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Evaluation of Different Functionalized CNTs for Development of Choline Amperometric Biosensor

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ABSTRACT

Choline oxidase (ChOx) was chosen as a model enzyme for evaluating the performance of CNTs' functional groups for development of enzyme electrodes. CNTs were functionalized with carboxylic acid, amine or amide groups. Carboxylic acid, amine and amide functionalized CNTs were obtained by acid treatment, ethylenediamine or tetraethylenepentamine chemically modification and ammonia plasma treatment, respectively. The CNTs with different functional groups were mixed with 1-butyl-3-methylimidazolium tetrafluoroborate as a typical room temperature ionic liquid. ChOx was adsorbed on the thus prepared nanocomposites containing different modified electrodes and its electron transfer and electroanalytical response towards choline was investigated. The resulting data showed that the ammonia plasma treated nanocomposite had higher apparent heterogeneous electron transfer rate constant (2.74 s^{-1}) than the others, indicating more facile and rapid rates of electron transfer; while, nanocomposites modified with tetraethylenepentamine functionalized CNTs showed the most sensitive response towards choline (1.09×10^3 A M^{-1} m^{-2}) with the lowest detection limit of 5.81×10^{-6} M. Consequently, tetraethylenepentamine modified electrodes were more convenient for choline biosensing applications.

Keywords: Carbon nanotubes, Functionalization, Ionic liquid, Choline oxidase

INTRODUCTION

Direct electron transfer between redox proteins or enzymes and underlying electrodes has attracted considerable attention in recent years [1-4]. This process not only can provide an ideal model for studying the kinetics and thermodynamics of biological redox reactions [5], but may also establish a foundation for fabricating third-generation biosensors [6]. These biosensors usually offer higher selectivity, because their potential windows are well-matched to the redox potential of the enzymes [7]. They are also advantageous for *in vivo* detection due to their simplicity and harmlessness [8]. However, establishment of direct electrochemistry between redox proteins and conventional electrodes is often impossible, because the enzymatic redox centers are usually deeply embedded into the low conductive amino acid chains, resulting in slow rates of electron transfer [9]. Besides, direct adsorption of enzymes on a conventional electrode surface leads to their denaturation or loss of bioactivity [10]. To overcome these drawbacks, it is necessary to find a host matrix, which enhances direct electron transfer between enzymes and underlying electrodes and also provides a favorable microenvironment to keep the enzyme bioactivity.

Carbon nanotubes (CNTs) have attracted a great deal of interest in electrochemistry due to their unique electrical, chemical, structural and mechanical properties [11,12]. These materials have been extensively used to achieve direct wiring of enzymes to electrode surface, promote electrochemical reactions and amplify signal for biosensor applications [13]. However, the lack of solubility of CNTs has imposed great limitations to the application of CNTs [14]. Surface modification or functionalization of CNTs is often carried out to facilitate their applications [15]. Functional groups covalently attached to the outer surface of CNTs are able to modify the stacking and salvation properties of nanotubes [16]. Moreover, it is accepted that the electronic properties of semiconducting tubes are

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heavily increased by doping due to covalent modification of their carbon framework [17]. Three functional groups that are commonly attached to CNTs surfaces are carboxyl, amine and amide groups. Although functionalized CNTs have been intensively investigated in many fields, to the best of our knowledge, no reports have been published to date on the effect of CNTs' functional groups on the electrochemical and electrocatalytic properties of immobilized enzymes.

Room temperature ionic liquids (RTIL) are substances composed of two asymmetrical ions with opposite charges that preserve their liquid state at room temperature [18]. They have unique chemical and physical properties at ambient temperature such as high ionic conductivity, good solubility, wide potential window, good stability, low toxicity and negligible vapor pressure [18,19]. They are also compatible with biomacromolecules and even whole cells [20]. The use of RTIL related composites, especially RTIL/CNTs nanocomposites, as electrode modifiers may enhance the sensor sensitivity by increasing the electrical conductivity [21-23].

In the present report, based on our previous experiences [24-27], choline oxidase (ChOx) was chosen as a model enzyme for considering the effect of CNTs' different functional groups. For this purpose, CNTs with different functional groups including carboxylic acid, amine and amide were mixed with 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM]BF_4) as a typical RTIL. The thus prepared RTIL/CNTs nanocomposites were then used for comparing the effect of fuctional groups on amperometric detection of choline using the nanocomposites based biosensors.

MATERIAL AND METHOD

Reagents

ChOx (E.C.1.1.3.17) from Alcaligenes species (11 U mg^{-1}), choline chloride and [BMIM]BF_4, as a typical RTIL, were purchased from Sigma (St. Louis, MO, USA). Multi-walled CNTs, prepared by chemical vapour deposition, were obtained from Chengdu Organic Chemicals Co. Ltd. (Chengdu, China). Potassium phosphates (K_2HPO_4 and KH_2PO_4), Nitric acid (HNO_3), N,N-dimethylformamide (DMF) and tetrahydrofuran were purchased from Merck (Darmstadt, Germany). Ethylenediamine (EDA) and tetraethylenepentamine (TEP) modifier agents were obtained from Fluka (Buchs, Switzerland). Thionyl chloride ($SOCl_2$) was supplied by Acros Organics (Geel, Belgium). Unless otherwise stated, all samples were prepared in 0.2 M phosphate buffer, pH 7.0, called as phosphate buffer solution (PBS).

Apparatus and Measurements

The electrochemical experiments were carried out using a computerised Potentiostat/Galvanostat (model 263-A, EG&G, USA) equipped with a PowerSuite software package. All experiments were performed at room temperature (25 °C) in a conventional electrochemical cell. A three electrode system was used, where a silver/silver chloride (3 M KCl solution, from Metrohm) served as the reference electrode. A glassy carbon (GC) electrode (from Azar Electrode, Uromia, Iran) and a platinum wire electrode were used as working and auxiliary electrode, respectively. Fourier transform infrared (FT-IR) spectra were recorded using a FT-IR spectrometer (Model Nexus 870, Thermo Nicolet Co. USA).

CNTs Functionalization

Preparation of HOOC-CNTs. For preparation of HOOC-CNTs, 6 mg of CNTs was dispersed in 10 ml of HNO_3 (35%) for 6 h, with the aid of ultrasonic agitation. The suspension was then diluted with deionized water and filtered using a polytetrafluoroethylene filter (0.45 μm, PTFE, Schleicher & Schuell). The CNTs were then rinsed with water until a neutral pH value was reached. The resulting filter cake was dried under IR lamp. Then, 2 mg of carboxyl functionalized CNTs was dispersed in 1 ml DMF with the aid of ultrasonic agitation and used for further treatments.

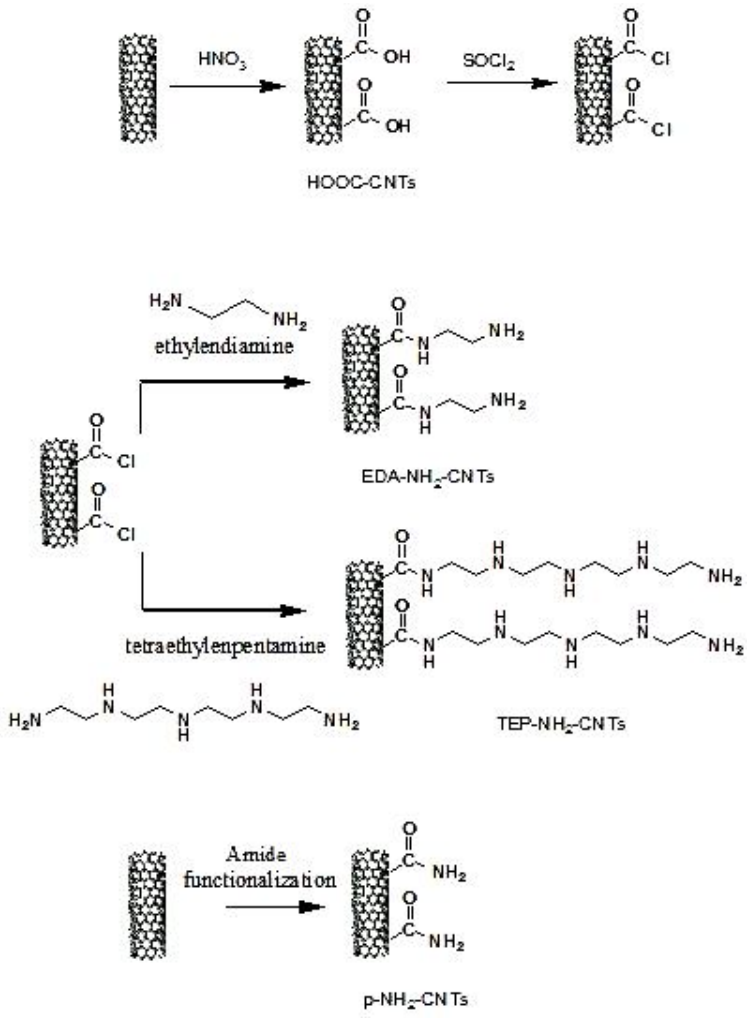
Chemical preparation of NH_2-CNTs. 20 mg of the HOOC-CNTs was stirred in 2 ml of $SOCl_2$ at room temperature (25 °C) for 12 h. Then, the acyl-chlorinated CNTs were divided into two parts: one part reacted with 2.5 ml EDA for 26 h (EDA-NH_2-CNTs) and the other was treated with 2.5 ml TEP (TEP-NH_2-CNTs) in the same condition. Finally, the residues were washed with ethanol followed by anhydrous tetrahydrofuran to remove any excess amine. The products were dried overnight under IR

lamp.

Amide functionalization of CNTs. The plasma treated amide functionalized CNTs (*p*-NH_2-CNTs) was kindly provided by Professor A. Khodadadi (School of Chemical Engineering, College of Engineering, University of Tehran, Tehran, Iran) and used without further treatment. To prepare p-NH_2-CNTs, CNTs were first treated by He plasma and then exposed to NH_3 [28]. The general strategy for the preparation of functionalized CNTs is described in Scheme 1.

Immobilization of Enzyme

Prior to use, GC electrodes were carefully polished with 0.3 and 0.05 µm alumina slurry sequentially. The electrodes were dried in air after being washed with water. To obtain the CNTs/GC electrode, 2.0 µl of each functionalized CNTs (HOOC-CNTs, EDA-NH_2-CNTs, TEP-NH_2-CNTs or *P*-NH_2-CNTs) in DMF was spread evenly onto the surface of GC electrodes and dried in air. Then, the CNTs/GC electrodes were immersed in pure solution of [BMIM]BF_4 for 10 h at 4 °C. The thus prepared RTIL/CNTs/GC electrodes were then immersed in a stock solution of 6 g l^{-1} ChOx (in PBS 0.2 M, pH 7.0) for 10 h at 4 °C. By this process, ChOx was immobilized on RTIL/CNTs/GC electrodes. The modified electrodes were stored at 4 °C in PBS (pH 7.0), when not in use.



Scheme 1. Strategies for the synthesis of functionalized CNTs

Determination of the Effective Electrode Area

The effective surface area of the modified GC electrodes was estimated from the cyclic voltammograms obtained by inserting each electrode in 4.0 mM $K_3[Fe(CN)_6]$ (probe molecule) solution containing 0.1 M KCl, at various scan rates. Under semi-infinite linear diffusion condition and at 25 °C, the dependence of peak current (I_p) on the scan rate (ν) could be described by the Randles-Sevcik equation (Eq. (1)):

$$I_p = (2.69 \times 10^5)\, n^{3/2} A D^{1/2} C \nu^{1/2} \quad (1)$$

where n represents the number of electrons participating in the redox reaction, A is the effective surface area of the electrode (cm^2), D is the diffusion coefficient of the probe molecules in the solution ($cm^2\ s^{-1}$), C is the concentration of the probe molecule in the bulk solution (mol cm^{-3}), ν is the scan rate (V s^{-1}) and I_p is the peak current of the redox couple. Using the slope of I_p *vs.* $\nu^{1/2}$, A was calculated, since D, n and C were constant [29].

RESULTS AND DISCUSSION

Characterisation of Functionalized CNTs

The existence of different functional groups on the surfaces of CNTs was investigated by FT-IR spectroscopy. Figure 1a shows the FT-IR spectrum of the pristine CNTs. The broad peak at around 3400 cm^{-1} is assigned to the presence of -OH groups on the surface of the CNTs and is believed to be resulted from either the relative humidity in the ambient atmosphere of the CNTs or oxidation of the raw material during purification [30]. In the FT-IR spectrum of HOOC-CNTs (Fig. 1b), the peaks at ~1700, 1150 and 3370 cm^{-1} correspond to C=O [31], C-O [31] and O-H stretching

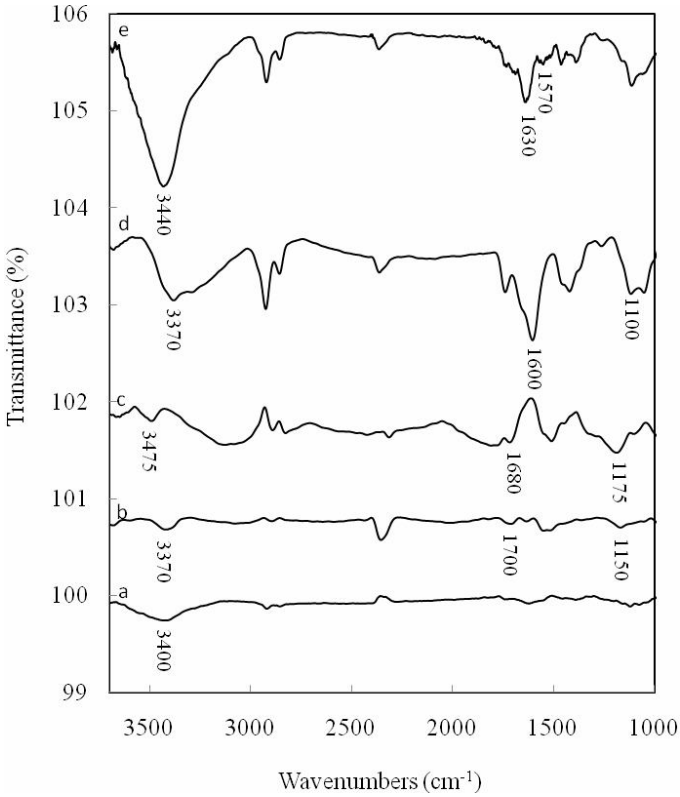


Fig. 1. FT-IR spectra of (a) pristine CNTs (b) HOOC-CNTs (c) EDA-NH_2-CNTs (d) TEP-NH_2-CNTs and (e) *p*-NH_2-CNTs.

vibration [32] of the carboxyl group, respectively. Therefore, carboxyl groups were successfully grafted on CNTs by acid treatment.

The spectrum of EDA-NH_2-CNTs (Fig. 1c) shows a peak at 1680 cm^{-1} that corresponds to the amide C=O stretch. The peak at 1175 cm^{-1} can be assigned to C-N stretching of amide groups, while the peaks at 3475 cm^{-1} is ascribed to N-H stretching vibration of amine and amide groups. The features of these spectra indicate that the EDA had been attached onto CNTs successfully [31]. The FT-IR spectrum of the TEP-NH_2-CNT (Fig. 1d), exhibits the bands at 3370, 1600 and 1100 cm^{-1} attributed to the N-H stretching vibration of amine and amide groups, C=O (corresponding to the amide carbonyl group) and C-N stretching vibration, which confirm that the surface of CNTs was functionalized with TEP [31]. In the FT-IR spectrum of *p*-NH_2-CNTs (Fig. 1e), the existence of peaks at 3440, 1630 and 1570 cm^{-1} correspond to N-H stretching, amide C=O stretching and in-plane N-H vibrations, Respectively, which indicates the attachment of amide groups on the CNTs surfaces [33].

Direct Electrochemistry of ChOx on RTIL/CNTs/GC Electrodes

As explained in Experimental Section, by combination of RTIL with the CNTs having carboxylic acid, amide or amine functional groups (the latter prepared by two different ways), four types of nanocomposites were prepared on GC electrodes. Thereafter, the electrochemical behaviour of ChOx on four different nanocomposites was investigated by cyclic voltammetry in PBS. Figure 2 shows the comparison between cyclic voltammograms of different modified electrodes. As seen, in the absence of enzyme, GC electrodes modified with different RTIL/CNTs nanocomposites exhibited no peaks (Fig. 2A). But when ChOx was immobilized on the modified electrodes, in all cases, a pair of well-defined redox peaks was observed (Fig. 2B, curves a-d). So, the observed redox peaks could be attributed to FAD/$FADH_2$ redox couple of ChOx. On the other hand, ChOx adsorbed on bare GC electrode, showed no electrochemical redox peak (Fig. 2B, Curve e). This indicates that all RTIL/CNTs nanocomposites could play an important role in facilitating electron transfer between ChOx and GC electrode. Our previous work represented a dual role for RTIL/CNTs nanocomposites: CNTs play as promoter for protein electron transfer at electrode surface, while RTIL acts as a biocompatible matrix by which the native structure of ChOx could be preserved [27]. The surface coverage of ChOx immobilized at GC electrode surface (Γ) was calculated according to the Faraday's law (Eq. (2)).

$$\Gamma = Q/nFA \quad (2)$$

Where, Q is the integrated charge involved in the reaction, obtained as the cathodic peak surface area, n is the number of electrons transferred, F is Faraday's constant and A is the effective electrode surface area calculated according to the method explained in Experimental Section. The value of Γ for different modified electrodes is reported in Table 1 (last row).

Figure 3 shows the effect of scan rate (ν) on cyclic voltammograms of ChOx immobilized on different RTIL/CNTs/GC electrodes. In all cases, the cathodic (I_{pc}) and anodic (I_{pa}) peak currents increased linearly with increasing scan rate from 0.01 to 1 V s^{-1}, suggesting a surface-controlled electrode process. This result indicates that not only the enzyme was successfully immobilized on all types of nanocomposites, but also the nanocomposites could establish an electronic communication between enzyme and electrode surface. The apparent heterogeneous electron transfer rate constant (k_s) was calculated according to Laviron Equations [34]. Comparison of electrochemical parameters of different modified electrodes including k_s, formal potential ($E^{o'}$), peak-to-peak separation (ΔE_p) and Γ is summarized in Table 1.

As seen, among the four functional groups, the electrochemical parameters of ChOx ($E^{o'}$, k_s and Γ) were improved when the enzyme was immobilized on CNTs functionalized by plasma (*p*-NH_2-CNTs) and acid (HOOC-CNTs) treatments. Except the higher value of Γ at HOOC-CNTs, the values for $E^{o'}$ and k_s at *p*-NH_2-CNTs were predominant. The superiority in ChOx loading at HOOC-CNTs could be attributed to the fact that the interaction between enzyme and RTIL/CNTs nanocomposite is mainly governed by hydrogen bonding [26]. Hydrogen bonding in HOOC-CNTs is stronger than that in NH_2-CNTs, since oxygen is more electronegative than nitrogen. So, it is reasonable that higher amount of ChOx (Γ value) was

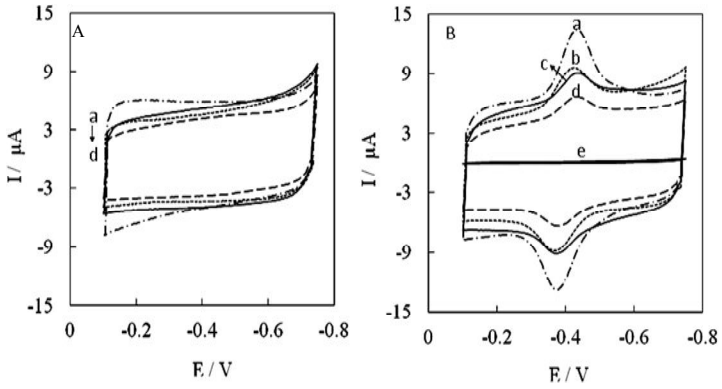


Fig. 2. A, CVs of RTIL/CNTs/GC electrodes. B, CVs of ChOx immobilized on RTIL/CNTs/GC electrodes. a to d, represent the CVs obtained by GC electrodes modified by CNTs having different functionalized groups: (a) HOOC-CNTs, (b) TEP-NH_2-CNTs, (c) *p*-NH_2-CNTs, (d) EDA-NH_2-CNTs, respectively. The experiments were carried out in 0.2 M PBS (pH 7.0) at scan rate of 0.1 V s^{-1}.

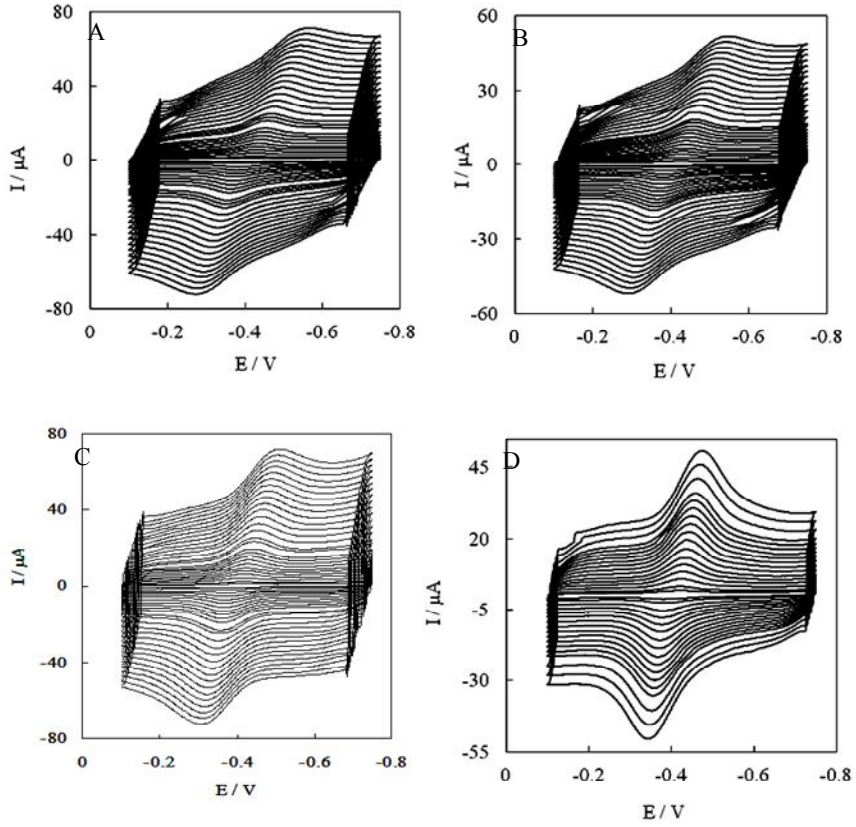


Fig. 3. CVs of ChOx immobilized on RTIL/CNTs/GC electrode in which different types of functionalized CNTs were used: (A) TEP-NH_2-CNT, (B) EDA-NH_2-CNTs, (C) *p*-NH_2-CNTs and (D) HOOC-CNTs at various scan rates. The selected scan rates (from inner to outer) are 10, 20, 40, 60, 80, 100, 125, 150, 175, 200, 225, 250, 275, 300, 350, 400 and 450 mV s^{-1}, respectively. The experiments were carried out in 0.2 M PBS (pH 7.0).

Table 1. Comparison between the Electrochemical Parameters Obtained for ChOx Immobilized at Different Functionalized CNTs

Eelectrochemical parameters	Type of functionalized CNTs			
	-COOH	*p*-NH_2	EDA-NH_2	TEP-NH_2
$E^{o'}$ (mV)	-403 ± 0.3	-396 ± 0.4	-405 ± 0.3	-404 ± 0.2
ΔE_p (mV)	58.6 ± 1.3	49.9 ± 1.5	54.2 ± 1.1	63.1 ± 1.4
k_s (s^{-1})	2.47 ± 0.07	2.74 ± 0.05	2.20 ± 0.06	1.81 ± 0.04
$\Gamma \times 10^{-10}$ (mol cm^{-2})	14.30 ± 0.28	6.80 ± 0.17	5.51 ± 0.13	5.93 ± 0.11

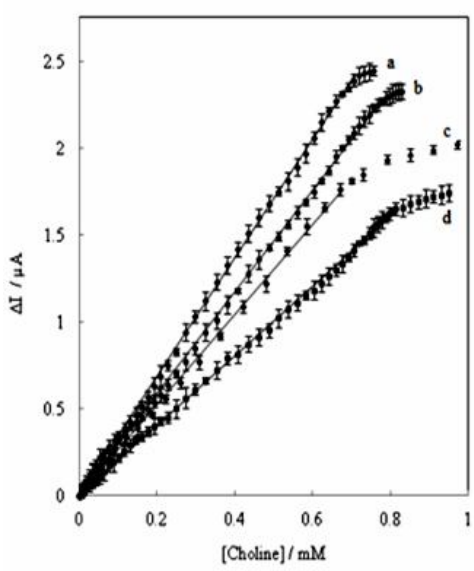


Fig. 4. The calibration plots for choline detection. The amperometric responses of rotating disk GC electrode, modified with ChOx/RTIL/CNTs, were obtained by successive addition of choline to the electrochemical cell containing 3 ml of PBS. In ChOx/RTIL/CNTs/GC electrodes, different functionalized CNTs were used: (a) TEP-NH_2-CNT, (b) EDA-NH_2-CNTs, (c) *p*-NH_2-CNTs and (d) HOOC-CNTs. The rotation speed was 500 rpm.

observed on HOOC-CNTs modified electrodes (Table 1). On the other hand, increase in enzyme loading results in limitation of charge transport through the film [35]. This is why the k_s value of ChOx at HOOC-CNTs is lower than that at *p*-NH_2-CNTs. This limitation could also be responsible for lower electron transfer reversibility of ChOx (ΔE_p) on HOOC-CNTs modified electrodes. Among the electrodes modified by -NH_2 groups, the electron transfer rate and reversibility of the redox reaction decreased by increasing the length of -NH_2 functional group. The length of -NH_2 containing functional groups varied in the order of: *p*-NH_2-CNTs < EDA-NH_2-CNTs < TEP-NH_2-CNTs (Scheme 1). Lower values of k_s and higher values of ΔE_p for ChOx on CNTs with longer functional groups may be due

Table 2. Comparison between the Analytical Parameters Obtained for ChOx Immobilized at Different Functionalized CNTs

Electroanalytical parameters	Type of functionalized CNTs			
	-COOH	*p*-NH_2	EDA-NH_2	TEP-NH_2
Sensitivity ($A\ M^{-1}\ m^{-2}$)	$6.35 \times 10^2 \pm 1.58$	$8.25 \times 10^2 \pm 1.06$	$9.30 \times 10^2 \pm 1.32$	$1.09 \times 10^3 \pm 1.72$
Linear range (M)	1.20×10^{-5}- 8.10×10^{-4}	1.18×10^{-5}- 6.70×10^{-4}	1.28×10^{-5}- 7.56×10^{-4}	7.90×10^{-6}- 7.05×10^{-4}
Detection limit (M)	$(8.26 \pm 0.019) \times 10^{-6}$	$(7.64 \pm 0.016) \times 10^{-6}$	$(6.78 \pm 0.013) \times 10^{-6}$	$(5.81 \pm 0.015) \times 10^{-6}$

to increasing distance between ChOx and CNTs, which makes the electron transfer between enzyme and CNTs more difficult.

Biocatalytic Activity of ChOx on RTIL/CNTs/GC Electrodes

The biocatalytic activities of ChOx/RTIL/CNTs/GC electrodes towards successive addition of choline were investigated by amperometry (Fig. 4). As seen, the biocatalytic activity of ChOx was different depending on the functional groups of CNTs.

The electroanalytical parameters of the modified electrodes for choline detection are summarized in Table 2. As shown in this table, compared to HOOC-CNTs/RTIL/GC, ChOx showed a better electroanalytical performance on NH_2-CNTs/RTIL/GC electrodes. The activity changes of proteins correlate well with the changes in protein-nanocomposite interaction. As explained in the Previous Section, hydrogen bonds that are the main interaction between enzyme and RTIL/CNTs nanocomposite are stronger in HOOC-CNTs modified electrodes. Stronger interaction may lead to more change in enzyme native structure and its activity. Therefore, weaker electroanalytical parameters were observed on HOOC-CNTs modified electrodes. Among NH_2-CNTs/GC electrodes, higher sensitivities and lower detection limits were achieved on longer -NH_2 containing CNT functional groups. In this case, the surface contact area of CNTs and ChOx decreases with increasing the length of the -NH_2 containing functional groups of CNTs. Consequently, less change in enzyme native structure and better electroanalytical parameters occurs on NH_2 containing CNT functional groups with longer lengths.

CONCLUSIONS

In the present report, the electrochemical and electrocatalytic behaviour of ChOx at RTIL/CNTs nanocomposites containing different functionalized CNTs were compared. The results showed that the type of CNT functional groups and their length could affect on the electrochemical behaviour of ChOx. The key point addressed in studying electrochemical and electrocatalytic properties of ChOx is the preference of using NH_2-CNTs rather than HOOC-CNTs. Besides, NH_2-CNTs with longer -NH_2 containing functional groups provide more suitable environment for ChOx activity. Therefore, the attractive biosensing characteristics such as lower detection limit, higher sensitivity and wider linear range were obtained by ChOx immobilized on TEP-NH_2-CNTs due to its longer chains. So, among the different enzyme electrodes, the ChOx/RTIL/TEP-NH_2-CNTs biosensor is more suitable for biosensing applications.

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