RESEARCH ARTICLE

Preparation of electrocatalytically active chitosan biopolymer films by solvent-dependant electrophoretic deposition

K. Aneesh · G. Ravikumar · Sheela Berchmans

Abstract Herein, it has been shown that electrocatalytically active films of the biopolymer chitosan could be electrophoretically deposited from strong acid (HCl) medium and could be used for the mediatorless electrocatalytic oxidation of nicotinamide adenine dinucleotide (NADH) at neutral pH. The chitosan-modified electrode alleviated electrode fouling effects and decreased NADH oxidation overpotential by ∼540 mV. Sensitivity and detection limit were found to be 1.049 µA µM^{-1} and 0.017 µM, respectively. The modified electrode exhibited a linear dynamic range between 39.8×10^{-6} and 23.4×10^{-5} M. The morphology of the electrochemically deposited chitosan films from solvents such as HCl and acetic acid was analyzed in detail using scanning electron microscopy. It was shown by zeta potential measurements that the chitosan molecules were stabilized to a greater extent in the presence of *p*-toluene sulfonic acid in HCl medium and led to a well-defined amperometric response for NADH oxidation. The proof of concept for ethanol sensing was demonstrated by coupling alcohol dehydrogenase enzyme to the chitosan-modified electrode using NADH transduction.

Keywords Chitosan · Electrophoretic deposition · NADH oxidation · Electrocatalysis

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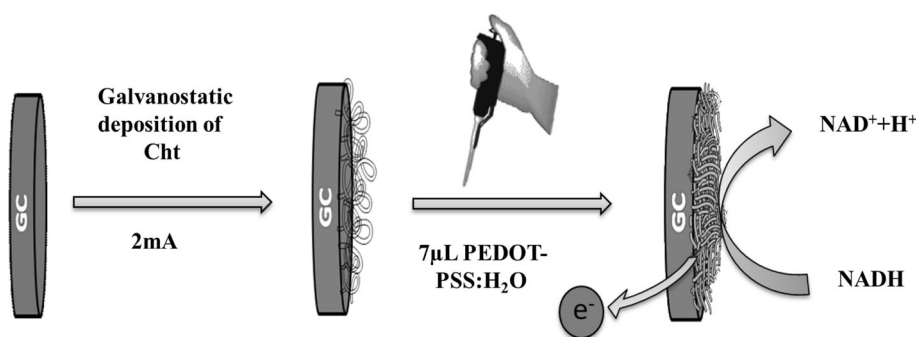e-mail: sheelaberchmans@yahoo.com; sheelab@cecri.res.in

1 Introduction

Chitosan, which is deacetylated chitin, has its chemical and physical properties influenced by the degree of chemical polymerization and the extent of deacetylation. The biocompatibility of chitosan makes it suitable for applications in biomedicine [1–3], gene delivery [4], drug delivery [5–7], enzyme immobilization [8–10], tissue engineering [11, 12], antifungal, antimicrobial anti-viral preparations [13–17], pesticide removal [18], pharmaceutical sciences [19, 20] etc.

The electrochemical oxidation of dihydronicotinamide adenine dinucleotide (NADH) to the enzymatically active NAD$^+$ in aqueous solution has received much interest because NADH behaves as an essential cofactor in the naturally occurring enzymatic reactions. [21] The NADH and its oxidized form, NAD$^+$, are ubiquitous biomolecules found in both eukaryotic and prokaryotic organisms. These two nucleotides, known as coenzymes, transfer hydrogen and electrons from one metabolite to another in many cellular redox reactions. Particularly, NAD$^+$ and NADH are involved in cellular energy metabolism, including glycolysis, tricarboxylic acid cycle and oxidative phosphorylation [22]. The NADH-dependent dehydrogenases catalyze the oxidation of various compounds of great interest in analytical chemistry, such as alcohols, aldehydes and carbohydrates. Since sensing these analytes using dehydrogenase enzymes is based on the electrochemical detection of enzymatically generated NADH, a highly selective and sensitive NADH transduction is desired. However, direct electrochemical oxidation of NADH to its corresponding oxidized form NAD$^+$ is accompanied by high over potential at platinum or carbon electrode [23]. The electrochemical sensing of NADH at low working potential in the micro/sub-micro molar level without any interference is a challenging task. Although the reversible potential of NADH/NAD$^+$ couple is estimated to be −0.32 V

Scheme 1 Schematic representation of the modification of glassy carbon electrode (GCE) surface and electrochemical detection of NADH

versus normal hydrogen electrode (NHE) at neutral pH, the direct oxidation of NADH at the unmodified electrode requires very high overpotential as large as 1.0 V [24]. The oxidation of NADH at bare glassy carbon electrode in phosphate buffer solution at pH 7 is known to occur at 640 mV versus saturated calomel electrode (SCE) [25]. Moreover, the unmodified electrodes suffer from (1) electrode fouling due to adsorption of oxidation products at the electrode surface [26] and (2) interference due to other electroactive compounds such as ascorbate (AA) and urate (UA). Many different approaches have been made to decrease the overpotential and to overcome the above-mentioned problems using various mediators and electrode modification methods [27–30]. However, the strong adsorption of not only NADH itself but also its oxidation product NAD^+ leads to electrode surface fouling resulting in a decrease in sensitivity, reproducibility and stability [31]. Mediators such as Quinone derivatives [32, 33], thionine derivatives [34], diamines [35], ferrocene [36], ruthenium complexes [37], catechols [38] and L-glutamate [39] derivatives adsorbed onto graphite or as electrodeposited films have been employed for accelerating the interfacial electron transfer between NADH and a solid electrode. However, improvements are still required to avoid the water-soluble mediator loss from the electrode surface in order to enhance the stability and reproducibility of the analytical measurements. In this direction, nanostructured carbon materials such as pyrolytic graphite [40], carbon nanofibers [41, 42], carbon microparticles [43] and especially carbon nanotubes [44, 45] have been used to reduce the high overpotential for NADH oxidation, minimize the surface fouling and to improve electron transfer kinetics without the help of redox mediators due to their edge-plane sites/defects present in the carbon materials. However, the preparation of nanostructured carbon materials involves carefully controlled experimental conditions and often involves high temperatures and expensive reagents. In this work, we describe a new method to effectively catalyze the oxidation of NADH in the absence of electron transfer mediators at neutral pH, using the natural bio polymer chitosan by galvanostatically depositing the same on a glassy carbon electrode under ambient conditions (Scheme 1). Also we report our new findings related to the solvent-dependant electrochemical deposition of chitosan

and a proof of concept for ethanol sensing using NADH transduction.

2 Materials and methods

2.1 Chemicals

Nicotinamide adenine dinucleotide, chitosan (From crab shells-highly viscous), alcohol dehydrogenase (ADH), PEDOT-PSS and *p*-toluene sulfonic acid monohydrate (PTSA) were obtained from Sigma-Aldrich and used as received. Hydrochloric acid (Rankem) and phosphate buffer solution (PBS, 0.1 M) of pH 7.0 were used as supporting electrolytes. Phosphate buffer solution, PBS (pH 7.0), was prepared using KH_2PO_4 (Merck) and NaOH (Merck). All the solutions were prepared using deionized water (Millie-Q system) with a resistivity of 18.2 MΩ.

2.2 Instrumentation

Cyclic voltammetry and chronoamperometry experiments were performed using Autolab PGSTAT 302 N. GPES software of version 4.9.007 was used in this analysis. FT-IR was performed on Bruker Tensor 27 FT-IR Spectrometer. All electrochemical experiments were carried out in a cell containing 5.0 ml of 0.1 M PBS solution (pH 7) at room temperature using a platinum wire as auxiliary, a normal calomel electrode (NCE) as reference and the Glassy carbon electrode (GC) as working electrodes. Zeta potential measurements were made using Beckman Coulter Delsa Nano C particle size analyzer. The samples were initially filtered through 0.4-μm syringe filter and then used for measuring zeta potential.

3 Procedure

3.1 Preparation of 0.5 % chitosan solution

0.05 g of chitosan was accurately weighed and transferred to 10 ml of 0.5 M HCl. It was sonicated continuously for 1 h followed by stirring for 2 h. The solution was filtered

through an 11-μm filter. The filtered solution was kept at 4 °C.

3.2 Preparation of chitosan-modified glassy carbon electrode

The GC (3 mm in diameter) was polished to a mirror-like finish with 1.0, 3.0 and 0.05 micrometer alumina slurry followed by thorough rinsing in doubly distilled water. The electrodes were sonicated in acetone, and doubly distilled water, and then allowed to dry at room temperature. Electrophoretic deposition of chitosan on GCE was carried out galvanostatically from a solution containing 0.5 % chitosan in (1) 0.5 M HCl (2) 0.5 M HCl containing 0.1 M p-toluene sulfonic acid (PTSA) (3) 0.5 M acetic acid. In the case of HCl, a positive current density of 2 mA cm^{-2} was applied for the electrophoretic deposition and in the case of acetic acid, a negative current density of -2 mA cm^{-2} was used for electrophoretic deposition. After five minutes of electrophoretic deposition, the electrode was taken out and dried at room temperature. Then, 7 μl of PEDOT-PSS: H$_2$O (1:2) was applied over the modified GC electrode to make the chitosan layer stable. The modified electrode will be designated as PEDOT-PSS/ Cht/GC hereafter. For FT-IR analysis, the deposited chitosan was peeled off from the surface of the glassy carbon electrodes, dried, powdered and used.

3.3 Immobilization of alcohol dehydrogenase

Alcohol dehydrogenase was immobilized by dropcasting the enzyme from a stock solution containing 10 mg ml^{-1} of the enzyme on the chitosan layer. The electrode was completely allowed to dry before the experiments.

4 Results and discussion

4.1 Electrophoretic deposition of chitosan

The electrophoretic deposition of chitosan from mild acidic solution was first reported by Wu et al. [46]. From the literature reports, we gather that chitosan can be electrodeposited from acid solution by applying a current density which produces localized pH changes at the interface where proton reduction occurs and this acts as the driving force for the electrochemical deposition [47]. Our experiments with the electrochemical deposition reveal a solvent-dependent deposition of chitosan on the glassy carbon electrode. We have observed a zeta potential value of -55.83 mV for a 0.5 % chitosan solution in HCl and a value of 16.1 mV for a 0.5 % solution of chitosan in acetic acid (Supporting information). This observation reveals

that chitosan solution is highly stabilized in the presence of HCl, and the solution is stabilized by negatively charged chloride ions surrounding the cationic chitosan. Literature reports often suggest chitosan to be cationic. However, our experiments reveal that the chitosan solution is highly stabilized by chloride ions, and the solution is anionic. Hence, it facilitates electrophoretic deposition of chitosan by application of positive current densities. When acetic acid is used as the solvent for the dissolution of chitosan a positive zeta potential value of 16.1 mV is observed indicating chitosan is cationic in presence of acetic acid. However, a lower value suggests that the solution is not highly stabilized as in the case of HCl. Most of the amino groups present in the polymeric backbone are perhaps acetylated in presence of acetic acid resulting in the lowering of cationic protonated amino groups which leads to a low value of zeta potential. Hence, in the case of chitosan dissolved in acetic acid, we could electrochemically deposit chitosan by applying a negative current density. We further noticed that the chitosan solution in HCl can be further stabilized in the presence of p-toluene sulfonic acid (PTSA). When PTSA is present in 0.1 M concentrations, the zeta potential value simply shoots up to $-2,835$ mV, which indicates that PTSA can stabilize HCl solution of chitosan to a tremendous extent (scheme 2).

4.1.1 FT-IR analysis of the deposited material

Figure 1 shows the FT-IR spectrum of (a) chitosan (b) electrophoretically deposited 0.5 % chitosan from 0.5 M acetic acid and (c) electrophoretically deposited 0.5 % chitosan from 0.5 M HCl. Typical bands of chitosan viz., the broad band centered at 3,446 cm^{-1} due to the overlapped stretching vibrations of OH and NH groups, the band corresponding to C=O vibration (1,650 cm^{-1}) of acetyl groups, the band at 1,332 cm^{-1} due to the combination of NH deformation, the CN stretching vibration and the band due to the stretching of C=O at 1,089 cm^{-1} can be seen in spectra of deposited materials. This confirms that the electrophoretically deposited material is chitosan.

4.2 Morphological characterization using SEM

Morphological characterization is carried out using scanning electron microscope. Figure 2 shows the surface morphology of galvanostatically deposited 0.5 % chitosan in HCl. The surface is highly porous and looks like a woven mat. The porosity probably confers better electron transfer properties to the electrochemically deposited film. SEM image of chitosan in acetic acid medium appears like a smooth white deposit which can be clearly seen by naked eye. SEM image does not reveal any special microscopic

Scheme 2 Stabilization of chitosan in **a** HCl + PTSA, **b** HCl and **c** acetic acid

(a)   **(b)**   **(c)**

$R-\overset{+}{N}H_2-C-CH_3$
            ||
            O

Cross linking site

PTSA anion

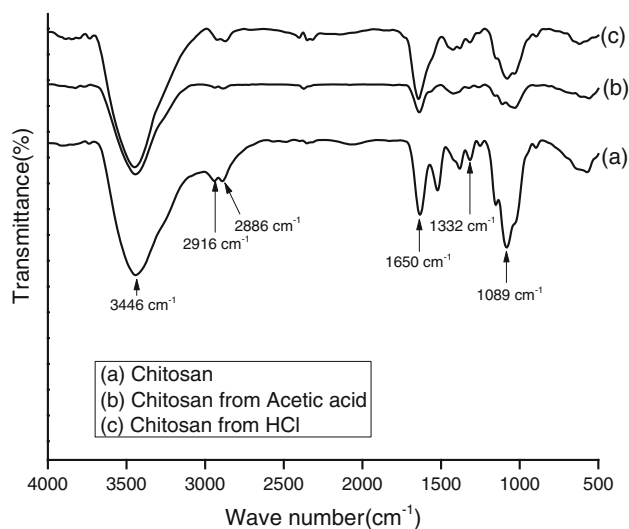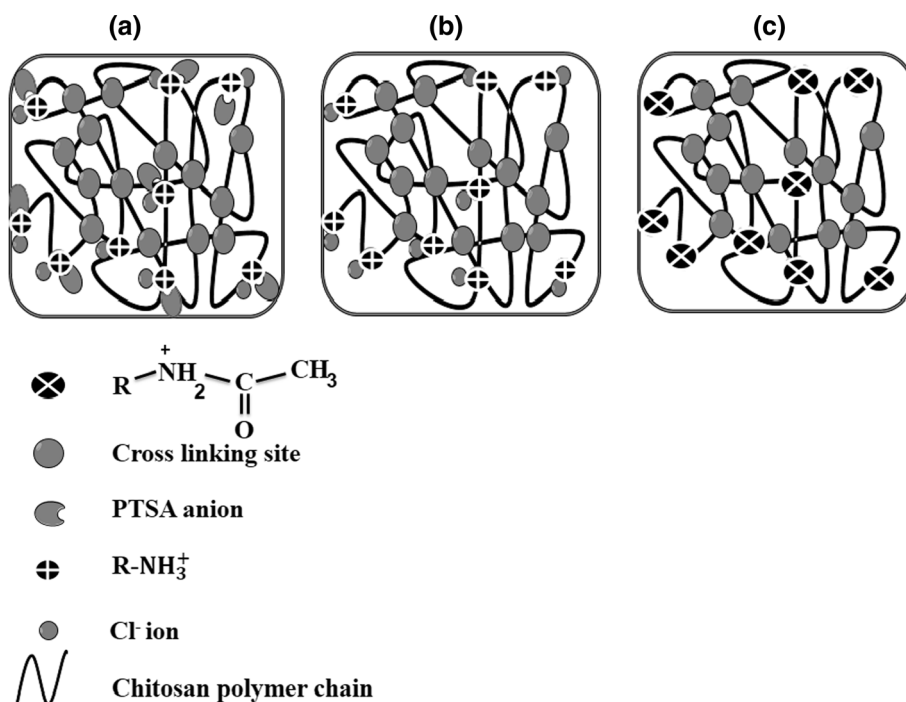$R-NH_3^+$

Cl⁻ ion

Chitosan polymer chain

Fig. 1 FT-IR spectrum of **a** chitosan **b** chitosan deposited from 0.5 M Acetic acid **c** chitosan deposited from 0.5 M HCl

features. Thick, nonporous deposit formed in presence of acetic acid is mainly responsible for its absence of catalytic activity toward NADH oxidation. In the case of electrochemical deposition of chitosan prepared from HCl containing 0.1 M PTSA, the SEM image shows bead-like structure for the electrochemically deposited chitosan. This film is also catalytically active toward the oxidation of NADH and leads to a stabilized amperometric response.

4.3 Voltammetric characterization

Figure 3a shows the cyclic voltammograms of the PEDOT-PSS/Cht/GC electrode in 0.1 M PBS at pH 7 at a scan rate of 20 mV s^{-1}. Each addition corresponds to 20 μL of 10 mM NADH. Two distinct peaks can be seen after the addition of NADH. The peak close to 100 mV is due to the catalyzed oxidation of NADH at the chitosan-modified electrode, and the oxidation peak at 550 mV is due to the direct oxidation of NADH at the glassy carbon surface. As seen from the SEM image, the chitosan deposited from HCl is porous, and hence, we are also able to see the direct oxidation of chitosan in this film. The overlayer of PEDOT-PSS makes the chitosan film stable for repeated measurements.

Figure 3b shows the cyclicvoltammograms obtained for the electrocatalytic oxidation of NADH on a glassy carbon electrode modified with electrodeposited chitosan in HCl containing 0.1 M PTSA. The electrocatalytic oxidation of NADH occurs at around 100 mV, and the catalytic current gradually increases with an increase in concentration of NADH. In this case, we do not observe the direct oxidation of NADH, and this film is perhaps impervious and completely covers GC surface. In the case of chitosan films prepared from acetic acid, we do not observe any catalytic effect (Supporting information). The voltammetric results suggest that the PEDOT-PSS/Cht/GC electrode can facilitate amperometric detection of NADH at low overpotential.
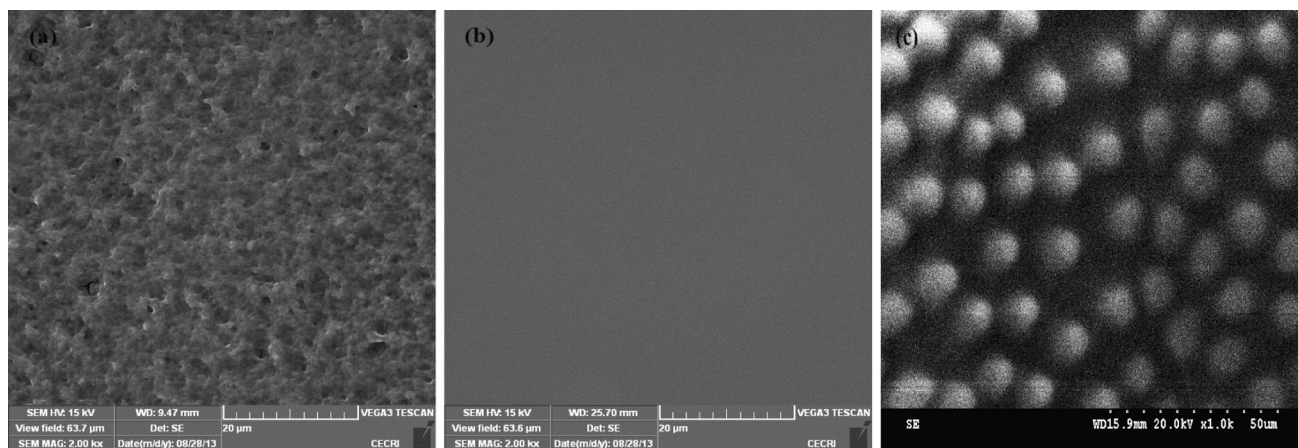
Fig. 2 SEM images of galvanostatically deposited 0.5 % chitosan in **a** 0.5 M HCl **b** 0.5 M acetic acid **c** 0.5 M HCl + 0.1 M PTSA
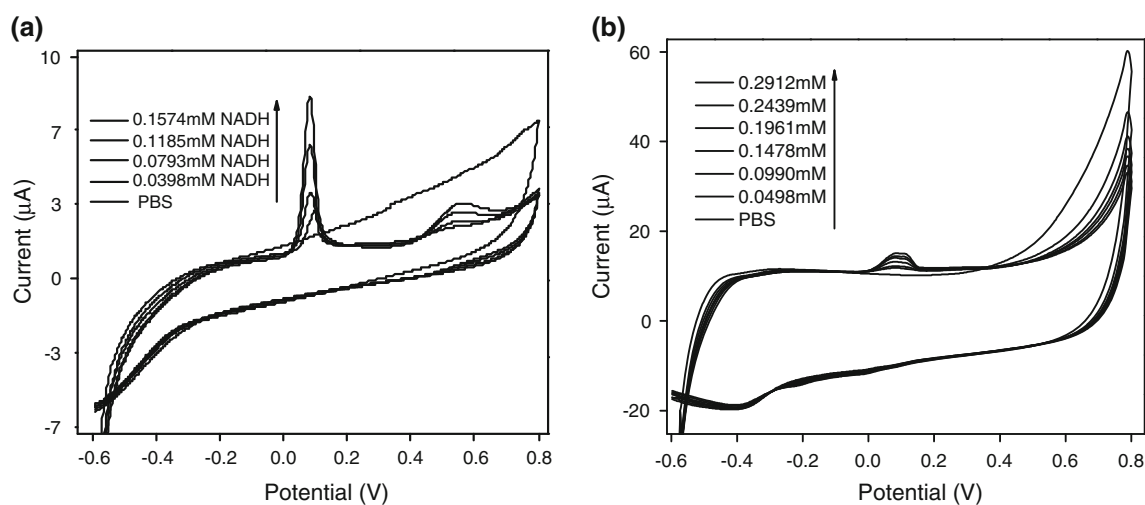

Fig. 3 Cyclic voltammograms of the PEDOT-PSS/Cht/GC electrode in 0.1 M PBS at pH 7 **a** in the absence of **b** in the presence of *p*-toluene sulfonic acid. Scan rate: 20 mV s^{-1}
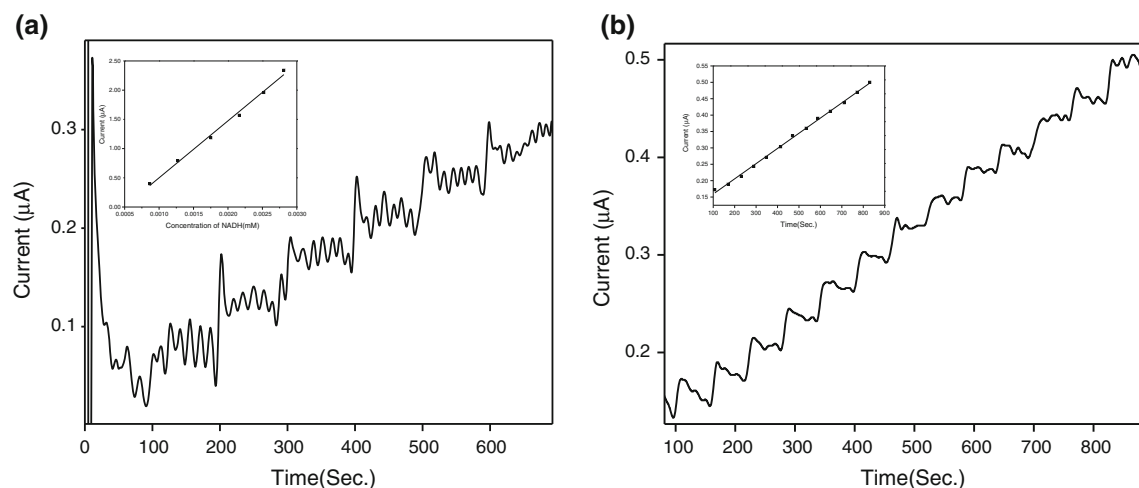

Fig. 4 Chronoamperometric determination of NADH using a PEDOT-PSS/Cht/GC electrode **a** in the absence of *p*-toluene sulfonic acid; $E_{applied} = 100$ mV **b** in the presence of *p*-toluene sulfonic acid; $E_{applied} = 120$ mV

4.4 Amperometric detection of NADH

Figure 4a shows an amperometric response at the PEDOT-PSS/Cht/GC electrode (E = +0.100 V) during the successive addition of NADH aliquots (10 mM) into a stirred PBS (pH 7.0). From the calibration plot, the sensitivity is found to be 1.048 μA μM^{-1} and detection limit is found to be 0.017 μM. Figure 4b shows the amperometric response for NADH in the case of chitosan films prepared from HCl in presence of 0.1 M p-toluene sulfonic acid. The amperometric response is highly stable. Sensitivity and detection limit are nearly the same in both the cases. Table 1 compares the over potential for NADH oxidation at the chitosan-modified glassy carbon electrode with those reported in the literature. It is found that when compared to many other methods, the potential for NADH oxidation on PEDOT-PSS/Cht/GC electrode is very low, indicating the efficient oxidation of NADH.

4.5 Electrochemical sensing of ethanol based on NADH transduction

Figure 5a shows the voltammetric response of the enzyme-modified PEDOT-PSS/Cht/GC electrode. By immobilizing alcohol dehydrogenase onto the PEDOT-PSS/Cht/GC electrode, we can show that the modified electrode can be used for sensing alcohol. The voltammetric currents show a well-defined proportional increase for every addition of ethanol showing scope of the modified electrode for sensing alcohol. The corresponding calibration plot is depicted in Fig. 5b.

5 Conclusions

This work brings about effectively the capability to tune the electrochemical deposition of chitosan from different

Table 1 Comparison of electrochemical NADH detection in the absence of mediators

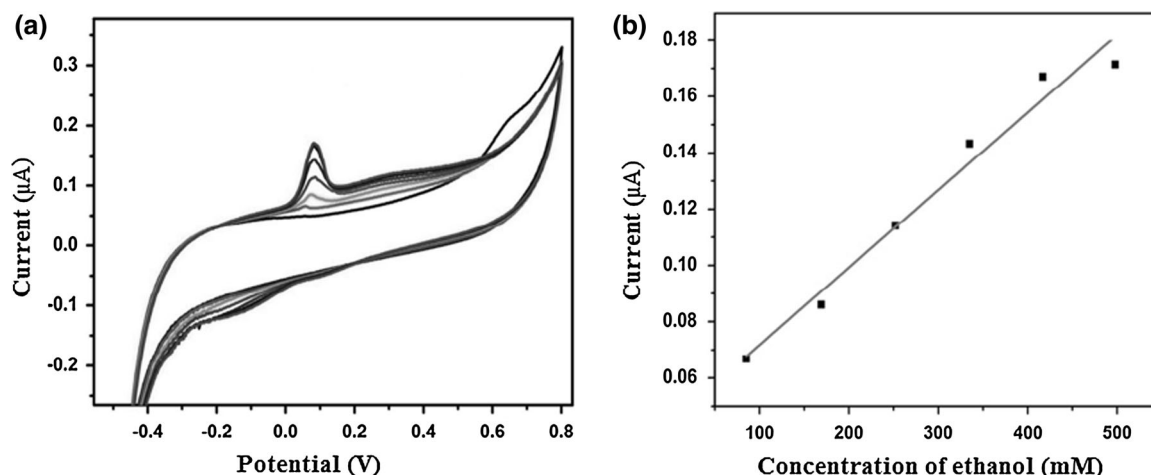| SI. no.	Electrode modification protocol.	Over potential for NADH oxidation (with respect to NCE in bracket)	References
1	CNF/GC	300 mV versus Ag/AgCl (217 mV)	[42]
2	SPCE	420 mV versus Ag/AgCl (337 mV)	[48]
3	CNF-CPE	450 mV versus Ag/AgCl (367 mV)	[49]
4	Pd/CNF-CPE.	500 mV versus Ag/AgCl (417 mV)	[50]
5	GC/PFIL-AuNPs	530 mV versus Ag/AgCl (447 mV)	[51]
6	CMM/GC	300 mV versus SCE (217 mV)	[52]
7	DA/GO/GC	100 mV versus Ag/AgCl (17 mV)	[53]
8	PTh/MWCNTs/CILE	220 mV versus Ag/AgCl (137 mV)	[54]
9	DMF-exfoliated graphene	390 mV versus Ag/AgCl (307 mV)	[55]
10	GO/SPCEs	410 mV versus Ag/AgCl (327 mV)	[56]
11	PGE/QH2	300 mV versus Ag/AgCl (217 mV)	[57]
12	PEDOT-PSS/Cht/GC	100 mV versus NCE	This work

Fig. 5 a Cyclic voltammograms showing the response for different additions of ethanol (each addition corresponds to 83 mM) at a scan rate of 100 mV s^{-1}, **b** linear response between the concentration of ethanol and the corresponding voltammetric currents

solvents based on the zeta potential values. This concept can be used to prepare electrocatalytically active chitosan films on solid electrode surfaces. Further, we have demonstrated the mediatorless electrocatalytic oxidation of NADH on chitosan films deposited from HCl solutions at very low overpotential with very good sensitivity of detection. The results are reproducible with the same electrode with very less loss in efficiency indicating that the electrode fouling effects by the oxidation products of NADH is minimized. Using this approach, it is possible to decrease the overpotential of NADH oxidation by about 540 mV. Sensitivity and detection limit achieved are 1.049 $\mu A\ \mu M^{-1}$ and 0.017 μM, respectively. We have also established the proof of concept for the detection of ethanol by coupling alcohol dehydrogenase enzyme to the chitosan-modified electrode.

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