-carboxy PIn-carboxylated PVC and 5-carboxy PIn-carboxylated PVC-MWNT composites for immobilization of urease and studied morphological properties. After composite formation film became porous with carboxylated PVC. Due to MWNT surface area increased which is good for immobilization of urease. This matrix was further applied for covalent immobilization in the presence of coupling agent DCC and NHS. Treatment of DCC and NHS was carried out in cyclohexane; immobilization was carried out in aqueous solution of the enzyme in distilled water.

2.4.4 Mechanism of potentiometric urea biosensors based on conducting polymer:

Potentiometric measurements are based on monitoring potential values under a zero current flow regime (under equilibrium), in order to determine the analytical concentration of desired components after catalytic reaction between biomolecule and analyte in the sample solution [43, 70-72]. Source of potential may be a physical phenomena not involving explicit redox reactions but having non-zero free energy initial conditions because of enzymatic reaction and ionic gradient. For instance ion concentration gradient over the surface of sensing membrane is one of such phenomena and ionic potential similar to dipolar potential. Potentiometric sensor either symmetric or asymmetric depends on the homogeneous and heterogeneous interface between the dipolar layers of charges. Conducting polymer or solid state ion selective electrodes (ISEs) based potentiometric sensor falls in the asymmetric type because ionic dipolar layer is generated at the interface of liquid and solid phases [73]. Since the biochemical reaction between analyte and urease change pH due to the formation of ammonia and carbondioxide, it can be analysed using pH sensitive or H+ exchanger moieties through potentiometric mode of detection [74]. In addition, potentiometric sensor for detection of ammonia, and ammonium ion generated by enzymatic reaction, using ISE are being studied [75]. The major problem for pH sensitive electrode is that the sensor response is strongly dependent on the buffer capacity of the sample. This is because the pH change produced in the course of the enzymatic reaction is suppressed by the buffer used. The general problem of ammonium sensitive electrode is

interference by Na+, K+ and Ca+ ion, which are present in the serum and other biological derive fluid. Potentiometric response is affected by various parameters viz. concentration of the buffer, pH of the buffer, loading of the enzyme, temperature, stirring rate etc. Sensor gives best response at an optimum condition of these parameters [1, 76]. Polymers both intrinsically conducting and non conducting, have played a prominent role in pH sensitive potentiometric sensor as well for urea biosensor. Some of the ureabiosensor based on pH sensitive conducting polymer using potentiometric technique is shown in table 3.

Electrode	Sensitivity	Mode of sensing	Lower detection limit	Linear Dynamic range	Reference
Polypyrrole (Ppy)-Bovine serum albumin	~25mV	pH, Chronapotentiometry	6.6×10^{-6}M	6.6×10^{-6}-7.5×10^{-4}	T. Ahuja et. al. [82]
Poly(N-vinyl carbazole)/stearic acid	~50mV	pH Potentiometry	1mM	10-68mM	R. Singhal et. al. [64]
Polyaniline-Tetraphenyl borate doped-PVA	~26.6mV	pH Potentiometry	20µM	~60µM-to mM	P. C. Pandey et. al. [44]
Electroactive polypyrrole/poly anion complex	110mV/decade	pH Potentiometry	0.02mM	0.3-30mM	Osaka et. al. [77]

Table 3. pH transducer based urea biosensor with different conducting polymers

We developed potentiometric urea biosensor fabricated using 5-carboxy PIn-Carboxylated PVC composite based on solid state pH transducer. A classical two electrode cell required for the estimation of urea by potentiometric technique having one H+ ion sensitive electrode coated with enzyme urease and reference electrode. Total response of Urease/PIn-PVC/Pt biosensor electrode gave 130±10mV/ change in urea concentration of 10^{-6}-10^{-1} M (24.6 mV/decade) as shown in figure 6. The linearity obtained for the above biosensor in the range of 10^{-5}-10^{-1}M. Lower detection limit of the biosensor was 10^{-5} M concentration of urea. The apparent Michaelis-Menten constant K_m is an indicator of the enzyme-substrate reaction kinetics and used to evaluate the biological activity of the immobilized enzyme. Two linear ranges were obtained viz., 10^{-6}-10^{-4}M and 10^{-3} to 10^{-1}M. The reciprocal of apparent Michaelis-Menten constant (lower concentration region) in the present study was found to be -$1/K_{mapp}$=-5.276882x10^{-6} for Urease/5-carboxy polyindole-carboxylated PVC electrode in the lower concentration range (when the enzyme was 20µl, optimum loading of enzyme), which is lower than any other urea sensor reported in literature so far.
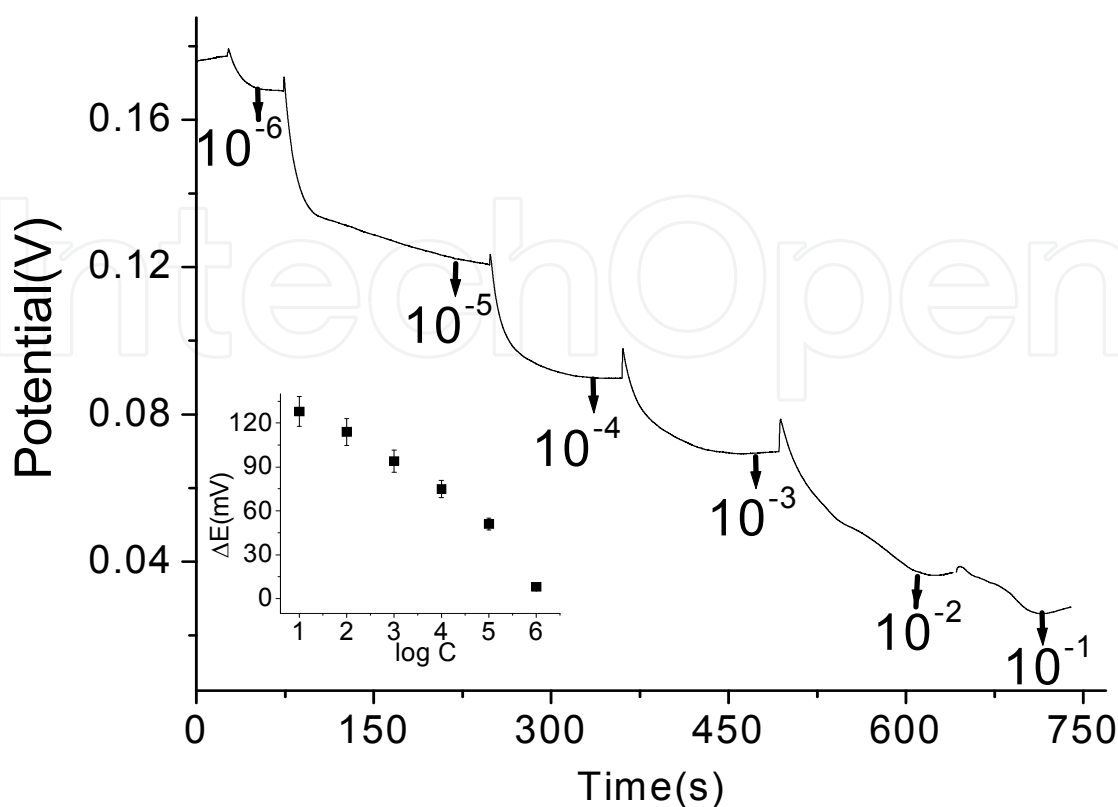
Fig. 6. Potentiometric response of the biosensor (Urease (20µl) /PIn-PVC/Pt) to increasing urea concentration (10^{-6}-10^{-1})M. 1 mM Tris–HCl buffer (pH 7.0) calibration curve (Inset)

2.4.4.1 Carboxylated-MWCNTs-Conducting polymer composite based urea biosensor:

CNTs are used to increase the biosensor sensitivity because for their electron relay properties and at times due to interaction with analyte products. 5-carboxy PIn-carboxylated PVC- carboxylated MWNT composite was used for urea biosensor. The response for the urea increased up to 152±10mV for urea concentration in the range of 10^{-6}-10^{-1} M. In the presence of MWCNTs biosensor showed slope of 28.4 mV/decade of urea concentration (for 10^{-6}-10^{-1} M concentration of urea) in well accordance with its super Nerstien pH response of the same composite as shown in figure 7.

3. Effect of enzyme loading on urea biosensor response:

The amount of urease over the transducing membrane affects activity, response time and linear dynamic range due to thickness of sensing membrane and amount of enzyme. Activity of the biosensor was used to estimate the minimum enzyme unit required for the construction of the biosensor. In our biosensor we found that the lower and higher urea concentration responses of the biosensor can be altered by changing the loading of the urease over the electrode. When the loading was less (5-10 µl) biosensor was more sensitive in the lower concentration (10^{-6}-10^{-3}M) range and vise versa.
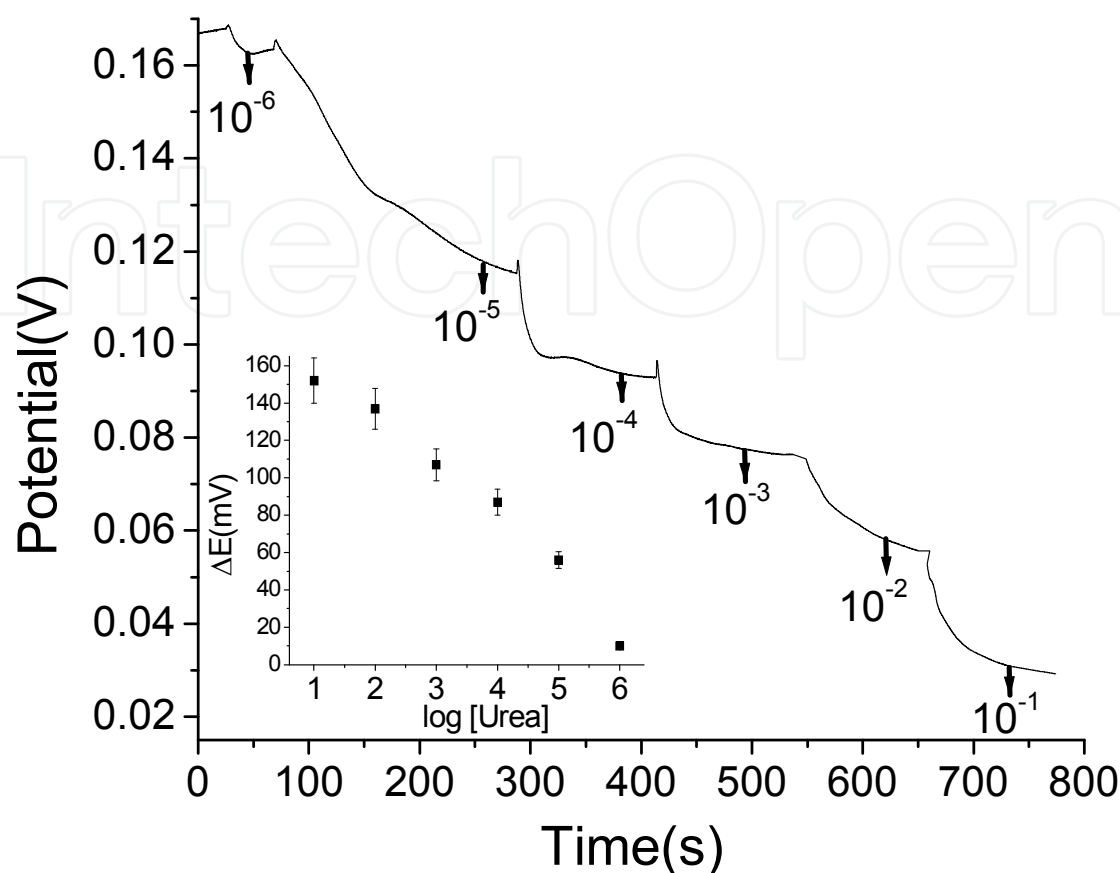
Fig. 7. Potentiometric response of the biosensor (Urs(20µl) /PIn-PVC-MWCNT/Pt) to increasing urea concentration (10^{-6}-10^{-1})M. 1 mM Tris–HCl buffer (pH 7.0) calibration cure (Inset)

4. Stability of the urea biosensor:

Stability of the conducting polymer based biosensor depends on the chemical stability (for long term usage) and electrochemical stability (for short term usage) of the conducting polymer layer over the solid substrate and enzyme layer immobilized with the polymer matrix. Stability of both of the components over the conducting electrode directly or indirectly affects the overall performance of the biosensor. Stability of the conducting polymer is measured by two parameters, firstly their inherent stability of the conductive and electroactive material (related to it functioning) and secondly their stable deposition over the electrically conducting substrate (physical stability) [28]. The ion exchange into and out of the conducting polymer creates concomitant dynamic change in the polymer (due to expansion and contraction) and causes interfacial shear stress there by causing leaching of the polymer from the surface. The interaction between polymer film and underlying

electronic conducting substrate is primarily hydrophobic in nature and is supported by some weak forces. Several attempts have been made to improve the adhesion of conducting polymer onto the conducting substrate for long term applications by using rough surface electrode, giving thiol modified polymer and by providing functional group over the substrate with the help of some chemical [78-82]. Through functional group chemistry, a stable deposition of the polymer has been achieved. Long term stability of the enzyme over the matrix is an important factor for consideration in the construction of the biosensor, since this is not only beneficial for the biosensor transport but it also reduces measurement cost. In addition due to the efficient coupling it is easy to achieve monolayer (thin and regular) deposition of the enzyme over the polymer which is good for the biosensor sensitivity. This could be achieved only; there would be strong and efficient bonding between enzyme and immobilizing material [69]. Biosensor developed here is highly stable and sensitive when enzyme immobilization is processed and stored at 5^0C. When the biosensor developed at room temperature sensitivity of the biosensor was in the range of 10^{-4}-10^{-1} M and at 5^0C it was responsive in broader range (10^{-6} - 10^{-1}M). The stability experiment was carried out in 0.1M urea concentration for two months, keeping biosensor electrode at 5^0C. It was found that the biosensor was stable for ~2 months with 75% degradation of the enzyme, similar to the recently reported urea biosensor having immobilization through carbodiimide coupling reaction.

5. Estimation of urea in biological sample

Urea is an important parameter which is frequently analyzed in biological samples such blood serum, urine and milk. Urea concentration in the above constituents above an optimum quantity directly affects the human health. The normal range of urea in blood is between 1.7- 8.3 mmol/l and levels increased up to 100 mM/l under pathophysiological condition affect the normal functioning of kidney [75]. The proposed urea biosensor was performed in human serum after 50 times dilution using standard addition method. The 5-carboxy polyindole/PVC electrode immobilized with 20 ul urease enzyme was used for the estimation of blood urea in the blood serum of healthy volunteers using three point calibration methods. The concentration of the urea in the blood was found to be 9.5×10^{-5} M (in 50 times diluted) or 4.75×10^{-3} M (in real sample) using standard addition method as shown in figure 8.

In this chapter potentiometric pH sensors and urea biosensors using conducting polymer composite and nanocomposite materials are discussed especially based on carboxyl functionalized polyindole conducting polymer. We have tried to identify the role of various materials and there capability to influence the performance of a sensing device based on pH transducer. We have also considered the effect of the different materials, processing, immobilization and morphology of the conducting polymer composite systems on sensing property. Finally we close our discussion along with work done by our group. The sensors and biosensors developed are compared with earlier known sensors and found to be more sensitive and stable over the others. We not only reported improved pH solid state sensor and urea biosensors based on such pH transducers but also showed the importance and potential of the novel carboxyl functionalized polyindole conducting polymer composites and nanocomposites for future direction for sensor and biosensor technology.
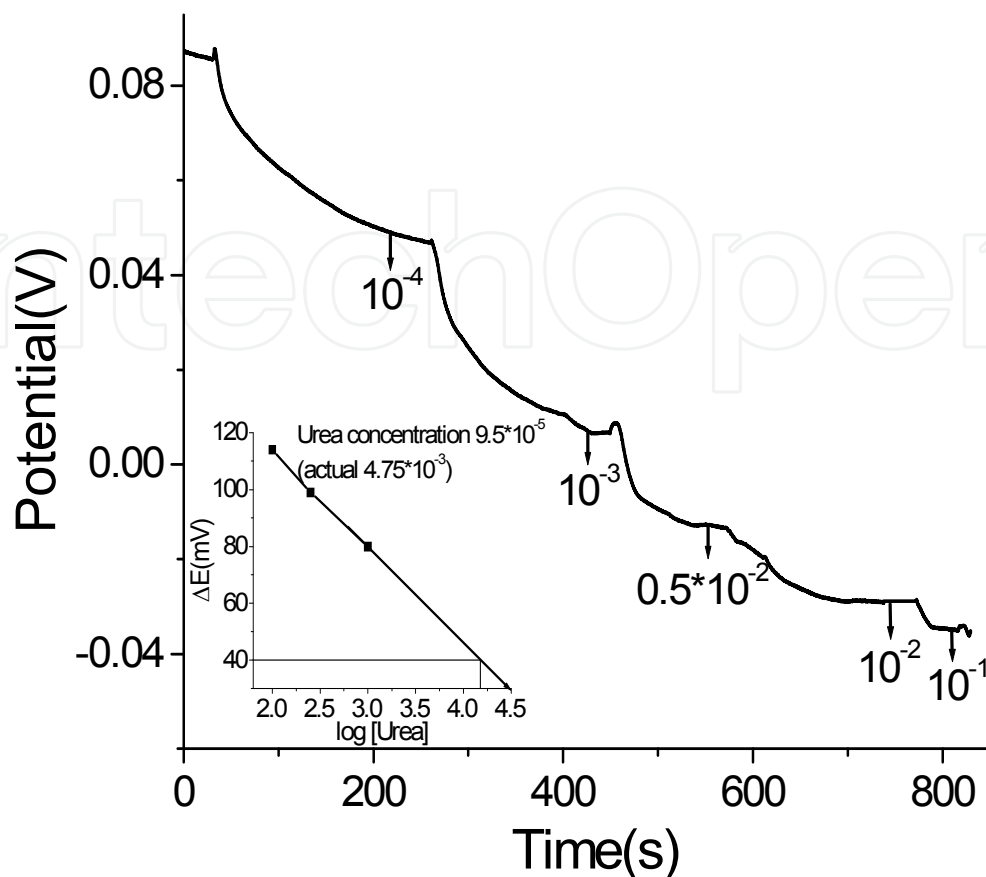
Fig. 8. Potentiometric response in blood serum. Three point calibration curve of this response to estimate urea concentration (Inset)

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Biosensors

Edited by Pier Andrea Serra

A biosensor is defined as a detecting device that combines a transducer with a biologically sensitive and selective component. When a specific target molecule interacts with the biological component, a signal is produced, at transducer level, proportional to the concentration of the substance. Therefore biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. Bringing together researchers from 11 different countries, this book covers a wide range of aspects and issues related to biosensor technology, such as biosensor applications in the fields of drug discovery, diagnostics and bacteria detection, optical biosensors, biotelemetry and algorithms applied to biosensing.

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