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Poly(*o*-anisidine)–anion composite films as sensing platform for biological molecules

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Abstract

Polyanisidine films doped with two different anions, viz., perchlorate and paratoluene sulphonate anions are prepared electrochemically on a gold substrate. Polyanisidine films doped with the anions aid in retaining the redox conductivity of the polyanisidine films. This behaviour is similar to that of polyaniline films. Unlike polyaniline films, even smaller ions like perchlorate and paratoluene sulphonate are sufficient for retaining the redox conductivity of the film, which may be primarily due to the compact, non-permeant films formed during electropolymerization. The films are found to be suitable for the immobilization of glucose oxidase enzyme and are found to be an efficient sensing matrix for glucose. The films also exhibited catalytic activity towards the oxidation of NADH. These studies indicate the usefulness of polyanisidine–anion films as sensing platform for biomolecules.

Keywords: Poly(*o*-anisidine); Electrochemical polymerization; Effect of anions; Biomolecular sensing; Glucose; NADH

1. Introduction

Molecular engineering of enzymes on electrode surfaces is a fascinating area of research due to its potential applications in biotechnology and biosensors. Preparation of a stable sensing platform containing the biocatalyst (enzyme or microorganism), without the loss of its biocatalytic activity, is the prerequisite for the fabrication of a biosensor. Thus, deposition of enzyme(s) in ordered mono- or multi-layers assumes vital significance in the design of biosensors and bioelectronic devices. For immobilizing biocatalysts on electrode surfaces, there are many methods, which are based on, for example, Langmuir–Blodgett films [1]; self-assembled monolayers (SAM) [2–4] or multilayers [5,6], antigen–antibody interactions [7,8], avidin–biotin interactions [9], surfactant films [10], electrostatic adsorption of hyper-branched polyelectrolytes [11] and redox dendrimer layers [12]. However, these films, except SAMs, are not stable enough because they were constructed mainly based on the electrostatic or weak inter-molecular interactions. On the other hand, polymer films can be used as matrices to immobilize enzymes without loss of their electrocatalytic activity. Electrochemical polymerization is a preferred methodology for forming the sensing matrix of a biosensor. The polymeric film resulting from electropolymerization of a monomer, which is itself a redox mediator, generates high catalytic currents in electro-enzymatic reactions. The glucose biosensors based on the non-conducting poly(*o*-phenylenediamine) [13,14], poly(ethacridine) [15], overoxidized polypyrrole [16], poly(phenol) [17] and poly(*o*-amino phenol) (POAP) films [18,19] have showed good selectivity.

The non-conducting polymers, due to their good selectivity and fast response, have attracted much interest in the development of the biosensors. Although non-conducting polymer films exhibit excellent selectivity properties, their use is still a matter of concern because of their relative high detection limit and the low response current. To increase the current response, ferrocene, an electron transfer mediator has been used in the biosensor based on non-conducting polymer films of phenol and its derivatives and an enhanced amperometric current response for hydrogen peroxide has been observed [19].

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It is well known that for many technological applications, it is desirable to prepare polyaniline (PAni) thin films. PAni-based films can be deposited by electrochemical polymerization, making them ideal for sensing platforms. The PAni films are also electronically conducting, ensuring rapid propagation of charge through the film, which is advantageous for the construction of bioelectronic devices. However, PAni films, losing their conductivity at neutral pH is a major problem that has to be overcome. The conducting emeraldine form of PAni deprotonates at around pH 5, forming the insulating emeraldine base. Interestingly, it has been shown [20] that the pK_a of the emeraldine form can be shifted to pH 7 or 8 by incorporation into the PAni film of polymeric counter anions, such as poly(vinylsulphonate) or poly(acrylate) in place of small anions, such as chloride or bisulphate. This is because for the small anions, deprotonation of the emeraldine state occurs with loss of both the proton and the anion from the film. In contrast, in PAni–poly(anion) composite films, deprotonation can occur only if the loss of the proton is coupled to the ingress of another cation into the film because the polyanion is itself trapped inside the film [20]. It is important to increase the processability of the PAni by using substituent groups in the monomer or in the polymer backbone. The substituted polyanilines offer greater processability for application in electronic and optical device technologies. Substituent groups (–CH_3, –OCH_3, –OC_2H_5, etc.) in the monomer or the polymeric chain of polyanilines decrease conductivity due to a significant increase in electronic localization but result in enhanced processability due to increased solubility in organic solvents.

In this work we have used the monomer, *o*-anisidine(*o*-methoxy aniline) to form a polymer matrix for the immobilization of glucose oxidase. *o*-Anisidine undergoes electropolymerization to yield compact nonporous films and in the presence of dopant anions, the redox activity can be retained in the film. Hence, poly-*o*-anisidine (POA) films have good selectivity associated with the non-conducting polymers and significant redox activity due to dopant anions and thereby preclude use of any independent redox mediators for the enzymatic sensing.

Anisidine (*o*-methoxy aniline) is a monomer that has not yet been explored for the fabrication of biosensors. However, limited attempts have been made to polymerise anisidine to prepare corrosion resistant coatings [21] and to fabricate a potentiometric sensor for K^+ ion [22]. Composite films of anisidine with nafion, carbon nanotubes and TiO_2 have been prepared and evaluated for their conducting properties to explore their use as biosensing platforms [23–25]. But these reports do not mention sensing of any analyte by the above composite films. Unlike aniline, anisidine yields non-conducting, polymeric films even in acidic state as the –OCH_3 substituent decreases the conductivity of the polymer. It has been shown for the first time in this work that doping of anions leads to increase in the redox activity of POA films similar to that of PAni films. Since highly compact films are formed, even small anions are trapped inside the film. Even in presence of small dopant anions like perchlorate and *p*-toluene sulphonate (PTS), the film exhibits good redox conductivity. The redox activity of the emeraldine base is found to be shifted to neutral pH. Further, the non-conducting and compact nature of the film makes it less prone for interferences. Thus, the electropolymerized film of *o*-anisidine offers itself as an ideal matrix for immobilization of enzymes. In this communication, we discuss the electropolymerization of *o*-anisidine doped with PTS and perchlorate and the utility of the POA film as a sensing platform for NADH. Moreover, the POA film was immobilized with glucose oxidase and then evaluated for its response to glucose.

2. Experimental

2.1. Reagents

o-Anisidine (OA) was purified by distillation before use and the following chemicals were used as received. Glucose oxidase (GOx) (Sigma), β-(D)-glucose (Sigma), NADH (Acros Organics), $LiClO_4$, *p*-toluene sulphonic acid (PTSA). Buffer solutions were prepared using Millipore water.

2.2. Apparatus

Cyclic voltammograms (CV) were recorded using either the Advanced Electro-chemical System, PARSTAT 2263 or a Wenking Potentiostat LB75M coupled to a scan generator VSG 72 and a X-Y/t recorder (Rikadenki). A gold disk electrode ($\varphi = 3$ mm) was used as the working electrode. Pt foil and a calomel electrode (1N KCl) were used as counter and reference electrodes, respectively. Scanning electron micrographs (SEM) were recorded using the instrument Hitachi S3000H.

2.3. Fabrication of composite electrodes

Electropolymerization of OA was carried out in 10 ml of buffer containing 30 μl OA by sweeping the potential between −0.5 V and 0.8 V (vs. normal calomel electrode) for 15 cycles on a gold disk electrode ($\varphi = 3$ mm). The polymerization was carried out in phosphate buffer (pH 6.5) and acetate buffer (pH 5.0). The dopants used during polymerization are $LiClO_4$ and PTSA (1% by weight). The polymer-modified electrodes are referred to as POA–ClO_4 and POA–PTS composite electrodes, respectively depending on either perchlorate or *p*-toluene sulphonate (PTS) used for doping.

Immobilization of GOx was carried out by electropolymerizing OA from either phosphate or acetate buffer solution containing GOx (1 mg/ml) and any one of the dopant anions. The modified composite electrodes thus prepared are referred to as POA–ClO_4–GOx and POA–PTS–GOx electrodes.

3. Results and discussion

Fig. 1 depicts the cyclic voltammograms (CV) recorded during the electrochemical polymerization of OA from neutral phosphate buffer solution containing (a) no dopant anion and (b) PTS anions, as dopant. In the absence of dopant anions, the CVs are featureless. In presence of PTS anions, there are two pairs of redox peaks. The first pair of peaks (I_a 0.21 V and I_c 0.17 V) corresponds to emeraldine–leucoemeraldine and the second pair (II_a 0.38 V and II_c 0.36 V) to pernigraniline–emeraldine

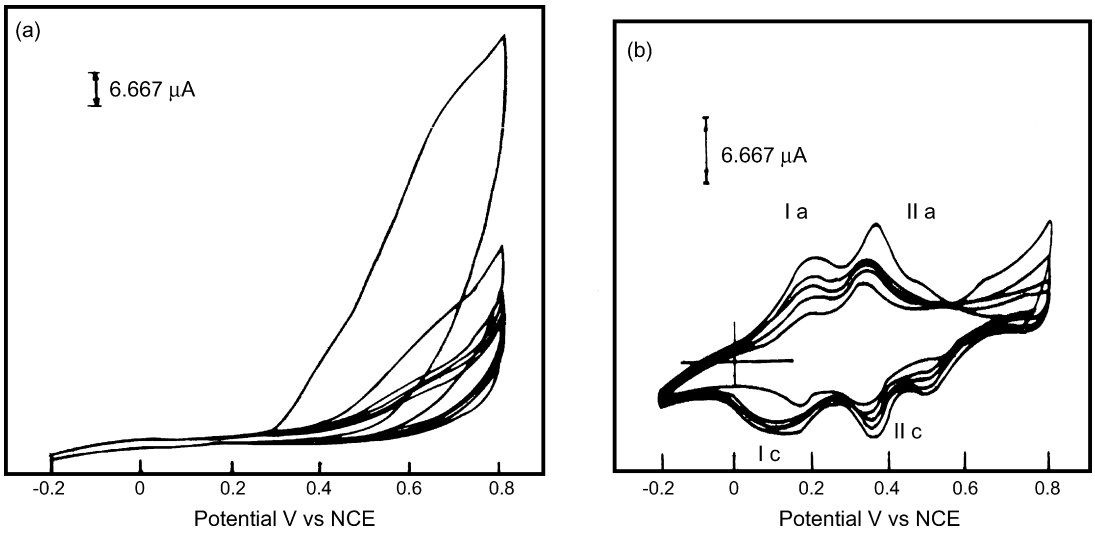


Fig. 1. Cyclic voltammograms showing the electropolymerization of OA from phosphate buffer (10 ml; pH 6.2) containing 30 μl of OA. (a) No dopants and (b) in presence of PTS anions (1%). First, 2nd, 3rd, 5th, 17th and 18th cycles are shown in (a) and 1st, 3rd, 5th, 10th and 20th cycles are shown in (b). Scan rate: 50 mV/s.

redox transformations. This shows that the electropolymerization behaviour of OA is similar to that of aniline. Further, the electrode modified with PTS-doped POA film, when subjected to voltammetric scan in neutral phosphate buffer exhibits only one pair of redox peaks (cf. Fig. 2b), which do not correspond to any of the two redox peaks that were observed during the electropolymerization process. This behaviour is analogous to that observed for PAni film in neutral buffer and the pair of redox peaks, as in the case of PAni films, could be attributed to the merger of the two pairs of redox transitions mentioned above.

Interestingly, in acidic solution, the POA–PTS electrode shows only one pair of redox peaks (I_a and I_c) and an irreversible anodic peak (II_a) (cf. Fig. 5a). However, in acidic solutions also, the PAni film exhibits two pairs of redox peaks [26,27].

When ClO_4^- is used as the dopant, only one pair of quasi-reversible peaks is observed during electropolymerization of OA. The POA–ClO_4 electrode when subjected to voltammetric scan in neutral phosphate buffer exhibits only one pair of redox peaks (cf. Fig. 2a). In acidic solution, only one pair of quasi-reversible redox peaks is observed during electropolymerization and also during the voltammetric scan of the modified electrode in buffer solution.

The behaviour of POA films is marginally different from that of PAni. It is known [26,27] that at highly acidic pH, PAni exhibits two sets of redox peaks. The more cathodic pair is due to leucoemeraldine–emeraldine redox transformation and second one corresponds to redox exchange between emeraldine and pernigraniline forms of PAni. As pH increases, the peaks merge and a single redox pair is observed. Polyaniline exhibits redox conductivity even in the absence of dopants. However the redox conductivity is lost at higher pH. The conducting emeraldine form of PAni deprotonates at around pH 5, forming the insulating emeraldine base. Interestingly, it has been shown [20] that the pK_a of the emeraldine form can be shifted to pH 7 or 8 by incorporation into the PAni film of polymeric counter anions, such as poly(vinylsulphonate) or poly(acrylate) in place of small anions, such as chloride or bisulphate. This is because for the small anions, deprotonation of the emeraldine state occurs with loss of both the proton and the anion from the film. In contrast, in PAni–poly(anion) composite films, deprotonation can occur only if the loss of the proton is coupled to the ingress of another

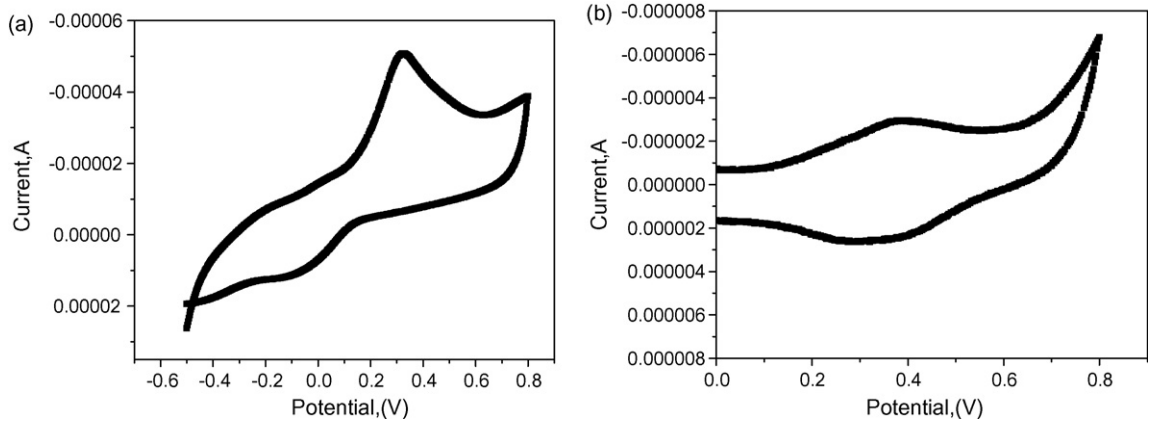


Fig. 2. Cyclic voltammograms representing the response of (a) POA–ClO_4 and (b) POA–PTS electrodes in phosphate buffer (pH 6.2). Scan rate: 50 mV/s.

cation into the film because the polyanion is itself trapped inside the film. Similar observation is seen in the case of POA also.

Fig. 2 depicts the CV of the POA–ClO_4 and POA–PTS electrodes in phosphate buffer recorded at 50 mV/s. Quasi-reversible peaks appear at 0.327 V (anodic) and −0.118 V (cathodic) for the perchlorate-doped film while in the case of PTS-doped film, the peaks appear at 0.376 V and 0.318 V. As pH of the electrolyte decreases, the reversibility increases. This is the behaviour normally expected for a PAni-based polymer. OA is essentially aniline substituted with a methoxy group and its polymerization behaviour is similar to that of aniline. The above pair of peaks corresponds to emeraldine–leucoemeraldine redox transformation.

Fig. 3 shows the CV of the POA–ClO_4–GOx electrode and its catalytic effect on glucose oxidation in acetate buffer (pH 5.0). The peak current increases with increasing concentrations of glucose and the peak potential shifts anodically with concentration. The increase in anodic peak current followed by a decrease in the cathodic peak current indicates the reaction is catalytic in nature. The electrode can be reused for two to three trials without much change in the current.

Fig. 4 represents the CV of the POA–ClO_4–GOx electrode and its catalytic effect on glucose oxidation in phosphate buffer (pH 6.2). With increase in concentration of glucose, the catalytic current increases; however, the increase is relatively smaller than that noticed at pH 5.0. Since the pK_a of GOx is 4.5, the enzyme moieties tend to remain negatively charged in both buffer solutions. At the same time, the OA molecules undergo protonation in both the buffer solutions and in acetate buffer it will be relatively more positively charged. Hence due to electrostatic interactions, it is likely that a less amount of enzyme is getting immobilized at a higher pH, which results in a lower catalytic current at pH 6.2.

Fig. 5 shows the CV of the POA–PTS–GOx electrode in acetate buffer (pH 5.0) containing glucose. The curve a depicts the CV response in the absence of glucose, while curves b, c and

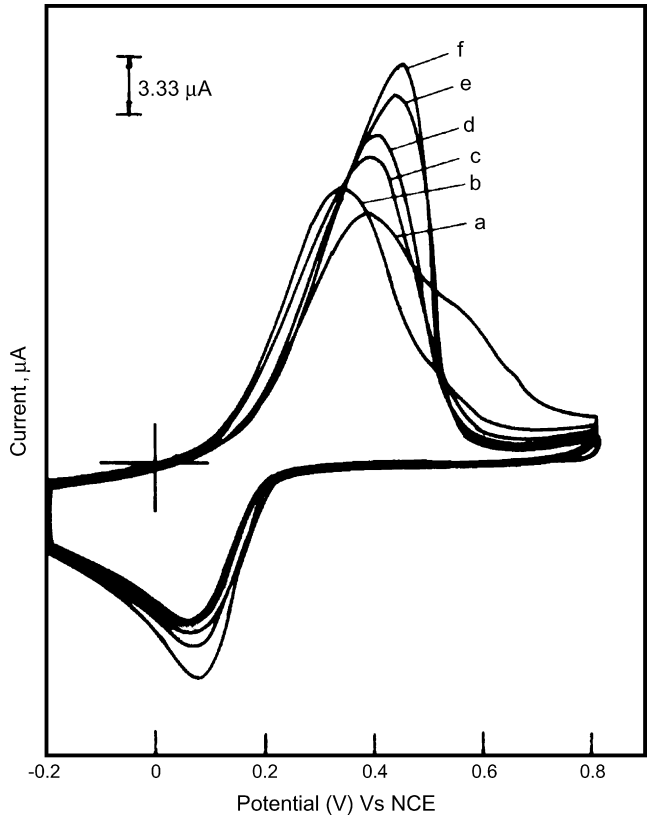


Fig. 3. Cyclic voltammograms for the oxidation of different concentrations of glucose at POA–ClO_4–GOx electrode in acetate buffer (pH 5.0): (a) blank, (b) 2.5 mM, (c) 5.0 mM, (d) 7.5 mM, (e) 10.0 mM and (f) 12.5 mM. Scan rate: 50 mV/s.

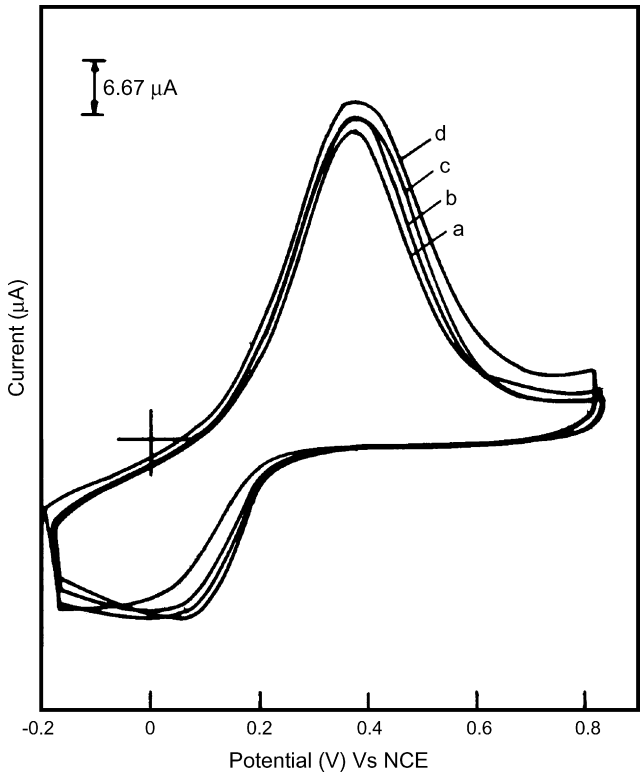


Fig. 4. Cyclic voltammograms for the oxidation of different concentrations of glucose at POA–ClO_4–GOx electrode in phosphate buffer (pH 6.2): (a) blank, (b) 2.5 mM, (c) 5.0 mM and (d) 7.5 mM. Scan rate: 50 mV/s.

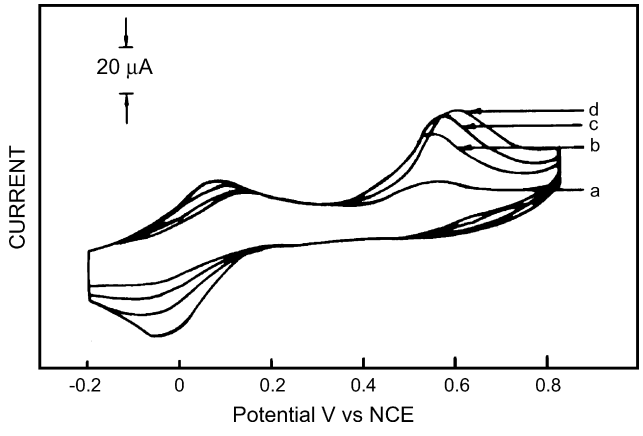


Fig. 5. Cyclic voltammograms depicting the response of POA–PTS–GOx electrode in acetate buffer (pH 5.0) by the additions of glucose. Scan rate: 50 mV/s. The concentration of added glucose at (a) 0 mM, (b) 2.5 mM, (c) 5.0 mM and (d) 7.5 mM.

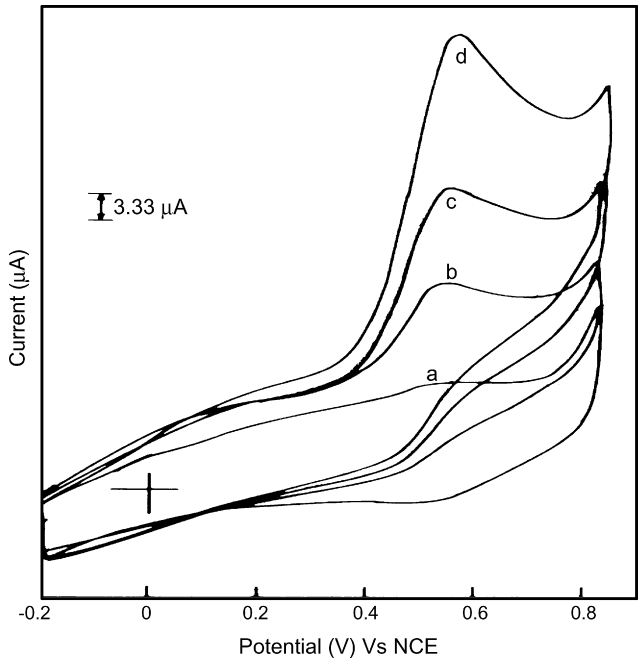


Fig. 6. Cyclic voltammograms for the electrocatalytic oxidation of NADH on a POA–PTS electrode in phosphate buffer (pH 6.2). Concentration of NADH at (a) 0 mM, (b) 1.25 mM, (c) 2.5 mM and (d) 7.5 mM. Scan rate: 50 mV/s.

d correspond to voltammograms resulting from successive additions of 2.5 mM each of glucose. It can be seen that compared to POA–ClO_4–GOx electrode, the redox peak for POA–PTS–GOx electrode occurs at a lower potential (0.08 V) and hence the electrocatalysis of glucose oxidation is more favourable at the latter electrode. An irreversible peak appears at 0.530 V which is likely to be due to Emeraldine base (50% oxidized form) of the polymer. The added glucose undergoes oxidation at this potential also.

Fig. 6 depicts the CV for the catalytic oxidation of NADH on POA–PTS electrode in phosphate buffer (pH 6.2). NADH is oxidized at around 0.52 V, which is distinctly cathodic compared to the oxidation potential for NADH on a bare Au electrode. Higher catalytic currents compared to literature reports indicate the facile kinetics of oxidation at the POA–PTS electrode. On a bare electrode, NADH oxidation proceeds at high overpotentials and leads to fouling of the electrode surface. This can be understood because the one-electron oxidation product, $NADH^{\bullet+}$ is difficult to form and highly unstable. Moreover, no fouling is observed on the modified electrode after NADH oxidation.

Fig. 7a shows the amperometric response of the POA–PTS electrode to oxidation of NADH. The concentration of NADH in the electrolytic medium is increased with successive additions of 1.25 mM of NADH each. With each addition, a nearly proportionate increase in current is observed, thereby indicating the capability of the modified electrode for sensing NADH. Fig. 7b shows the calibration curve for the amperometric estimation of NADH using the POA–PTS electrode.

Fig. 8 shows the SEM picture of the POA–ClO_4 and POA–PTS electrodes. It is observed that the PTS-doping leads to the formation of strips of polymer closely packed together while ClO_4-doped film presents a flake-like structure.

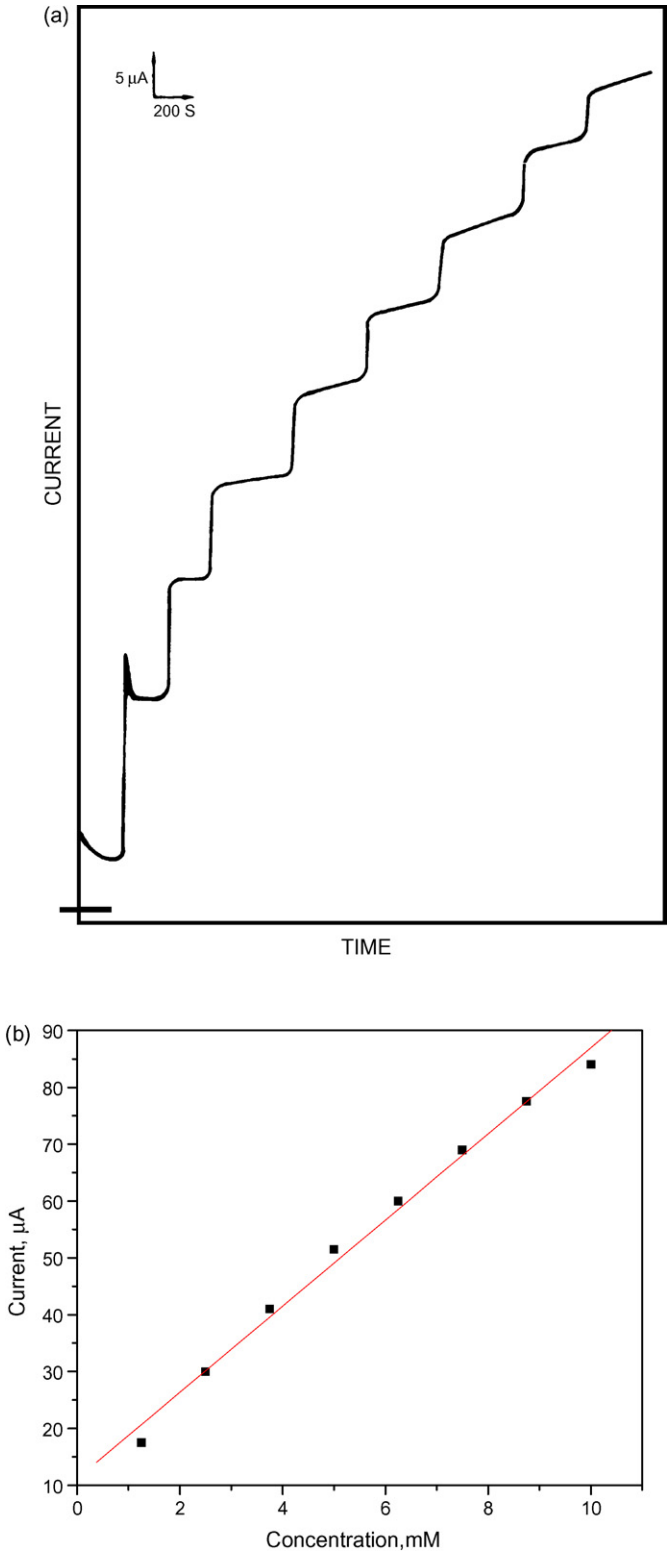


Fig. 7. (a) Amperometric response for NADH oxidation at POA–PTS electrode in phosphate buffer (pH 6.2). Each addition corresponds to 1.25 mM of NADH. Bias potential: 0.55 V.

Mostly, the amperometric biosensors for glucose are based on the electrochemical oxidation of hydrogen peroxide, which is formed during the enzyme-catalysed oxidation of glucose by dissolved oxygen. In the absence of oxygen, glucose oxidation

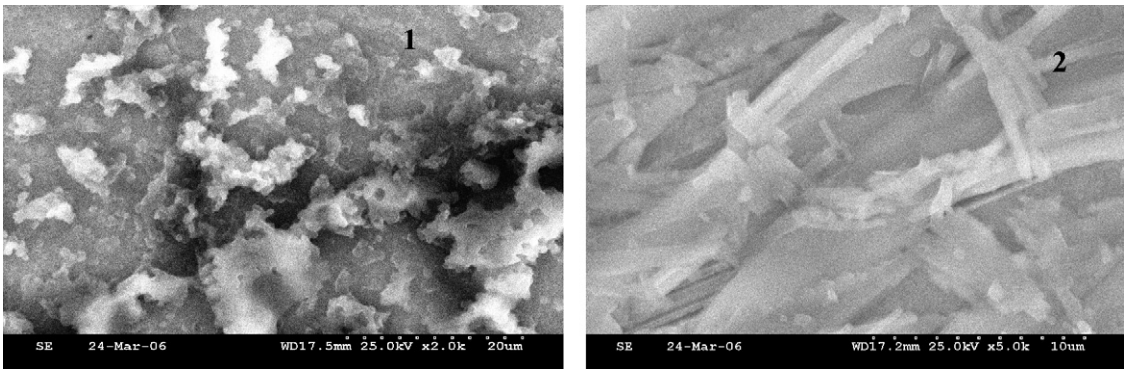
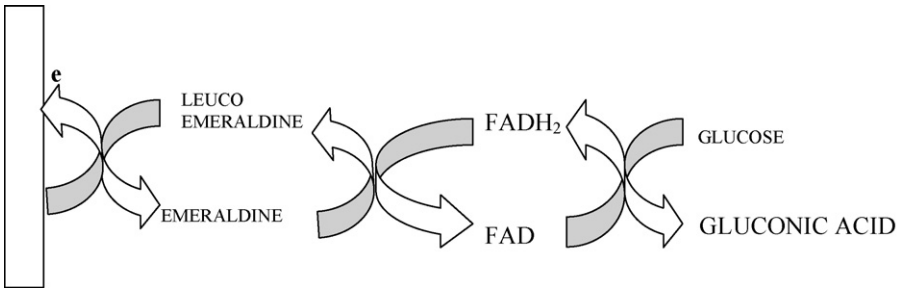


Fig. 8. SEM images of POA films prepared with the dopants: (1) perchlorate and (2) PTS anion.

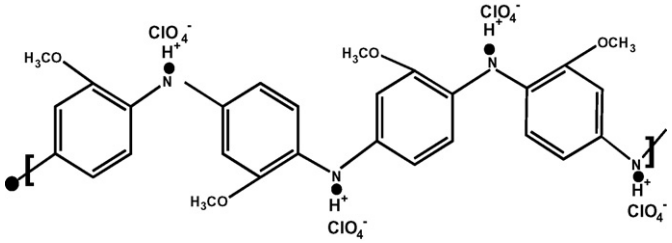


Scheme 1. Mediated electron transfer on a POA-modified electrode during glucose oxidation.

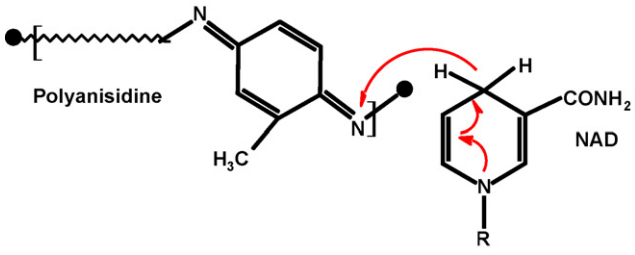
can be mediated by electron transfer shuttles, which transfer electrons between the enzyme and the substrate (Scheme 1). The reactions are carried out strictly in the absence of oxygen to avoid oxygen-aided enzymatic oxidation. The isoelectric point of GOx enzyme is 4.5. Hence at pH 5.0, the anionic enzyme molecules can get trapped inside the POA film. As already discussed, at pH 6.2, the concentration of enzyme molecules trapped inside the polymer matrix is likely to be less and hence the catalytic response observed was lower compared to pH 5.0.

The immobilization of the enzyme with the retention of their catalytic activity is important for biosensor application. It is known [21] that POA film is mostly non-conducting in nature. It has been demonstrated that even smaller anions could be trapped inside the POA films and they aid in maintaining the redox conductivity of the film. The anions stabilize the protonated form of the polymer as shown in Scheme 2 and hence redox conductivity is retained at higher pH also. The redox conductivity enables wiring of the enzymatic activity to the electrode.

The oxidation of NADH proceeds via either hydride or hydrogen atom transfer followed by electron transfer. An efficient



Scheme 2. Structure of protonated POA stabilized by perchlorate anions.



Scheme 3. Facile transfer of hydrogen from NADH to POA.

mediator for NADH oxidation should have the structure, which allows hydride transfer from NADH to the oxidized form of the mediator. The conducting emeraldine form of POA contains *para*-quinonimine groups, which fulfill the structural requirements for NADH oxidation, as shown in the following scheme (Scheme 3).

In the case of NADH, the oxidation takes place at 0.5 V. A high sensitivity is observed. The use of POA as an immobilization matrix has been explored for the first time. With POA film, a higher overpotential for NADH oxidation is observed when compared to that on a PAni film. Nevertheless, the relatively non-conducting and compact nature of POA film has resulted in good selectivity associated with higher catalytic currents for the oxidation of NADH, which increases the sensitivity of the technique.

4. Conclusions

In this work we have demonstrated that the non-conducting polyanisidine films can be made redox-active in presence of

smaller anions like perchlorate and *p*-toluene sulphonate as dopants and they serve as sensing platforms for NADH and glucose. POA-anion composite films efficiently wire the GOx enzyme to the electrode surface. In the case of NADH, the catalytic oxidation at POA-modified electrode occurs at a higher potential compared to PAni electrode. However, this shortcoming is more than compensated by the inherent selectivity associated with the non-conducting POA films. No fouling was observed at the electrode surface. The compact, non-conducting polyanisidine films, with the redox conductivity kept in tact by the trapped anions show promise as good biosensor platforms.

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