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Review

Recent advances in polyaniline based biosensors

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ABSTRACT

The present paper contains a detailed overview of recent advances relating to polyaniline (PANI) as a transducer material for biosensor applications. This conducting polymer provides enormous opportunities for binding biomolecules, tuning their bio-catalytic properties, rapid electron transfer and direct communication to produce a range of analytical signals and new analytical applications. Merging the specific nature of different biomolecules (enzymes, nucleic acids, antibodies, etc.) and the key properties of this modern conducting matrix, possible biosensor designs and their biosensing characteristics have been discussed. Efforts have been made to discuss and explore various characteristics of PANI responsible for direct electron transfer leading towards fabrication of mediator-less biosensors.

Contents

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1. Introduction

Biosensors are analytical devices that integrate biological sensing elements like enzymes, antibodies, nucleic acids, cells, etc. with electronic transducer equipped with an electronic amplifier. Biosensors represent conceptually novel approach to real-time, on-site, simultaneous detection of multiple hazardous agents. They provide speedy interactive information about the desired sample and found to have applications in various fields, e.g. clinical diagnostics, environmental monitoring, bioprocess monitoring, food and agricultural product processing, etc. Recently, IUPAC has given the specific definition of biosensor: it is a self-contained integral device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological element (Thevenot et al., 1999). Biosensor has three major components: (i) a bio-recognition element such as deoxyribonucleic acid (DNA) or enzyme or antibody, etc. for the recognition of an analyte also called bioreceptor, (ii) an immobilization surface such as a conducting polymers (CP) (Gerard et al., 2002), nanomaterials (Luo et al., 2006), sol–gel films (Ansari et al., 2008), and self-assembled monolayers (Arya et al., 2009), etc., used for immobilization of biomolecule and (iii) a transducer unit for conversion of biochemical reaction product into a recognizable signal (Fig. 1). Bioreceptor and transducer together may be referred as biosensor membrane.

The history of biosensor began in 1962 with the development of first enzyme based glucose sensing device by Clark and Lyons (Clark and Lyons, 1962). This novel biosensing device relied on a thin layer of glucose oxidase (GOx) entrapped over an oxygen electrode via semi-permeable dialysis membrane. Measurements were based on the monitoring of the O_2 consumed by the enzyme-catalyzed reaction as shown below

$$\text{Glucose} + O_2 \overset{\text{Glucose oxidase}}{\longrightarrow} \text{Gluconic acid} + H_2O_2 \quad (1)$$

Though the current scenario of biosensors is much advanced than the Clark enzyme electrode, the researchers are facing new challenges and are struggling to find solutions to the instability of desired biomolecules. Until recently, the majority of biosensors are based on one or more biomolecules used in conjunction with an electrode. The redox reaction could be detected electrochemically by measuring the loss or formation of substrate or product, respectively, by the use of a small mediator species that shuttles between the biomolecule and the electrode, or less commonly by direct electron transfer (ET) between the biomolecule redox site and the electrode (Murphy, 2006). Direct ET can be difficult to achieve, since the redox site of a biomolecule is often hidden deep inside the biomolecule. Recent advances in achieving direct ET have been made by modification of biomolecules or electrode surfaces through the use of novel conducting materials as mediators and design of functional biointerfaces (Hartmann, 2005). Thus, highly conductive organic transducers are gradually emerging for the development of next generation biosensor design for highly reliable, stable and robust field-based diagnostic devices. There has been considerable interest towards the development of biosensor probes for detection of biologically significant molecules using CP. Hoa et al. (1992) have recently described a new generic concept based on the fact that the conductivity of this class of polymers is very sensitive to chemical potential of the microenvironment within a polymer matrix. And these polymers thus act as both immobilization matrices as well as the physicochemical transducer to convert a chemical signal into an electrical signal.

In general, CP are polymers with delocalized π-electron system with a wide band gap in their pristine state (Smilowitz et al., 1993) and can be made electrically conducting by doping. Doping in CP refers to the oxidation or reduction of π-electronic system, p-doping and n-doping, respectively, and can be effected chemically or electrochemically. To maintain electro-neutrality, doping requires incorporation of a counter ion. The doped and undoped states have different electronic, optical, physical, chemical, and electrochemical aspects. Thus, reversible interchange between redox states in CP gives rise to the changes in its properties including polymer conformation, doping level, conductivity, and colour. These make the CP suitable for applications to electrochromic devices (Beaujuge and Reynolds, 2010), energy storage devices (Cheng et al., 2009), actuators (Fuchiwaki et al., 2009), sensors (Hatchett and Josowicz, 2008), etc. Polyaniline (PANI), polypyrrole, polythiophene, and polyacetylene are some of the most explored and interesting CP and have been used for a variety of applications.

Among various conducting polymers, PANI has attracted much attention due to its unique and controllable chemical and electrical properties (Kang et al., 2004), its environmental (Pruneanu et al., 1999), thermal (Wang et al., 1995) and electrochemical stability (Chiang and MacDiarmid, 1986), and its interesting electrochemical, electronic, optical and electro-optical properties (Heeger, 2001). Moreover, PANI is known to have a broad range of tunable properties emanating from its structural flexibility leading to many applications in different fields such as anti-corrosive coatings, energy storage systems, gas sensing, as well as electrochromic and electrocatalytic devices. Chandrakanthl and Careem (2000) have claimed that PANI has the highest environmental stability and is recognised as the only conducting polymer that is stable in air (Sergeyeva et al., 1996). PANI has been found as an interesting material for sensor and biosensor interfaces since it acts as an effective mediator for electron transfer in redox or enzymatic reactions and can also be used as a suitable matrix for immobilization of biomolecules (Luo and Do, 2004). The former role is possible due to the inherent electroactivity of PANI. PANI has gained much popularity in biosensor applications, partially due to its favourable storage stability and simple synthetic procedures with good processibility. Advantages of PANI in the field of biosensor are indicated by impressive signal amplification and elimination of electrode fouling. Besides this, PANI exhibits two redox couples in the convenient potential range to facilitate an efficient enzyme–polymer charge transfer. Recent reports have elucidated the role of PANI as enzyme amplifier to provide signal amplification in the recognition process (Sergeyeva et al., 1996; Kim et al., 2000; Grennan et al., 2003; Morrin et al., 2003). The use of polyaniline as an enzyme switch, which yields "on" and "off" responses, has also been recently demonstrated (Iribe and Suzuki, 2002). From economic point of view, aniline monomer is less expensive than other monomers used for the synthesis of other conducting polymers.

The goal of this review is to describe at length the role of PANI for biosensor application in a comprehensive fashion. It covers PANI as active component mediating or transducing responses that indicate the presence of an analyte.

2. Polyaniline

PANI, a CP of semi-flexible polymer family, was discovered over about 150 years ago. Recently, PANI has captured attention of scientific community due to the discovery of its high conductivity and low cost. Therefore researchers are continuously exploring its applications including those in biosensors because of a number of useful features such as 1) direct and easy deposition on the sensor electrode, 2) control of thickness, 3) redox conductivity and polyelectrolyte characteristics, 4) high surface area, 5) chemical specificities, 6) long term environmental stability and 7) tuneable properties. The different tuneable properties of PANI that make it a suitable candidate to be used in various applications have been summarized in Table 1.

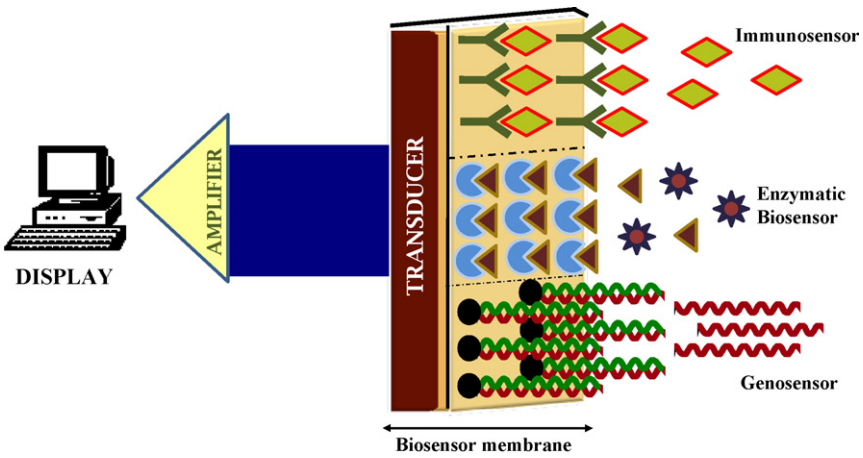

Fig. 1. Schematic of a biosensor.

2.1. Structure of PANI

The structure of PANI has been illustrated in Fig. 2. It consists of '*n*' reduced (benzenoid diamine) and '*m*' oxidised (quinoid diamine) repeating units where the oxidation state can be defined by the value of *m*. Table 2 shows different redox forms of PANI.

Leucoemeraldine (LE) is fully reduced state. Pernigraniline (PG) is fully oxidized state with imine links instead of amine links. The emeraldine form of PANI, often referred to as emeraldine base (EB), is either neutral or doped, with imine nitrogens protonated by an acid. EB is regarded as the most useful form of PANI due to its high stability at room temperature and moreover its doped form

Table 1
Various tuneable properties of PANI.

Tunable properties of PANI	Different approaches to tune these properties	Applications	References
Conductivity	(i) *By monitoring the solution pH*: PANI shows its electrical conductivity only in its protonated (emeraldine salt) form i.e. at low solution pH values (≤3). (ii) *Doping*: conductivity of PANI also depends on the type of dopant and degree of doping. A higher basicity of the dopant anion results in a lower conductance of PANI. In the case of organic dicarboxylic acid dopants, a greater quantity of dopant results in lower yield and conductivity. (iii) *By changing the oxidation state*: conductivity can also be controlled either chemically or electrochemically by changing the oxidation state of PANI. (iv) *By composite formation*: conductivity of PANI can be tuned via incorporating different materials like carbon nanotubes (CNT), gold nanoparticles (GNPs), etc. (v) *Thickness of PANI film*: the resistance of the PANI electrode has been found to increase exponentially with increasing film thickness. (vi) *By regulating PANI morphology*: the morphological changes in PANI during the protonation–deprotonation and oxidation–reduction processes also affect the conductivity of the resultant film.	This tunable property makes PANI a promising material for many applications including in batteries, sensors, actuators, electromagnetic. Shielding, antistatic coatings, corrosion protection, electro-optic and electrochromic devices.	Genies et al., 1990 MacDiarmid et al., 1987 Chandrasekhar, 1999 Hu et al., 2007 Grennan et al., 2006 Saraswathi et al., 1992
Electrochemical behaviour	*By composite formation*: the electrochemical characteristics or the redox behaviour of PANI can be monitored by integrating with electro-active materials like CNT, GNPs, Fe_2O_3 nanoparticles, graphite oxide etc. inside this conducting matrix. *By using different dopants*	Chemical sensor and biosensor fabrication. Actuators	Wu et al., 2005 Zhang et al., 2009 Dhand et al., 2008
Electrochromism	PANI exhibits multiple colour transition (pale yellow–green–blue–violet) depending on both: the oxidation state and pH via protonation–deprotonation process.	Organic or polymer light emitting diodes. Electrochromic displays and smart windows.	Watanabe et al., 1987 Rodrigues et al., 1991 Gaponik et al., 1999
Porosity	Porosity can be controlled by doping and dedoping processes.	Separation membranes.	Anderson et al., 1991

Table 2
Different oxidation states of PANI.

Form of polyaniline	Redox state	Oxidised groups (*m* value)	Reduced groups (*n* value)	Colour
Leucoemeraldine	Fully reduced	0	1	White/Clear
Emeraldine	Partially oxidized	0.5	0.5	Green/Blue
Pernigraniline	Fully oxidized	1	0	Blue/Violet

(emeraldine salt; ES) is electrically conducting. LE and PG are poor conductors, even when doped with an acid. These forms may be inter-converted by chemical and/or electrochemical oxidation or reduction.

2.2. Conductivity in PANI

PANI is a p-type semiconductor and thus majority charge carriers in PANI are holes (Bhadra et al., 2008). The delocalized π-bonds available in this system are responsible for its semi-conducting properties. PANI has semi-crystalline, heterogeneous system with a crystalline (ordered) region dispersed in an amorphous (disordered) region (Kulikov et al., 2002). It is similar to a quasi-metallic island surrounded by non-metallic amorphous zone. When PANI is doped with an acid, a polaron is formed through successive formation of bipositive species, bipolaron structure, and more stable polaron structure as shown in Fig. 3. This polaron structure is responsible for electrical conduction through hopping mechanism in its crystalline region and this hopping may be intra-chain or inter-chain (Stafstrom et al., 1987). In polaron structure, a cation radical of one nitrogen acts as a hole and this hole acts as charge carriers. The electron from the adjacent nitrogen (neutral) jumps to this hole and it becomes electrically neutral. Consequently, the holes start to move (Fig. 3). However, in bipolaron structure, this type of movement is not possible since two holes are adjacently located (Fig. 3). In LE or PN structures, the electronic environments of all nitrogen atoms along the polymer chain are similar. Protons from a dopant can be attracted by any nitrogen atom and there may be a few (more than two) protonated nitrogen or free nitrogen atoms situated side by side across the chain. Hence, there is a less chance for chain regularity, creating less chance for the formation of a polaron. As a result, protonated LE or PN are insulating in nature.

2.3. PANI: an efficient material for biosensing application

PANI is interesting as a material for sensor and biosensor interfaces because it can act as an effective mediator for electron transfer in redox or enzymatic reactions (Shi et al., 2004). The role of PANI as mediator is possibly due to the delocalized redox charges present over a series of conducting grains (polarons) in its crystallite ES-I phase (Wolter et al., 1998). PANI is considered to be an attractive polymer since it exhibits two redox couples in right potential range to facilitate an enzyme–polymer charge transfer and thereby acts as self-contained electron transfer mediator (Fig. 4). Consequently, no additional diffusional mediators need to be added to the sensing system for electron transfer. This has advantages for long-term stability of the sensor, since confinement of mediator to the sensor surface prevents it from leaching into solution, thus eliminating the requirement for a containment membrane. Such moves towards reagentless systems facilitate the use of sensors as stand-alone devices.

PANI offers a myriad of opportunities to couple analyte receptor interactions, as well as non-specific interactions, into observable (transducible) responses. In particular, PANI's transport properties, electrical conductivity or rate of energy migration, provide enhanced sensitivity. It is the best known semi-flexible rod CP system with chemical and structural flexibility surrounding its amine nitrogen linkages for effective binding and immobilization of biomolecules. Immobilization of a biological component on a matrix refers to restricting the gross movement of biomolecule and to keep it in a relatively defined region of space resulting in enhanced stability and reusability of biological component. The various methods can be utilized for the immobilization of desired biomolecules on the PANI surface (Supplementary data 1). Besides this, control over the shape and dimensions of PANI (Ding et al., 2007) by varying synthesis or processing conditions is likely to result in desired physical and electrochemical properties for biosensing application.

Nanostructures of PANI e.g. nanowires, nanospheres, nanorods, and nanotubes have provided a new ground to improve its characteristics and offer the possibility of enhanced performance wherever a high interfacial area between PANI and its environment is important (Forzani et al., 2004). For example, in sensor applications, nanostructured PANI has greater sensitivity and faster response time relative to its conventional bulk counterpart due to higher effective surface area and shorter penetration depth for target molecules (Zhao et al., 2009). The morphology of nanostructured PANI plays an active role in enhancement of activity of desired catalyst. Sreedhar et al. (2009) have recently studied

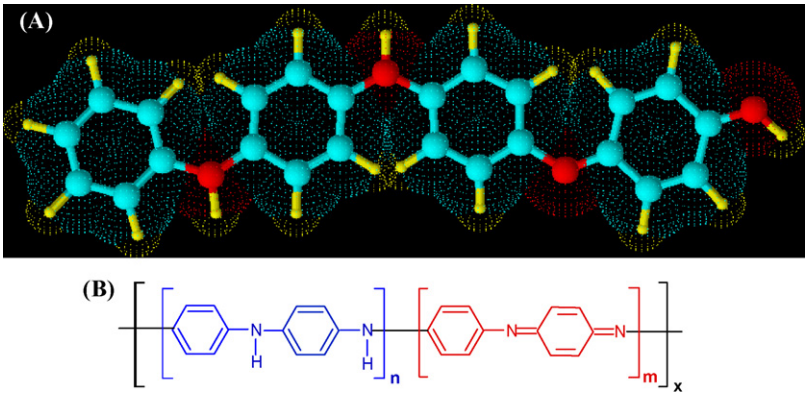

Fig. 2. (A) 3D and (B) 2D structures of polyaniline.

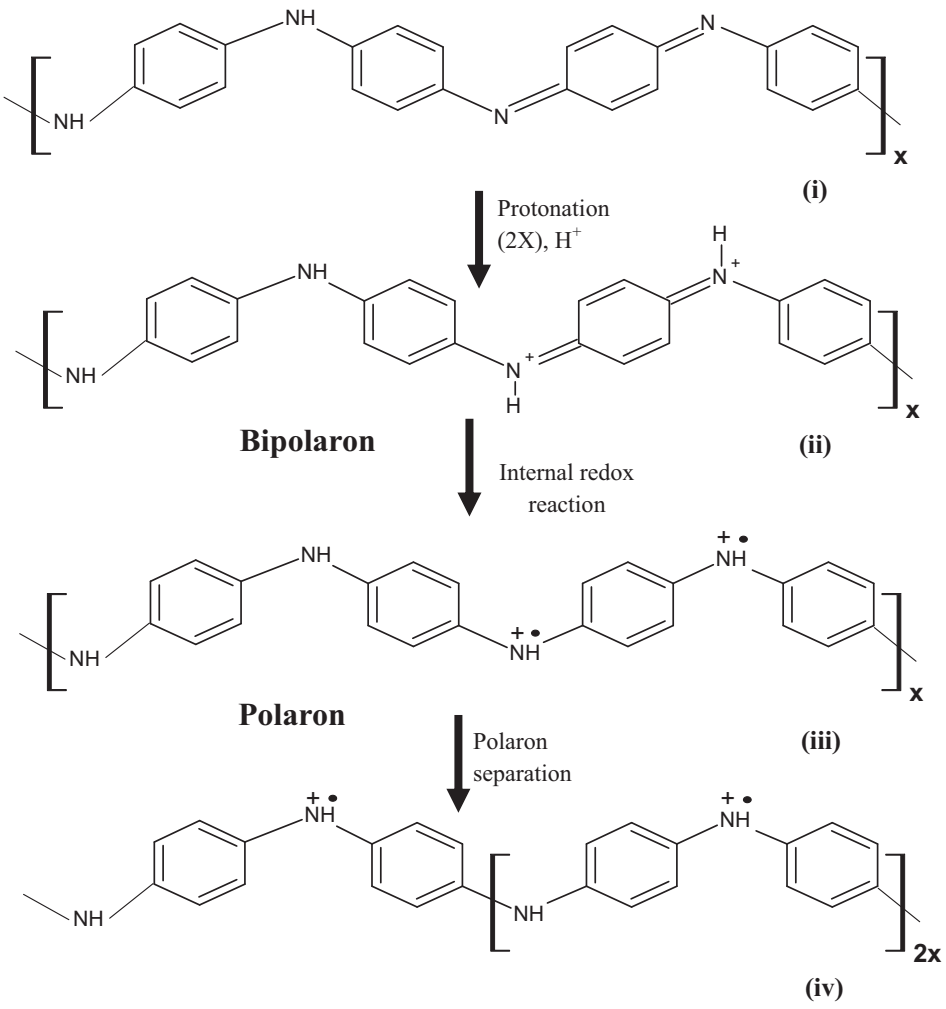

Fig. 3. Chemical structures of emeraldine (i) before protonation (emeraldine base), (ii)–(iv) after 50% protonation: (ii) formation of bipolaron, (iii) formation of polaron and (iv) separation of two polarons.

the activity profile of the oxidation of 4-bromothioanisole using PANI nanospheres (S), nanorods (R), nanotubes (T). These investigations have revealed that the activity varies as S < R < T, that has been attributed to enhanced surface area in case of PANI nanotubes (PANI-NT). Similarly, Dhand et al. have investigated the performance of chemically fabricated PANI nanospheres and electrophoretically deposited nanostructured PANI as platform towards the fabrication of cholesterol biosensor. These studies have revealed that PANI nanospheres show better detection range with faster response time and shelf-life that may be attributed to the large aspect ratio of these films (Dhand et al., 2010). The integration of nanostructures and biomolecules may lead to new hybrid systems that couple the recognition or catalytic properties of biomaterials with attractive electronic and structural characteristics. The use of PANI nanostructures also helps to overcome the processibility issues associated with PANI.

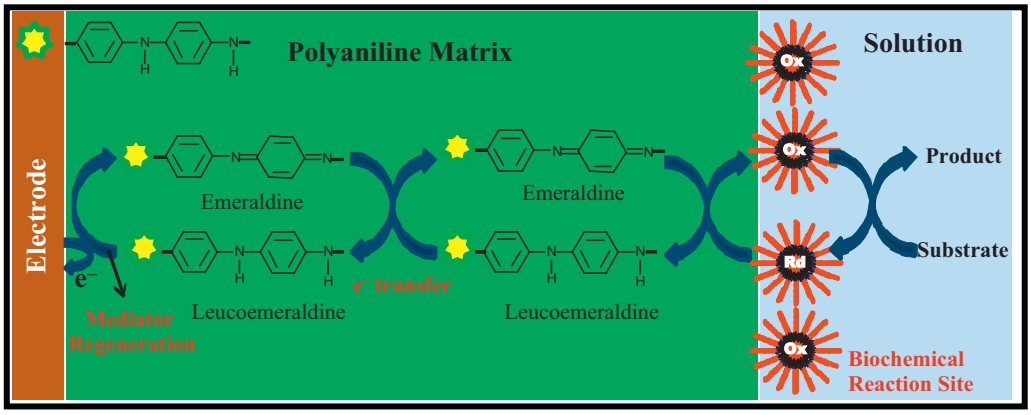

Fig. 4. Schematic of electron transfer from the biochemical reaction site to the electrode through conducting PANI network in an amperometric biosensor.

3. Classification of enzymatic biosensors based on PANI as immobilization platform

Enzymatic biosensors utilize the bio-specificity of an enzymatic reaction on an electrode surface that can be detected and quantified using various transduction techniques. The concept of enzyme based sensor devices was presented first by Clark and was realized as a commercial clinical analyser (Clark and Lyons, 1962). Most enzyme-based biosensors employ a class of enzymes known as oxido-reductases mainly oxidases and dehydrogenases. Their general reaction sequences can be described by the following reactions:

$$^{1}S_{red} + E_{ox} \rightarrow {}^{1}S_{ox} + E_{red} \quad (2)$$

$$^{2}S_{ox} + E_{red} \rightarrow {}^{2}S_{red} + E_{ox} \quad (3)$$

If GOx is the enzyme employed, then $^{1}S_{red}$, $^{1}S_{ox}$, $^{2}S_{ox}$, and $^{2}S_{red}$ correspond to glucose, gluconic acid, O_2 and H_2O_2, respectively. To make reaction (3) rate limiting, a large excess of $^{2}S_{ox}$, typically O_2, is required. Thus, the overall rate of reaction can be measured either by monitoring the rate of consumption of $^{2}S_{ox}$ (O_2) or the rate of formation of $^{2}S_{red}$ (H_2O_2). The advantage of the former approach is the relative ease of separation of O_2 from other electro-active species through the use of a gas-permeable membrane. The disadvantage of this approach is that it is more complicated, requiring two measurements (concentration of O_2 in the presence and absence of enzymatic reaction). Measurement of H_2O_2 formation has the advantage of being simpler, especially for small sensors, however, with the disadvantage that a number of *in vivo* endogenous species (ascorbate and urate) are electroactive at the applied potential required for H_2O_2 oxidation. Monitoring of peroxide reduction is also not possible because of the inability to find a potential at which peroxide, but not oxygen, is reduced.

It is apparent that two redox couples are necessary to carry out the enzyme-catalyzed redox reaction, one of which is the analyte. Thus, the other ($^{2}S_{ox}$) has to be introduced in some fashion. It is often desirable for biosensor to be "reagentless". This means that a clever method for delivering the second substrate (cofactor) must be devised or $^{2}S_{ox}$ must initially be present at sufficiently elevated levels that it does not affect sensor response. This is the reason for overwhelming preference for oxidases, because oxygen is often present at the required levels. If the enzyme belongs to dehydrogenase class, it will typically use NAD (nicotinamide adenine dinucleotide) as a cofactor. Either NAD or NADH is monitored electrochemically or by some other means.

$^{2}S_{ox}$ can be eliminated if the redox centre of the enzyme is coupled directly to the electrode, thus making the electrode the "sink" for electrons needed to complete Eq. (2). In other words there is a need to wire the redox centre of enzyme directly to an electrode with sufficiently high efficiency to prevent completion with O_2. The excellent conductivity and electrochemical behaviour of PANI makes it a suitable candidate to be used for electric wiring of enzyme with the electrode for direct ET and thus provides a non-diffusionally mediated redox active system.

3.1. PANI based peroxide biosensors

PANI based peroxide sensors can be fabricated by combining the exquisite selectivity of HRP with the excellent PANI stability to produce sensors with high sensitivity. Several PANI based peroxide sensors have been reported (Morrin et al., 2005a; Kathleen et al., 2006). Mathebe et al. (2004) have fabricated H_2O_2 biosensor by immobilizing HRP on electropolymerized films of PANI by making use of electrostatic interactions between PANI backbone and enzyme. This electrochemically active PANI matrix serves as an efficient non-diffusional mediator, shuttling electrons between redox active centre of enzyme and the electrode surface. Michira et al. (2007) have synthesized and characterized a novel anthracene sulfonic acid (ASA)-doped PANI nanomaterial and have explored its application for amperometric biosensor for H_2O_2 and erythromycin. The studies reveal that this self-doped PANI/ASA matrix acts as an effective electron mediator as it provides direct electrical communication between the enzymes (HRP, cytochrome $P_{450}3A_4$) and the platinum electrode. Further, the low apparent Michaelis–Menten constant (K_m^{app}) value of this bioelectrode is indicative of formation of stable enzyme–substrate complex (Michira et al., 2007). Wang et al. (2009a) have reported H_2O_2 biosensor constructed by cross-linking with HRP and PANI, electrochemically deposited in the presence of ionic liquid, using glutaraldehyde on F-doped tin oxide (FTO). Appropriate porosity configuration of this PANI matrix is found to enhance the stability of thus fabricated bioelectrode. The activation energy of HRP catalytic reaction for PANI–HRP biosensor is larger than that of native HRP, which will result in higher activity for the immobilized HRP at higher temperatures (Wang et al., 2009a).

Iwuoha et al. (1997) have examined amperometric behaviour of HRP in biosensor format where polyvinyl sulphonate (PVS) doped aniline is electropolymerized onto the surface of glassy carbon electrode (GCE). Biomolecules are then doped onto the polymer surface by electrostatic interactions with polymer backbone (Iwuoha et al., 1997). The influence of the thickness of PANI film deposited electrochemically onto screen-printed electrode (SPE) surface has been described in terms of its influence on a variety of amperometric sensor characteristics like time to reach steady state, charging current, catalytic current, background current and signal/background ratio (Kathleen et al., 2006). The sensitivity and limit of detection of polymer-based biosensor are dependent on the background current exhibited by polymer (Lyons et al., 1993). If the background current of a sensor based on these PANI/PVS films is too large, then the contribution from the reduction of H_2O_2 by HRP is difficult to measure. High background currents could limit the contribution from the enzyme catalytic reaction, thereby decreasing sensitivity of the sensor. Therefore, thinner polymer films are preferable, especially where lower levels of analytes are to be detected (Mu and Xue, 1996).

The good biocompatibility of PANI nanostructure enables it to become a simple and effective platform for the integration of proteins/enzymes and electrodes, providing analytical access to a large group of enzymes for a variety of bioelectrochemical applications. Morrin et al. (2005b) have described fabrication of uniform array of nanoparticulate PANI nodules using dodecylbenzenesulfonic acid (DBSA) as dopant for H_2O_2 detection using HRP as biosensing element. This effective biosensor format exhibits higher signal-to-background ratio and shorter response time. The authors show a comparison between the characteristics of the optimized nanoPANI/DBSA film with that of PANI/PVS film in terms of a platform for biosensing. The results indicate nanoPANI/DBSA film as a more efficient matrix in terms of protein immobilization and require concentration of protein six-fold lower than PANI/PVS layer for monolayer coverage. Luo et al. (2007) have prepared a highly nodular, cauliflower-like nanostructured electrode through the electropolymerization of aniline on SPE with subsequent deposition of polystyrene (PS) nanoparticle template and further growth of PANI around these templates. The high sensitivity of this biosensor is probably due to the unique nanostructure on the electrode surface, as the cauliflower-like nanostructured PANI can contact with more enzyme molecules and thus can be more efficient in assisting electron transfer from the enzyme to the electrode surface.

3.2. PANI based glucose biosensors

Diabetes mellitus is a worldwide public health problem and is reflected by blood glucose concentration higher or lower than normal range of 80–120 mg dL^{-1}. The diagnosis and management of diabetes mellitus thus requires a tight monitoring of blood glucose levels. Glucose biosensors account for about 85% of entire biosensor market. Such huge market size makes diabetes a model disease for developing new biosensing concepts. Wang et al. have recently reported a glucose biosensor based on direct ET of GOx entrapped electrochemically into the inner wall of highly ordered PANI-NT synthesized using anodic aluminium oxide (AAO) as template. The direct ET from the GOx to the electrode (Pt) is indicated by the generation of pair of well-defined symmetrical redox peaks in CV of GOx-PANI-NT/Pt. No redox peak is observed in PANI-NT/Pt electrode, suggesting that nano-PANI cannot undergo the redox reaction in this potential range. This biosensor exhibits advantages of ease of construction, enhanced electrocatalysis indicated by high sensitivity (97.18 ± 4.62 μA mM^{-1} cm^{-2}), fast response time (3 s), efficient preservation of enzyme activity, and effective discrimination to common interfering species (Wang et al., 2009b).

A glucose nanosensor based on conducting polymer/enzyme nanojunctions has been described by Tao et al. They have fabricated this nanojunction by bridging a pair of nanoelectrodes separated with a small gap (20–60 nm) with PANI/GOx. The signal transduction mechanism of the sensor is based on the change in the nanojunction conductance as a result of glucose oxidation in the polymer redox state. Due to the small size of nanojunction sensor, the enzyme is regenerated naturally without the need of redox mediator, and yields very fast response (<200 ms). These features make the nanojunction sensor potentially useful for *in vivo* detection of glucose (Forzani et al., 2004). A novel glucose biosensor, with high sensitivity and selectivity, has been fabricated by self-assembling GOx and Pt-DENs on nanofibrous PANI. The resulting biosensor exhibits excellent amperometric response to glucose and possesses biocompatible performance with the possibility of minimizing enzyme denaturing (Xu et al., 2008). Xian et al. (2006) have reported the electrical contacting of gold nanoparticles (Au NP) with the glassy carbon through PANI nanofibers which enables this nanocomposite structure to be used as an excellent matrix for electrocatalysis and enzyme (GOx) immobilization. Zhou et al. (2005) have reported results of similar studies using platinum (Pt) microparticles for glucose detection and found the bioelectrode to be highly stable and reproducible. Ramanathan et al. (1995) have utilized Langmuir Blodgett (LB) films of PANI for a glucose biosensor fabrication. Recently, Nemzer et al. (2010) have demonstrated the role of PANI both as host polymer and redox indicator for glucose oxidase. Thus, this smart material provides a biosensing route that eliminates the need of redox dyes during optical detection of glucose.

PANI-tosylate and PANI ferricynide films, prepared by self-ion-exchange in HCl, has been investigated by Verghese et al. (1998) for high GOx loading due to its significant change in the film porosity. The importance of this ion self-exchange technique has been used to develop reagentless PANI-based biosensor. Borole et al. (2004) have reported a comparative study of glucose sensor by entrapping GOx in PANI, poly (*o*-toluidine) (POT), and its co-polymer. The amperometric response reveals that PANI-GOx electrode is much more preferable for use because of the comparatively fast response. A new glucose biosensor has been developed by *in situ* electropolymerization of aniline into micro-porous PANI-coated platinum electrode in the presence of GOx. The technology combines the advantage of micro-porous polymer material and electropolymerization in biosensor construction. Thus prepared bioelectrode exhibits good selectivity, sensitivity, stability and shows no apparent loss of activity after 100 consecutive measurements and intermittent usage for 100 days (Xue et al., 2005). Inverse opals of PANI doped with poly(styrene sulphonate) (PSS) have been prepared by electropolymerization method by using silica spheres as sacrificial templates. Poly (diallyldimethylammonium chloride) (PDDA)/GOx multilayer has been self-assembled via self-assembly method in the hole's wall of as-synthesized ordered porous PANI for biosensing application. The inverse opal of PANI architecture offers a microenvironment for increasing the quantity of GOx that contacts the electrode directly, hence, facilitate direct ET between enzyme and electrode, leading to higher sensitivity and short response time (Yang et al., 2008).

Shi et al. (2004) have reported that poly (aniline-boronic acid) wires generated on double standard DNA (dsDNA) template could facilitate electrical communication between electrode and GOx reconstituted on polymer wires. However, the operating potential for detection of glucose at this electrode is +0.5 V (vs SCE) and at this potential many other electroactive species commonly coexisting in biological fluids, such as ascorbic acid, uric acid, and 4-acetamidophenol, can get oxidized and severely affect the selectivity of sensors. Zhao et al. (2009) have investigated PANI nanofibers as electrode material for direct ET of redox proteins/enzymes with GOx as a model. After immobilization on this nanofibre matrix, GOx remains in its natural structure and undergoes effective direct ET reaction with a pair of well-defined, quasi-reversible redox peaks. The electrode displays good features in electrocatalytic oxidation of glucose with good reproducibility and stability. Sukeerthi et al. have fabricated and compared the response of three different biosensor array structures of PANI; macro sensor (twin-wire electrode configuration) (Contractor et al., 1994), micro sensor (Sangodkar et al., 1996) and microtubular sensors (uses structure based on isoporous membranes) (Sukeerthi and Contractor, 1998); for glucose, triglyceride and urea. This microtubular sensor is found to have the highest sensitivity for all the three analytes. This enhanced sensing characteristic in case of PANI microtubules is attributed to shorter source-to-drain distance (10 μm) and more disordered structure of PANI that results in increased conductivity, transduction ability and supports effective enzyme loading.

Karyakin et al. (1999) have explored the advantages of PANI as potentiometric transducer for detection of bioanalytes like glucose, urea, etc. The potentiometric detection of enzyme activity is based on the measurement of change in pH in the enzymatic layer on sensor surface. The pH change is caused by oxidation of H_2O_2 produced by the enzymatic reactions (Eqs. (4) and (5)) for potentiometric detection of glucose.

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{Gluconic acid} + H_2O_2 \quad (4)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (5)$$

The chemically synthesized PANI shows reversible potentiometric response of approximately 90 mV per pH unit over the pH range from 3 to 9 (Hoa et al., 1992). Using the same fundamentals, Gaikwad et al. (2006) have fabricated potentiometric glucose biosensor by immobilizing GOx on electrochemically synthesized PANI film via glutaraldehyde. This bioelectrode is found to perform well in terms of dynamic range of detection and short response time. The same strategy has been used in urea sensor that exhibits detection limit of 10.5 mol L^{-1} (Karyakin et al., 1999). Urea detection can also be done via pH sensor based on PANI doped with tetraphenylborate (Pandey and Singh, 2001).

3.3. PANI based cholesterol biosensors

The alarming rise in the rate of clinical disorders such as heart disease, hypertension, arteriosclerosis, cerebral thrombosis, etc. due to abnormal levels of cholesterol in blood has stimulated pub-

lic concern. As biosensors have the ability to measure constantly the presence, absence or concentration of specific organic or inorganic substances in desired test specimens, the thrust to monitor cholesterol in blood is driving the need for development of efficient cholesterol biosensors.

Efforts are being made to fabricate reliable, accurate and efficient biosensors for cholesterol detection. Singh et al. (2006) have reported characteristics of PANI/ChEt/ChOx bioelectrode, fabricated by covalent immobilization of ChOx and ChEt on electrochemically deposited PANI matrix. This bioelectrode is found to detect cholesterol oleate in a wide concentration range (50–500 mg dL^{-1}) and has good thermal stability (46 °C). Wang and Mu (1999) have studied bioelectrochemical characteristics of ChOx immobilized onto an electrochemically deposited PANI film. These studies have revealed that the response current of the enzyme electrode at Triton X-100 concentration of 1% that is about twice as high as that at Triton X-100 of 5%. Khan et al. (2008) have fabricated cholesterol biosensor via covalent immobilization of ChOx on electrochemically prepared PANI film in presence of TX-100 (non-ionic surfactant). The incorporation of TX-100 in PANI has been found to result in improved biocompatibility and provide better conformation for effective immobilization of ChOx and resulting in high sensitivity and low K_m^{app} value.

LB films have been used for the fabrication of electrodes for cholesterol sensing applications, as the ordered arrangement of molecules and thickness at nanoscale are likely to result in highly sensitive sensor with ultra-fast response (Ohnuki et al., 2007). Moreover, use of the LB technique provides an opportunity to fabricate biosensors at molecular level (Zasadzinski et al., 1994). Matharu et al. (2007) have covalently immobilized ChOx onto LB monolayer of PANI-stearic acid (SA) prepared onto ITO coated glass plates via glutaraldehyde chemistry. It has been reported that these well-aligned, uniformly packed polymer chains (in PANI-SA LB film) act as molecular wires resulting in better electron-accepting behaviour over molecular oxygen from the reduced enzyme. Dhand et al. (2007) have reported the fabrication of nanostructured PANI film using novel electrophoretic technique for application to cholesterol biosensor. These nanostructured PANI derived bioelectrodes (ChOx/PANI/ITO) are found to detect cholesterol with good sensitivity, selectivity and very low K_m^{app} value revealing the importance of this electrophoretically deposited nanostructured matrix to enhance the enzyme (ChOx)–substrate (cholesterol) interactions. Covalent immobilization of ChOx onto polyaniline nanospheres (PANI-NS) prepared by morphological transformation of micelle polymerized camphorsulphonic acid (CSA) doped polyaniline nanotubes (PANI-NT) has recently been reported by Dhand et al. for cholesterol detection. Thus prepared ChOx/PANI-NS/ITO bioelectrode can detect cholesterol in wide concentration range with sensitivity as 1.3×10^{-3} mA mg^{-1} dL. Further, this PANI-NS based bioelectrode shows fast response time (10 s), low K_m^{app} (2.5 mM) and shelf-life of 12 weeks (Dhand et al., 2010).

3.4. Other enzymatic biosensor based on PANI

An impedimetric triglyceride biosensor has been developed by covalent immobilization of Lipase (LIP) onto electrophoretically deposited CSA doped PANI-NT film. A linear decrease in the R_{CT} value has been observed with increased tributyrin concentration and is assigned to the fact that LIP attached on LIP/Glu/PANI-NT/ITO bioelectrode hydrolyse tributyrin, leading to the generation of proton and fatty acid in addition to glycerol, resulting in decreased solution pH. The lowering of pH results in increased positively charged quinoid moieties on PANI-NT backbone due to high H^+ ions concentration resulting in enhanced charge transfer rate leading to decreased R_{CT} value. This PANI-NT based bioelectrode shows fast response time (20 s), high sensitivity (2.59×10^{-3} kΩ^{-1} mg^{-1} dL), and shelf life of upto 10 weeks (Dhand et al., 2009).

Bayramoglu et al. (2009) have proposed a new conductive fiber support based on PAN/PANI for immobilization of invertase enzyme. This study offers a novel and economical protocol for reversible enzyme immobilization on conductive composite fiber. An amperometric superoxide microbiosensor has been developed by electropolymerization of aniline with concomitant doping of superoxide dismutase on the surface of Pt electrode. The amperometric response to superoxide anion is obtained at a potential of 0.75 V for oxidation of H_2O_2 generated from disproportionation of superoxide anion (Kim and Jeon, 2001). A novel sensitive and stable amperometric phenol biosensor has been developed by co-entrapping PPO with *in situ* polymerization of PANI in PAN matrix. This biosensor can be used to detect benzoic acid as low as 2×10^{-7} M revealing high enzyme loading (Shan et al., 2007).

PANI has two redox couples associated with the polymer chain. Changes in both protonation and its oxidation state induce a marked change of its optical spectra in the visible region (Grummt et al., 1997). Some spectral changes also occur in infrared region and are described as very useful for sensor development, due to the fact that no biological materials could interfere in that region (Piletsky et al., 2000). Bossi et al. (2000) have reported development of the PANI-based assay in microplate format for the analysis of AA. Castelletti et al. (2002) have developed a capillary electrophoresis (CE) biosensor for measuring AA by coupling a PANI optical sensor and CE. Langer et al. (2004) have developed a choline sensor by immobilization of choline oxidase (ChO) to nanoporous PANI layers.

NADH is an important coenzyme that takes part in a number of dehydrogenase enzymatic reactions. They are known to play a key role in developing amperometric enzyme sensors that use dehydrogenase-dependent enzymes. However, direct oxidation of NADH at bare electrode in neutral environment normally requires high overpotential upto 1.0 V (Moiroux and Elving, 1978). Consequently, different redox mediators have been used to reduce overpotential for NADH oxidation. Among these, PANI has been found to be one of the suitable candidates. Bartlett et al. (1997) have reported successful oxidation of NADH at PANI coated electrode and have carried out kinetic analysis of the redox reactions involved in this process. In this work, the electropolymerization of PANI has been accomplished in presence of PVS counter ions that give rise to films that are both stable and electroactive at pH 7. These films have been shown to have efficient electrocatalytic surfaces for oxidation of NADH at 0.1 V vs. SCE and are both reproducible in their responses from film to film and exhibit good stability. Using this characteristic of PANI, Gerard et al. (1999) have fabricated pyruvate biosensor by immobilizing lactate dehydrogenase (LDH) on electrochemically prepared PANI film. The biosensing studies conducted on this bioelectrode reveal the response time as 90 s and shelf-life as about two weeks.

4. Classification of bioaffinity sensors based on PANI as immobilization platform

A bioaffinity sensor consists of a bioaffinity reagent in close proximity to the transducer. Measurement of a target analyte can be achieved by selectively converting molecular recognition occurring at analyte sensor interface from a nonelectrical domain to an electrical signal. Several bioaffinity reagents can be used to fabricate a sensor, including receptors, binding proteins, antibodies and nucleic acids immobilized on surface of transducer. The binding of analyte is detected through change in physical property at the sensing surface. High protein loading and stability, direct and intimate

contact with the bioaffinity reagents, and the modulation of analytical signals through the application of electrical potentials are some of features that make CP as suitable candidate for fabrication of bioaffinity sensors.

The basis for using PANI in sensing lies in its ability to be reversibly oxidized and reduced through the application of electrical potential. Electrical conduction in PANI is achieved through the formation of defects in the polaronic (one hole state) and bipolaronic forms (two hole state) (Stafstrom et al., 1987). When exposed to analytes, stoichiometry of the existing PANI energy levels is disturbed through the substitution of different species into the polymer lattice. This causes a change in the electrical conductivity. In other words, the interactions between the proteins, mainly negatively charged at neutral pH, and the delocalized positive charges along the polymer chains induce changes in the capacitance of the material. Such interactions are perhaps the basis of bioaffinity signal.

4.1. Polyaniline based DNA biosensors

Recently, the development of systems allowing DNA detection is motivated by applications in many fields: DNA diagnostics, gene analysis, fast detection of biological warfare agents, and forensic applications. There is thus a greater need for sensitive, accurate, rapid, and user-friendly approaches for gene analysis and detection. DNA biosensor (genosensors) and DNA microarrays (DNA chips) relies on the immobilization of a single-stranded DNA (ssDNA) probe onto a surface to recognize its complementary DNA target sequence by hybridization. Transduction of DNA hybridization can be measured optically, electrochemically, or using mass-sensitive devices. Various transducer surfaces comprising of graphite pencil electrodes (Wang and Kawde, 2001), gold electrodes (Kerman et al., 2003), polypyrrole (Wang et al., 1999), etc. have been reported for hybridization detection. However, formation of PANI–DNA complex has recently been demonstrated (Sergeev et al., 2003). Arora et al. (2007) have reported the fabrication of ultrasensitive BdC–avidin–PANI and BdE–avidin–PANI genoelectrodes specific for detection of *Escherichia coli*. These bioelectrodes are found to electrochemically detect and differentiate the presence of sub-femto molar complementary and non-complementary target sequences present in the sample.

DNA hybridization has been demonstrated onto ssDNA probe immobilized onto thin layer of PANI/PAA coated onto boron-doped diamond substrate using cyclic voltammetry (Gu et al., 2004). It has been reported that the ssDNA-modified PANI-intercalated graphite oxide nanocomposite onto carbon paste electrode can be used to monitor DNA hybridization with complementary ssDNA in the linear range of 275–551 $\mu g\,mL^{-1}$ and detection limit of 29.34 $\mu g\,mL^{-1}$ using square wave voltammetry (SWV) within 30 min (Wu et al., 2005). PANI nanowires have been fabricated onto Si substrates containing fully stretched DNA as growing templates using HRP enzyme (Ma et al., 2004). A recent report suggests that PANI nanowires electrochemically deposited onto GCE using electrochemical DNA hybridization show detection limit of 1.0×10^{-12} mol L^{-1} of complementary DNA with methylene blue as indicator (Zhu et al., 2006). Singh et al. (2009) have developed highly sensitive and specific nucleic acid functionalized nanostructured-PANI electrode for *Gonorrhoea* detection. This sensor shows improved detection limit of 0.5×10^{-15} M within hybridization time of 60 s. Prabhakar et al. (2008a) have reported nucleic acid sensors for hybridization detection with DNA and peptide nucleic acid (PNA) probes specific for *Mycobacterium tuberculosis* immobilized on electrochemically polymerized PANI coated onto gold electrode. The PNA–PANI/Au electrode exhibits improved specificity (1000 times) and detection limit (0.125×10^{-18} M) as compared to that of DNA–PANI/Au electrode (2.5×10^{-18} M).

4.2. Polyaniline based immunosensors

An effective combination of antibody–antigen specificity with transducer in a biosensor device could provide the basis of direct detection for a wide range of analytes with high selectivity and sensitivity. This analytical device works on changes in mass, heat, electrochemical, or optical properties. Sai et al. (2006) have reported a protocol for covalent immobilization of human IgG on PANI using glutaraldehyde as cross-linker and utilized for development of a piezoelectric immunosensor. This piezoelectric biosensor developed for detection of IgG is capable of differentiating the target analyte concentrations between 500 $ng\,mL^{-1}$ and 25 $\mu g\,mL^{-1}$ with nonspecific binding of ~10%. Karir et al. (2006) have employed a new method of preactivating the surface of polystyrene (PS) tubes with a layer of PANI and reveal its effect on immobilizing antibodies for use as a solid phase in a T_3 immunoassay. AFM analysis shows enhanced roughness in the case of PANI modified surface as compare to unmodified one, allowing more adsorption of antibodies to the surface.

Tsekenis et al. (2008) have described the development and characterization of PANI based label-less immunosensor for myelin basic protein (MBP) and its interrogation using an ac impedance protocol. A logarithmic relationship between the MBP concentration and impedimetric response is observed. Crombrugghe et al. (2008) have reported acrylic acid grafting on PANI surface to obtain carboxylic groups. These carboxylic functionalities are then used for covalent binding of ICHA antigen by EDC-NHS chemistry and are compared with direct physical adsorption of antigen. Considerable enhancement in the antigen binding is observed in the case of acrylic grafted PANI. A reagentless capacitive immunosensor, based on capacitance change of parallel plate capacitor (PPC), has been developed by covalently immobilizing anti-human IgG (anti-HIgG) on electrophoretically deposited nanostructured PANI film. The capacitance measurements indicate that dielectric medium of this biologically modified PPC (anti-HIgG/PANI/ITO) is sensitive to HIgG in $5–5 \times 10^5$ $ng\,mL^{-1}$ range and has detection limit of 1.87 $ng\,mL^{-1}$ (Bandodkar et al., 2009). An electrochemical impedance immunosensor has been developed for monitoring of intermolecular interactions between anti-human-AFP monoclonal IgG and α-fetoprotein (AFP) via electrochemically entrapping the antibody within the PANI matrix. The resulting immunosensor has a linear dynamic range of 200–800 $ng\,mL^{-1}$ of AFP (Miao and Guan, 2004).

For the point-of-care examination, an immunochromatographic assay system has been investigated by utilizing colloidal gold with PANI bound onto the metal surface as signal generator. Although gold is a widely used label for antibodies to produce colorimetric signals, the tracer does not lend itself for a suitable electric conduction along the gold particles due to the presence of protein barriers (e.g. immunoglobulin and blocking agent) against electron transfer. To overcome this problem, PANI has been introduced as a conductivity-modulating agent on gold surface after immobilizing an antibody specific to human albumin used as model analyte. This novel signal generator amplifies the conductometric signal 4.7 times as compared with plain gold (Kim et al., 2000). The acceptability of different PANI, having different oxidation states and molecular weight, and selectivity and sensitivity of sensor for IgG detection has also been studied (Sergeyeva et al., 1996).

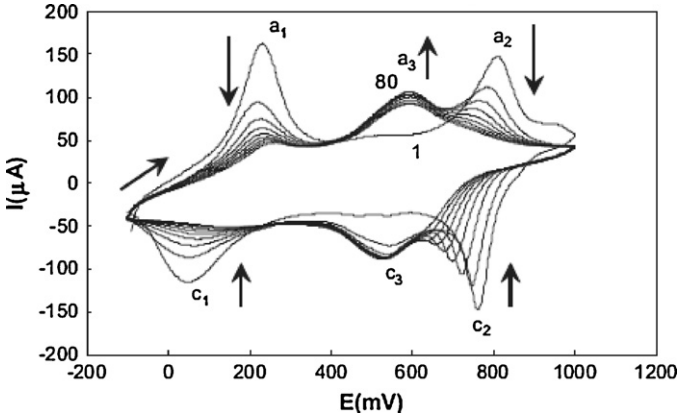

Fig. 5. Typical CVs of electrochemical degradation process of PANI/Cl^- deposited on Pt electrode.

5. Other biosensors based on PANI

PANI has been utilized for detection of insecticides, pesticides and toxicants present in the environment and biological samples. A nucleic acid sensor based on PANI has been fabricated by covalently immobilizing dsCT onto perchlorate (ClO_4^-) doped PANI film for the insecticide (cypermethrin/trichlorfon) detection. This disposable dsCT-DNA-PANI-ClO_4/ITO bioelectrode, stable for about 4 months, can be used to detect cypermethrin (0.005 ppm) and trichlorfon (0.01 ppm) in 30 and 60 s, respectively. The mechanism of interaction of insecticides with dsCT-DNA has also been proposed (Solanki et al., 2008). For the detection of organophosphorus pesticides, the entrapment of dsCT-DNA has been done in PANI-PVS film deposited onto ITO coated glass plate. These dsCT-DNA entrapped PANI-PVS/ITO bioelectrodes have been found to have response time of 30 s, stability of about 6 months and detection limit for chlorpyrifos and malathion as 0.5 ppb and 0.01 ppm, respectively (Prabhakar et al., 2008b). Malhotra et al. (2008) have fabricated a disposable dsCT-DNA-PANI/ITO bioelectrode to detect arsenic trioxide (0.1–10.0 ppm) within 10 s. Characteristics of the various PANI based biosensors reported in literature have been summarized in Supplementary Table 2.

6. Commercialization and challenges

A large number of reports are available related to the fabrication of PANI based electrode for biosensor application, though its commercialization is still a challenge. This is due to the aging effect, low electrochemical stability of PANI and lack of well optimized deposition techniques. PANI consists of conducting grains of ES-I separated by thin insulating barriers. These grains are composed of crystallites surrounded by "paracrystalline" and "amorphous" ES-I (Stafstrom et al., 1987). During the aging process, ES-I phase (conducting phase) progressively transforms into the insulating F phase which is first initiated at the periphery of grains and then expands towards the centre of the grains in a diffusion like manner. This results in continuous decrease in conductivity of PANI, which in turn influences its charge transfer properties and thus electrochemical stability (Wolter et al., 1998). Moreover, from chemical view point, the formation of damaged phase appears because of the oxidation of polymer backbone due to presence of O_2. This damaged phase appears as an additional peak around 0.5 mV between two main set of peaks around 0.2 V (associated with the oxidation of the leucoemeraldine to emeraldine) and 0.8 V (associated with oxidation of the emenalding to pernigraniline) during CV analysis of PANI (Fig. 5). This extra peak is due to the oxidation/reduction of soluble electrochemical degradation products, including p-benzoquinone (PBQ). This peak (a_3) increases slowly with increasing number of cycles at the cost of characteristics of PANI (0.2 (a_1) and 0.8 V (a_2)) as shown in Fig. 5 which is indicative of slow electrochemical degradation of PANI. The formation of PBQ initiates cross-linking reaction between linear polymer chains which interrupts the delocalization of both the charge and electrons along the polymer chain leading to the decrease in conductivity and charge transfer of PANI (Rahmanifar et al., 2006).

7. Conclusions

This review has described how the synchronization of various remarkable characteristics of PANI like controllable conductivity, charge transfer capability, good processibility, environmental stability, mechanical flexibility, etc. makes this matrix a novel platform for fabrication of variety of biosensors. Efforts have also been made to unravel the implications of PANI nanostructures (nanospheres/nanotubes/nanofibers) for electric wiring of biomolecules with the electrode for direct ET and thus providing a non-diffusionally mediated redox active system to generate mediator-free third generation biosensors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.10.017.

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