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Bioelectrochemistry 62 (2004) 29–35
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Electrochemical characteristics of the immobilization of calf thymus DNA molecules on multi-walled carbon nanotubes

Manli Guo[a,b], Jinhua Chen[b,*], Dengyou Liu[b], Lihua Nie[a], Shouzhuo Yao[a,*]

[a] State Key Laboratory of Chemo/Biosensing and Chemometrics, PR China
[b] College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, PR China

Received 4 June 2003; received in revised form 25 September 2003; accepted 2 October 2003

Abstract

Immobilization of DNA on carbon nanotubes plays an important role in the development of new types of miniature DNA biosensors. Electrochemical characteristics of the immobilization of calf thymus DNA molecules on the surfaces of multi-walled carbon nanotubes (MWNTs) have been investigated by cyclic voltammetry and electrochemical impedance analysis. The peak currents for $Fe(CN)_6^{3-}$/$Fe(CN)_6^{4-}$ redox couple observed in the cyclic voltammograms decrease and the electron-transfer resistance (R_{et}) obtained from the Nyquist plots increase due to the immobilization of DNA molecules (dsDNA or ssDNA) on the surfaces of MWNTs. Most of calf thymus DNA are covalently immobilized on MWNTs via diimide-activated amidation between the carboxylic acid groups on the carbon nanotubes and the amino groups on DNA bases, though the direct adsorption of the DNA molecules on MWNTs can be observed. Additionally, the interaction between DNA molecules immobilized on MWNTs and small biomolecules (ethidium bromide) can be observed obviously by cyclic voltammetry and electrochemical impedance analysis. This implies that the DNA molecules immobilized at the surface of MWNTs, with little structure change, still has the ability to interact with small biomolecules.

Keywords: Calf thymus DNA; Ethidium bromide; Multi-walled carbon nanotubes; Immobilization; Electrochemical characteristics

1. Introduction

Since the discoveries of multi-walled carbon nanotubes (MWNTs) and single-walled carbon nanotubes (SWNTs) in 1991 [1] and 1993 [2], respectively, much attention has been attracted because of their unusual structures and properties [3,4]. Various applications of carbon nanotubes have been investigated, such as batteries [5], tips for scanning probe microscopy [6], the oxidation of dopamine [7], electrochemistry of protein [8–10], chemical sensors [11,12], etc.

Both SWNTs and MWNTs have potential applications in developing new types of miniature biological devices including probes and sensors due to high electrical conductivity, nanometer size and so on. The first step towards achieving this goal involves the immobilization of bioactive molecules on the surfaces of carbon nanotubes through covalent or non-covalent bonds. Recently, several investigations have concerned the attachment of natural proteins to carbon nanotubes [13–16]. Sun et al. [14,15] have reported that bovine serum albumin (BSA) proteins can be covalently attached to SWNTs and MWNTs via diimide-activated amidation under ambient conditions, while the overwhelming majority (~ 90%) of the protein species in the nanotube–BSA conjugates remain bioactive. Dai et al. [16] have developed a controlled and nanotube-specific method for immobilizing proteins and small molecules onto non-covalently functionalized SWNTs.

On the other hand, DNA is an important and promising molecule with all the basic properties necessary for the assembly of nano-scale electronic devices. The construction of DNA electronic nano-devices is possible and will also be a predominant technique of new molectron with attendant benefits of miniaturization, low power requirements, high efficiency and low heat generation [17]. DNA immobilization has been paid great attentions and considered as a fundamental methodology for the construction of DNA biosensors. Lots of flat electrodes, such as gold [17], carbon paste electrode [18] and highly ordered pyrolytic graphite electrode [19], have been used to immobilize DNA. However, only a few investigations have focused on the immo-

* Corresponding author. Tel./fax: +86-731-8821818.
E-mail address: chenjh001@hotmail.com (J. Chen).

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doi:10.1016/j.bioelechem.2003.10.005

bilization of DNA on the surfaces of carbon nanotubes [20–23]. Amino-terminated DNA strands have been attached to the open ends and defect sites of oxidatively prepared SWNTs [20]. Kappes et al. [22] have covalently modified SWNTs with DNA and found that the SWNT–DNA adducts hybridize selectively with complementary strands with minimal nonspecific interactions with noncomplementary sequences. Compared with SWNTs, much cheaper MWNTs produced by the chemical vapor deposition (CVD) method are known to have more defects and can provide more sites for the immobilization of DNA. An MWNTs-enhanced electrochemical DNA biosensor has been fabricated by Fang and used in DNA hybridization detection [24]. Additionally, DNA molecules adsorbed on MWNTs via nonspecific interactions have also been observed [25,26]. In these works, the techniques applied to the study of the interaction between DNA and carbon nanotubes are usually scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal fluorescence microscopy, etc. [20–22]. According to our knowledge, the electrochemical characteristics on the immobilization process of DNA, especially the natural DNA, on the surface of MWNTs is still unclear.

In this work, the electrochemical characteristics on the immobilization of both double-stranded and single-stranded calf thymus DNA molecules on the surface of MWNTs have been investigated by cyclic voltammetry and electrochemical impedance analysis. MWNTs were treated with nitric acid prior to the immobilization of DNA in order to introduce carboxylic acid groups to the surfaces of carbon nanotubes. DNA molecules were covalently immobilized on MWNTs-modified electrode via diimide-activated amidation. Additionally, the interaction between DNA immobilized on MWNTs and small biomolecules, such as ethidium bromide (EB), has been investigated.

2. Experimental

2.1. Chemicals and instrumentation

Calf thymus DNA from Sino–American Biotechnology Company was used as received. The stock solution of double-stranded DNA (dsDNA) (around 2 mg/ml) was prepared with 1/15 M phosphate buffer solution (pH 7.0) containing 0.1 M NaCl and was stored at 4 °C. Single-stranded DNA (ssDNA) was obtained through heating dsDNA solution in a boiling-water bath for about 5 min and then rapidly cooling in an ice-water bath [27]. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxy-succinimide (NHS) were purchased from Merck and Sigma, respectively. EB from Amresco was used without further purification. Multi-walled carbon nanotubes (MWNTs) with the diameter about 70 nm, prepared by chemical vapor deposition (CVD) method [28] were obtained from Dr. Chen's group (Hunan University). Before use, MWNTs were refluxed in concentrated nitric acid for about 5 h, filtered and washed with double-distilled water until the filtrate became neutral, and then dried under vacuum. All other chemicals were of reagent grade. Double-distilled water was used throughout the experiments.

Cyclic voltammetry and electrochemical impedance measurements were carried out at a CHI660A electrochemical workstation. A gold wire (diameter, 0.2 mm), sealed in a glass tube with epoxy, was used as the working electrode. The reference electrode was a saturated calomel electrode (SCE), and a platinum electrode was used as the counter electrode. All experiments were carried out at room temperature.

2.2. Preparation of MWNTs-modified electrode

The gold electrode was cut with a sharp razor blade followed by cycling between 0.0 and 1.5 V vs. SCE in 0.5 M H_2SO_4 until a stable gold oxidation/reduction cyclic voltammogram was obtained. A 5 μl of the 0.5 mg/ml black suspension, which was prepared by dispersing purified MWNTs in double-distilled water with the aid of ultrasonic oscillation, was dropped on the gold electrode surface and then dried under vacuum at about 50 °C.

2.3. DNA immobilization and the interaction between DNA and EB

A 10 μl of a 1/15 M phosphate buffer solution (pH 7.0) containing 3 mg/ml EDC and 5 mg/ml NHS was dropped on the MWNTs-modified electrode surface and was evaporated to dryness for the activation of the carboxylic acid groups on the carbon nanotubes. After rinse, the electrode was immersed in the same phosphate buffer solution containing 2 mg/ml dsDNA (or ssDNA) for several hours. The interaction between EB and DNA was performed by immersing the DNA–MWNTs-modified electrode in a 1/15 M phosphate buffer solution (pH 7.0) containing 1 mM EB.

2.4. Electrochemical measurements

The electrochemical properties of the MWNTs-modified electrode were studied by cyclic voltammetry in 1/15 M phosphate buffer solution. A $K_3Fe(CN)_6/K_4Fe(CN)_6$ (10/10 mM) solution was used as a redox probe to study the interface properties of the electrode immobilized with DNA. 0.1 M NaCl aqueous solution was used as the supporting electrolyte. Electrochemical impedance measurements were performed at open circuit potentials in 10 mM $K_4Fe(CN)_6$ + 10 mM $K_3Fe(CN)_6$ + 0.1 M NaCl aqueous solutions. The frequency scan range was from 0.1 Hz to 100 kHz and the sinusoidal potential amplitude was 5 mV. The electron-transfer resistance was obtained through the nonlinear regression analysis of the semicircle portion on the Nyquist plot (Z_{im} vs. Z_{re}). All solutions were purged at least 5 min with nitrogen before electrochemical measurements.

3. Results and discussion

3.1. Electrochemical behavior of the MWNTs/gold electrode

In order to study the electrochemical characteristic of the MWNTs-modified electrode in media where the immobilization of DNA and the interaction between EB and DNA were performed, cyclic voltammogram of the electrode was recorded in 1/15 M phosphate buffer solution (pH 7.0) containing 0.1 M NaCl. For comparison, the cyclic voltammetric investigation for the bare gold electrode was also carried out. The corresponding results are shown in Fig. 1. As shown in Fig. 1b, a couple of redox peaks can be observed and become stable after several cycles when the gold electrode is modified with MWNTs. At a scan rate of 50 mV s^{-1}, the cathodic and anodic peak potentials were -0.109 and -0.004 V vs. SCE, respectively. As reported in literature [29], the couple of peaks correspond to the redox of the carboxylic acid group, which can be used to immobilize DNA with the aid of EDC and NHS via cross-linking reaction. Compared with the bare gold electrode, the background current of the MWNTs/gold electrode, which is the nature of capacitance, is apparently large. This implies that the real surface area of the MWNTs-modified gold electrode is significantly enhanced. It is benefit to the increase of immobilization mass of DNA on the electrode surface.

Considering the great importance of the stability of the MWNTs-modified electrode to further studies, cyclic voltammetry was carried out after the electrode was rinsed with double-distilled water and stored in air for about 24 h. As shown in Fig. 1c, no obvious change in the peak potentials and currents can be found, which indicates that the MWNTs film exists very stable on the surface of the gold electrode.

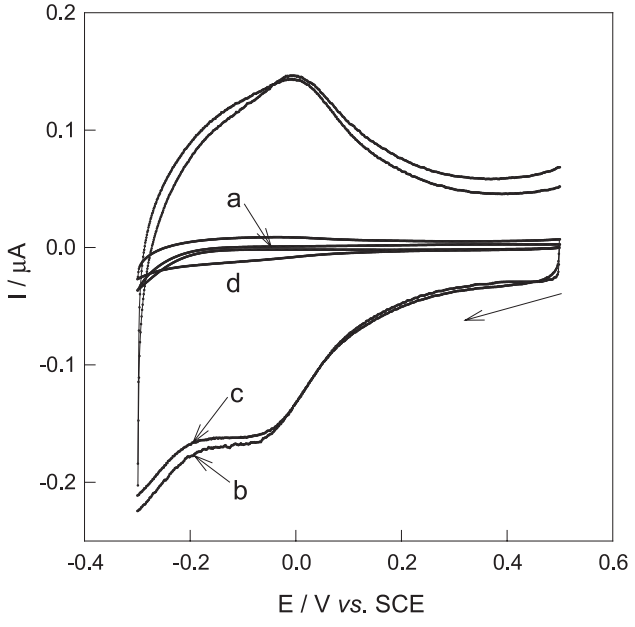

Fig. 1. Cyclic voltammograms for different electrodes in 1/15 M phosphate buffer solution (pH 7.0) containing 0.1 M NaCl (scan rate: 50 mV s^{-1}). (a) bare gold electrode; (b) MWNTs-modified gold electrode; (c) MWNTs-modified gold electrode stored in air for 24 h; (d) MWNTs-modified gold electrode treated with 2 mg/ml dsDNA with EDC/NHS activation for 3 h.

On the other hand, when DNA is immobilized on the MWNTs, it can be observed from Fig. 1d that the redox peaks of –COOH groups on carbon nanotubes disappear and the background current decreases significantly as a result of DNA coupling to MWNTs-modified electrode.

3.2. Cyclic voltammetry study on the immobilization of DNA onto MWNTs-modified electrode

It has been reported by Huang et al. [30] that calf thymus DNA can be attached onto carboxylate-terminated alkanethiol self-assembled monolayers preformed at gold surfaces with the aid of EDC and NHS through the formation of amide bond between the carboxylic groups and the amino groups on the DNA bases. Therefore, based on the aforementioned fact that carboxylic acid groups had been introduced to the carbon nanotubes during the purification process, EDC and NHS were used in this work to immobilize DNA on MWNTs-modified electrode.

Using $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox couple as the indicator, the immobilization of DNA on the MWNTs-modified electrodes has been studied by cyclic voltammetry. Fig. 2A shows the typical cyclic voltammograms about the immobilization process of dsDNA on MWNTs-modified electrode. After the electrode is treated with EDC, NHS and dsDNA, both the anodic and cathodic peak currents for the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox probe decrease. With the immobilization time increasing, the redox peak currents decrease rapidly. This may be explained as follows: after the immobilization of dsDNA on the carbon nanotubes, the phosphate groups negatively charged on dsDNA resist the access of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox couple to the electrode surface [31]. On the other hand, the DNA helices, which lie flat on the surface of the MWNTs due to the interaction between carboxylic acid groups and amino groups [31], may block the electron transfer between the redox probe and the electrode surface due to the low conductivity of DNA molecules.

The immobilization of ssDNA on the surface of MWNTs has also been carried out and the similar CV feature can be observed. To show the processes of DNA immobilization more clearly, the change of the anodic peak current (ΔI_{pa}) of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox probe after DNA immobilization on MWNTs is calculated and the relationships between ΔI_{pa} and the immobilization time are shown in Fig. 2B. From Fig. 2B, it can be observed that the anodic peak current of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox probe decreases quickly at the beginning of the immobilization process. When the immobilization time is longer than 3 h, no obvious change can be observed. This implies that the

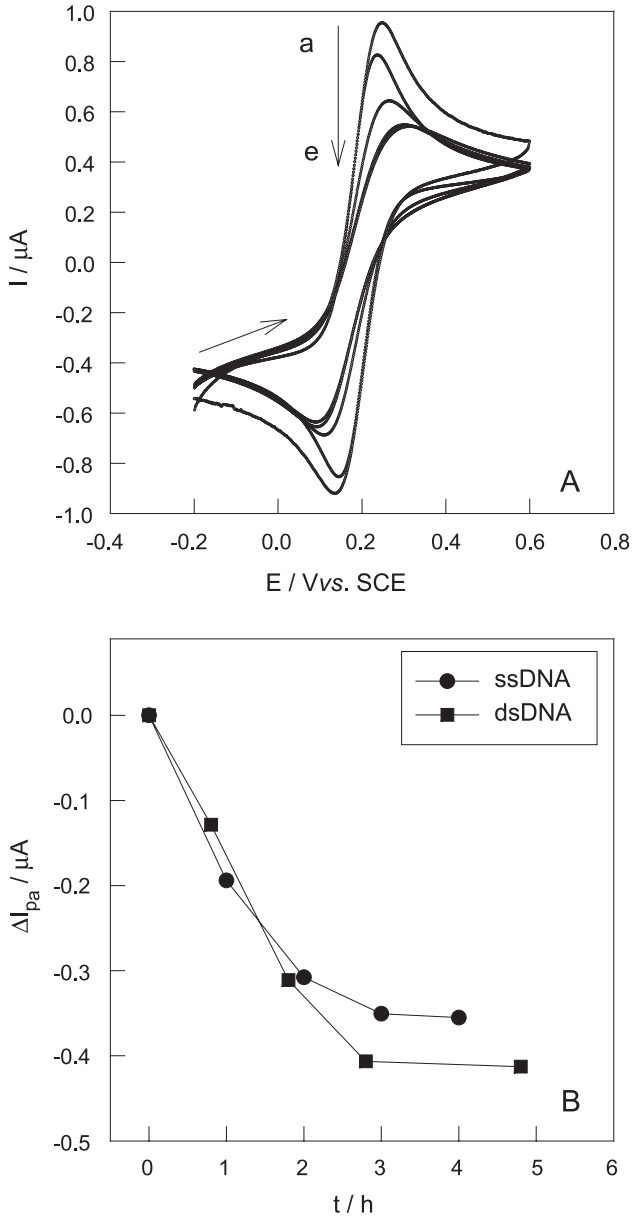

Fig. 2. (A) Cyclic voltammograms for MWNTs-modified gold electrode treated with 2 mg/ml dsDNA with EDC/NHS activation at different times. Supporting electrolyte solution is 10 mM $K_4Fe(CN)_6$ and 10 mM $K_3Fe(CN)_6$ containing 0.1 M NaCl. (a) 0 h; (b) 0.8 h; (c) 1.8 h; (d) 2.8 h; (e) 4.8 h. (B) Changes of the anodic peak current of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox couple at MWNTs-modified electrode immobilized with dsDNA (■) and ssDNA (●) for different time intervals.

saturation of DNA immobilization on MWNTs is reached. Therefore, the immobilization time of both dsDNA and ssDNA was chosen to be 3 h in the further studies. Additionally, it can be observed that the ΔI_{pa} for dsDNA is larger than that for ssDNA when the immobilization time is more than 2 h. This may result from that dsDNA lying flat on the electrode surface has more effective resistance to the access of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox probe to the electrode surface.

3.3. Electrochemical impedance characteristic of the immobilization of DNA onto MWNTs-modified electrode

Electrochemical impedance, the widely used and effective way to study the interface properties of the modified electrode, was also used to identify the immobilization of DNA on MWNTs-modified electrode. The corresponding Nyquist plots for the electrochemical impedance measurements are shown in Fig. 3. For MWNTs-modified electrode, a linear Nyquist plot (curve a), which indicates a diffusion-controlled process, can be observed. This implies that carbon nanotube modified electrode has high conductivity and the redox behavior of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ couple is controlled by diffusion process. After the electrode was treated with dsDNA with EDC/NHS activation for 3 h, the corresponding Nyquist plot (curve b) is a semicircle along with a straight line. The electron-transfer resistance, R_{et}, is estimated from the semicircle diameter and equal to 16.02 kΩ. This implies that the redox process of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ ion couple has been inhibited significantly due to the immobilization of dsDNA on the MWNTs surface. The results obtained from electrochemical impedance analysis method are in agreement with that from CV method.

To investigate whether the increase of the electron-transfer resistance was due to the direct adsorption of dsDNA on carbon nanotubes, a control experiment was performed by treating MWNTs-modified electrode with the same dsDNA solution without EDC/NHS activation for the

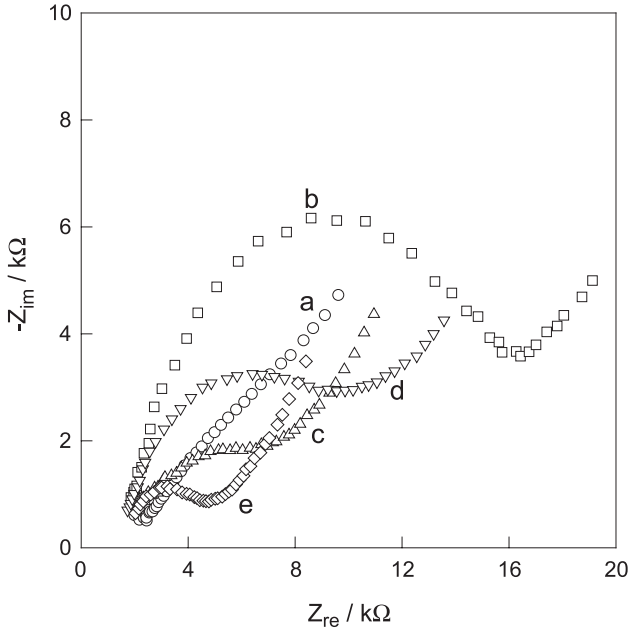

Fig. 3. Nyquist plots (Z_{im} vs. Z_{re}) for different electrodes in 10 mM $K_4Fe(CN)_6$ + 10 mM $K_3Fe(CN)_6$ + 0.1 M NaCl aqueous solutions. (a) MWNTs-modified gold electrode; (b,c) MWNTs-modified gold electrode treated with 2 mg/ml dsDNA with and without EDC/NHS activation, respectively; (d,e) MWNTs-modified gold electrode treated with 2 mg/ml ssDNA with and without EDC/NHS activation, respectively.

same time and the corresponding result is shown in Fig. 3c. As shown in Fig. 3c, a small electron-transfer resistance (6.58 kΩ) is obtained. This implies that the amount of dsDNA directly adsorbed on carbon nanotubes is relatively small and the immobilization of dsDNA on MWNTs-modified electrode through covalent bonds between the carboxylic acid groups on the carbon nanotubes and the amino groups on the DNA bases is dominant.

The electrochemical impedance analysis study on the immobilization of ssDNA on MWNTs has also been carried out and the corresponding Nyquist plot is shown in Fig. 3d. The characteristic of the Nyquist plot is similar to that in Fig. 3b. The corresponding electron-transfer resistance is about 11.41 kΩ, which is smaller than that for the dsDNA-modified electrode (16.02 kΩ). Such a result is consistent with that in the cyclic voltammetry study, and the reason has been mentioned above. On the other hand, the double layer capacitances for dsDNA and ssDNA modified electrodes can also be calculated from Nyquist plots and equal to 3.77 and 6.09 nF, respectively. This may result from the difference in the structure between dsDNA and ssDNA. For ssDNA-modified electrode, much supporting electrolyte solution can be involved in the double layer structure of the electrode due to the single-stranded structure of the ssDNA molecules. A similar control experiment has also been carried out for the MWNTs-modified gold electrode treated with ssDNA without EDC/NHS activation. Compared with Fig. 3d, a smaller semicircle diameter (5.08 kΩ) can be obtained from Fig. 3e, which confirms that ssDNA was immobilized on MWNTs mainly through covalent bonds.

As mentioned above, some DNA (ssDNA and dsDNA) can be adsorbed on the MWNTs modified gold electrode without EDC/NHS activation though DNA was immobilized on MWNTs mainly through covalent bonds. In order to identify whether DNA is adsorbed directly on the gold surface or MWNTs, a control experiment has also been performed by immersing the bare gold electrode in 2 mg/ml DNA solution for 3 h. No obvious changes for the electrodes with or without DNA (ssDNA or dsDNA) treatment have been observed in the corresponding cyclic voltammograms and electrochemical impedance spectra. This implies that no obvious adsorption of DNA (ssDNA or dsDNA) on the bare gold surface occurs and curves c and e in Fig. 3 were caused mainly by the adsorption of DNA on MWNTs.

3.4. Interaction between DNA and ethidium bromide

To investigate whether the characteristic of calf thymus DNA molecules has not changed after they were immobilized on the surfaces of MWNTs, the abilities of double-stranded and single-stranded calf thymus DNA molecules to interact with small biomolecules have been investigated. EB, a well-known DNA intercalator, was chosen and used as the DNA-binding reagent. The interactions between calf thymus DNA molecules (double-stranded or single-stranded) and EB have been studied by cyclic voltammetry and electrochemical impedance analysis.

Fig. 4A shows the cyclic voltammograms for the dsDNA–MWNTs-modified electrode before and after the interaction with 1 mM EB. After the electrode is treated with EB, the anodic peak current decreases obviously while the slight decrease of cathodic peak current can be observed. The split of the redox potentials changes from 0.108 to 0.189 V. The worse symmetry of peaks and the increasing of the peak-to-peak separation indicate that the irreversibility

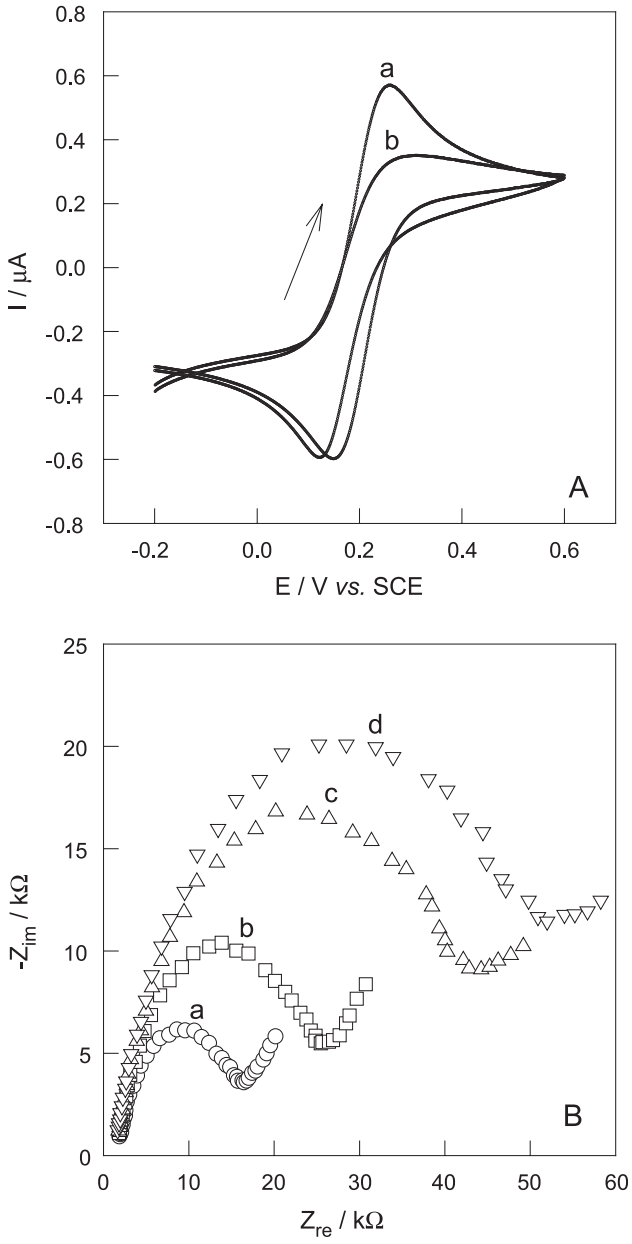

Fig. 4. (A) Cyclic voltammograms for dsDNA–MWNTs-modified electrode before (a) and after (b) the treatment with 1 mM ethidium bromide. (B) Nyquist plot for dsDNA–MWNTs-modified electrode treated with 1 mM ethidium bromide for (a) 0 min; (b) 2 min; (c) 7 min and (d) 18 min. Supporting electrolyte solution is 10 mM $K_4Fe(CN)_6$ and 10 mM $K_3Fe(CN)_6$ containing 0.1 M NaCl.

of the redox behavior of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ couple increases. These results imply that the intercalation of EB into DNA molecules between the stacked base pairs of the DNA double helix structure happens and the characteristic of double-stranded calf thymus DNA molecules has not changed obviously after they were immobilized on the surface of MWNTs.

The interactions between EB and dsDNA at different time intervals have also been investigated by electrochemical impedance analysis and the corresponding Nyquist plots are shown in Fig. 4B. After the dsDNA/MWNTs electrode is treated with EB, the semicircle diameter increases obviously, which also indicates that EB has interacted with DNA molecules immobilized on MWNTs. From Fig. 4B, it can also be observed that the semicircle diameter of the Nyquist plot increases with the interaction time increase. The electron transfer resistance, R_{et}, can be derived from the Nyquist plots and equal to 16.02, 25.73, 45.58, and 56.19 kΩ at 0, 2, 7, and 18 min, respectively. This also indicates clearly that more and more EB molecules intercalate into dsDNA.

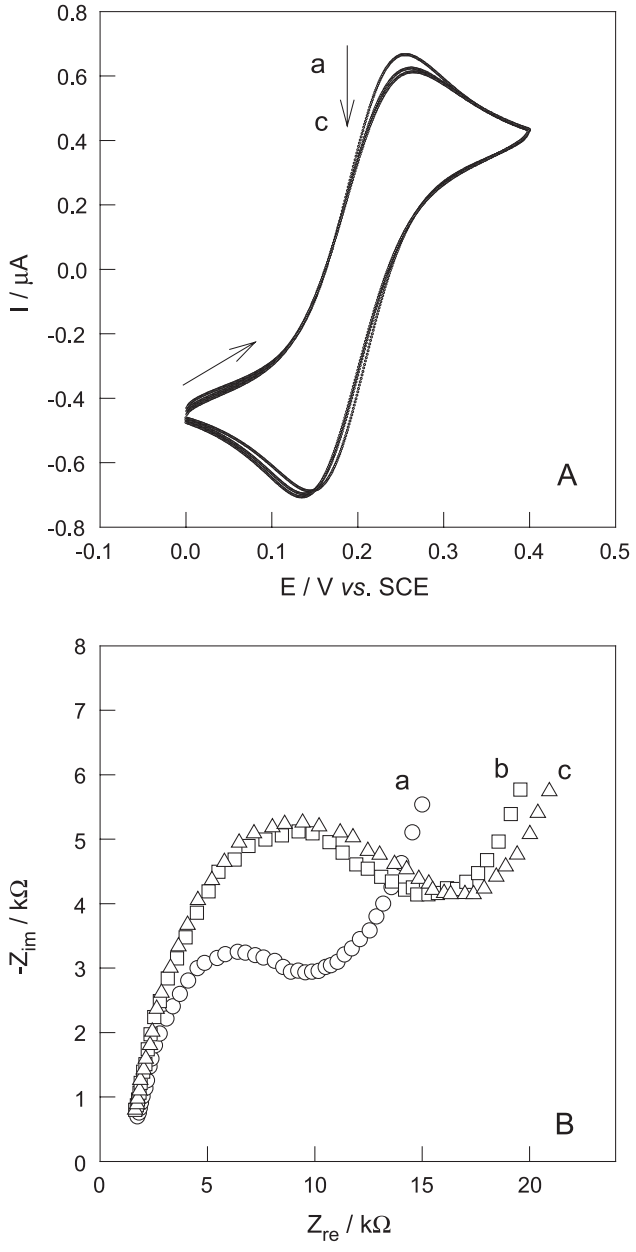

Fig. 5. Cyclic voltammograms (A) and Nyquist plot (B) for ssDNA–MWNTs-modified electrode treated with 1 mM ethidium bromide for (a) 0 min; (b) 10 min and (c) 20 min. Supporting electrolyte solution is 10 mM $K_4Fe(CN)_6$ and 10 mM $K_3Fe(CN)_6$ containing 0.1 M NaCl.

The interaction between EB and ssDNA immobilized on the surface of MWNTs were also studied with the same methods. The corresponding results are shown in Fig. 5. The features of cyclic voltammograms and Nyquist plots are similar to that in Fig. 4. However, there are still some differences between Figs. 4 and 5: (1) When DNA electrode is treated with EB, the change of the anodic peak current in Fig. 5 is much smaller than that in Fig. 4. (2) The change of the electron-transfer resistance for the interaction between ssDNA and EB (4.98 kΩ for 10 min) is much smaller than that for the interaction between dsDNA and EB (29.56 kΩ for 7 min). (3) For ssDNA, the saturation state for the interaction between ssDNA and EB will be reached quickly and no obvious changes for the cyclic voltammograms and electrochemical impedance spectra can be observed after about 10 min (Fig. 5). However, for the interaction between dsDNA and EB, no saturation state can be observed even when the interaction time is equal to 18 min (Fig. 4). The electron-transfer resistance increases continuously with the increase of the interaction time. These results imply that the interaction between ssDNA and EB is very weak and the direct adsorption of EB on the ssDNA-electrode surface may be dominant. This is in agreement with Fang's report [27].

4. Conclusions

The immobilization of biomolecules at the surface of carbon nanotubes has great importance in introducing carbon nanotubes into biological systems. Cyclic voltammetry and electrochemical impedance have been used to characterize the immobilization process of both double-stranded and single-stranded calf thymus DNA molecules on the surface of multi-walled carbon nanotubes (MWNTs). It has been found that most of calf thymus DNA are covalently immobilized on MWNTs via diimide-activated amidation between the carboxylic acid groups on the carbon nanotubes and the amino groups on DNA bases, though the direct adsorption of the DNA molecules on MWNTs can be observed. Additionally, the interaction between DNA molecules and small biomolecules (EB) can be observed obviously by cyclic voltammetry and electrochemical impedance analysis and the results are in good agreement with that reported in the literatures. This implies that DNA molecules immobilized at the surface of MWNTs still has the ability to interact with small biomolecules.

Acknowledgements

This work was supported by the National Natural Science Foundation of China under the grant 20275009 and the Foundation of Ministry of Education under the grant 2001-498.

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