Direct DNA Immobilization onto a Carbon Nanotube Modified Electrode: Study on the Influence of pH and Ionic Strength

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ABSTRACT

Over the past years, DNA biosensors have been developed to analyze DNA interaction and damage that have important applications in biotechnological researches. The immobilization of DNA onto a substrate is one key step for construction of DNA electrochemical biosensors. In this report, a direct approach has been described for immobilization of single strand DNA onto carboxylic acid-functionalized carbon nanotubes modified glassy carbon electrode. To do this, we first modified the glassy carbon electrode surface with MWCNT-COOH. The immersion of MWCNT-COOH/GCE in ss-DNA probe solution, with different pH and ionic strength, was followed by suitable interaction between amine group of ss-DNA bases and carboxylic groups of MWCNT-COOH. This interaction leads to successful ss-DNA immobilization on MWCNT-COOH that was confirmed by cyclic voltammetry, electrochemical impedance spectroscopy and atomic force microscopy. Immobilization of ss-DNA on the modified electrode increased the charge transfer resistant but decreased the peak current of redox probe ($[Fe(CN)_6]^{3-/4-}$). The result of cyclic voltammograms implicates that enhancements in the DNA immobilization are possible by adroit choice of low pH and high ionic strength. The standard free-energy of adsorption (ΔG°_{ads}) was calculated from electrochemical impedance spectroscopy data (-47.75 kJ mol^{-1}) and was confirmed covalent bond formation. atomic force microscopy topographic images demonstrate increased surface roughness after ss-DNA immobilization. Results offer a simple, rapid and low-cost of DNA immobilization strategy can be opportunities to design of novel nucleic acid biosensors.

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INTRODUCTION

In the recent years, there has been a burgeoning interest in the nucleic acid biosensor fabrication. These biosensors can be used in many fields of study involving clinical genetic testing and research-based molecular diagnostics. A biosensor is an analytical device that incorporates biological materials connected to a transducer. Transducer converts biochemical signal into a measurable analytical signal. The transducing system may be optical, piezoelectric or electrochemical [1].

In this context, electrochemical DNA biosensors have been the topic of considerable interest in many researchers for routine laboratory analysis. Electrochemical techniques have the advantageous of high selectivity, sensitivity, miniaturization capability and feasibility compared to the other methods [2]. A basic electrochemical sensor consists of oligonucleotide strands on a transducer surface, which hybrid with specific complementary target sequence (DNA or RNA). The event of hybridization between capture and

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target strands is detected and transformed into a current signal [3].

The immobilization DNA on solid surfaces is one of the most crucial steps toward fabricating DNA biosensor. This process has a great influence on DNA biosensor efficiency. Up to now, several kind of techniques have been adopted for surface-capturing DNA probes onto sensor surfaces based on electrostatic attraction [4], controlled potential adsorption [5,6], Langmuir–Blodgett technique [7], biotin–streptavidin interaction [8] and covalent binding [9].

Covalent immobilization utilizes surface bound functional groups that can react with DNA sequence. In the last decade, a number of approaches have been described for covalent immobilization of DNA onto different surfaces [10–12]. Amine coupling are commonly used through interaction between amine group of DNA bases and activated carboxylic groups on the electrode surface [13].

Recently, considerable attention has been devoted on the electrochemical biosensor based nanomaterials and nanostructures for signal amplifications [14]. Carbon nanotubes (CNTs) have attracted significant attention as components in electrochemical biosensors due to the intriguing properties such as mechanically strong, highly thermal conductivity and chemically stable nature of CNTs [15–17]. In particular, their high surface to volume ratio has led to great use of CNTs in bioanalytical applications [18]. Daniel et al. reviewed immobilization of DNA onto CNTs that can be employed for fabricating DNA biosensors [19]. The functionalization CNTs with different chemical groups is a prerequisite of covalent attaching biomolecule. In this order, carboxylic acid functionalized CNTs commonly applied because these functional groups are easily incorporated on the edge and side wall of CNTs via acidic oxidation treatment. For instance, carboxylic acid-functionalized multi walled carbon nanotubes (MWCNTs-COOH) was exploited for covalent DNA immobilization by Cai and coworkers [20]. They utilized 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxy succinimide (NHS) as activation agents for MWCNTs-COOH. EDC reacts with a carboxylic acid to form an active ester intermediate which is then displaced by an NHS ester. This more reactive ester then rapidly couples with primary amines. The amino-terminal end of oligonucleotides is able

to bind covalently with activated CNTs through the formation of amide bonds. However, the mentioned strategies suffer from some problems such as additional reagent for activation of surface and time-consuming. Major efforts have been devoted to development DNA biosensors that allow direct, simple and rapid attachment of DNA sequences onto the electrode surface.

In general, DNA is considered as negatively charged biomolecule because negative charge of its sugar phosphate backbone at physiological condition. So, an electrostatic repulsion exists between DNA molecules that lead to hinder adsorption of other DNA sequences on the immobilizing matrix. Consequently, immobilization step needs long time to form a stable DNA conjugate [21]. Therefore, in order to fabrication DNA biosensors, numbers of scientists have been working on reduces electrostatic repulsion between DNA molecules. Several early studies described fast DNA immobilization based on low pH induced and high salt concentration solution due to a decrease of repulsive interaction between DNA molecules [22–25].

Here we described a simple, efficient and rapid approach for ss-DNA immobilization on MWCNT-COOH modified glassy carbon electrode (GCE) based on amine coupling mechanism. The pH and ionic strength of ss-DNA probe solution play key roles for promoting DNA immobilization in our strategy. Cyclic voltammetry and electrochemical impedance spectroscopy (EIS) techniques and also atomic force microscopy (AFM) method was used for characterization of electrode surface which provide evidence for ss-DNA immobilization. The present study focuses on the covalent attachment of DNA probes on carbon electrodes that may provide a base for biosensing platform.

MATERIALS AND METHODS
Materials and reagents

MWCNTs with lengths of 50 μm was purchased from Nano times Company (Chengdu, China) and applied without additional purification. Potassium chloride (KCl), dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCl), tetrapotassium ferrocyanide trihydrate ($K_4[Fe(CN)_6].3H_2O$), tripotassium ferricyanide tetrahydrate ($K_3[Fe(CN)_6].4H_2O$), dimethyl-formamide (DMF), citric acid, and nitric acid were purchased from Merck. All solutions were prepared with autoclaved double distilled water. Synthetic ss-

DNA sequence was purchased from Bioneer Corporation (South Korea) that its base sequence was as follow: 5′-TAG CTT ATC AGA CTG ATG TTG A-3′. The phosphate buffer solution was prepared with 0.1 M K_2HPO_4 and KH_2PO_4 as supporting electrolyte (PBS, pH 7.0).

MATERIALS AND METHODS

Cyclic voltammograms were carried out using a potentiostat/galvanostat SAMA500 electrochemical analysis system (Isfahan, Iran). Impedance spectra were recorded by an Autolab system (model PGSTAT30) with the following parameters; AC voltage amplitude 0.005 V, voltage frequencies from 10^3 to 0.1 Hz. All experimental results were fitted to a Randle's equivalent circuit, using NOVA software, which allows to resistance extracting. Faradaic impedance measurements were made on different modified GCE using a conventional three electrodes-cell system operated at room temperature. A glass three electrode cell, a working GCE (Φ=2 mm), an Ag/AgCl (3 M KCl solution) reference electrode, and a platinum wire auxiliary electrode were employed. AFM topographic images were recorded using a Multi-Mode SPM instrument equipped with a DS 95-50-E scanner (Dualscope/ Rasterscope C26, DME, Denmark).

Working electrode preparation

In this study, MWCNT-COOH was prepared according to the literature [26]. In a typical procedure, 20 mg of MWCNTs were dispersed in 30 mL nitric acid solution (35%) by ultrasonic bath for 3 h. After that, the black solution was filtered with vacuum filtration equipped poly tetra fluoro ethylene (PTFE) membrane (pore size 0.45 μm) and washed thoroughly with distilled water until neutral pH, and then dried under the infrared lamp.

GCE was first polished mechanically on alumina slurry (5 and 0.5 μm, respectively) using a polishing cloth. The electrode was cleaned ultrasonically in ethanol and water for 5 min. MWCNT-COOH was dispersed into DMF (2 mg mL^{-1}) to provide a black suspension. Next, 1 μL suspension of MWCNT-COOH was dropped onto the surface of cleaned GCE and allowed to dry at room temperature.

DNA probe immobilization onto the electrode surface

In order to immobilize ss-DNA, MWCNT-COOH modified GCE (MWCNT-COOH/GCE) was immersed in ss-DNA probe solution for 120 min at room temperature. The probe solution for ss-DNA immobilization was 10 mM K_2HPO_4-citric acid, 1.0 M NaCl containing 1.0 μM oligonucleotide sequence (pH 5.4). All DNA modified electrodes (DNA/MWCNT-COOH/GCE) were adequately rinsed with autoclaved double distilled water prior to use for removing unbound ss-DNA probe.

RESULTS AND DISCUSSION
Characterization of MWCNT-COOH

FTIR spectroscopy was done to assess polar functional groups that generated on the MWCNT after chemical oxidation. The FTIR spectrum exhibits a number peaks at specific wave numbers which can be correlated with bond types. The infrared spectra of carboxylic acid-functionalized MWCNTs are presented Fig. 1. One peak at 3430 cm^{-1} could is assigned to stretching vibration of hydroxyl groups. The stretching vibration of C=O was appeared around 1628 cm^{-1} [27, 28]. So, FTIR spectra confirm the presence of carboxylic acid groups on the surface of MWCNTs.

Cyclic voltammetric studies on DNA immobilization

Cyclic voltammetry is one of the most popular electrochemical methods that often used for attaining information about electrochemical processes taking place at electrode-solution interface. Cyclic voltammograms of MWCNT-COOH/GCE in a blank PBS pH 7.0 were recorded before and after immobilization of ss-DNA to evaluate its immobilization (Fig. 2). The MWCNT-COOH/GCE shows higher background current in comparison to electrochemical response of bare GCE (curves a and b). This increasing reveals good electron transfer ability and also effective surface area enhancement as well as provide active binding groups for binding of oligonucleotides. As
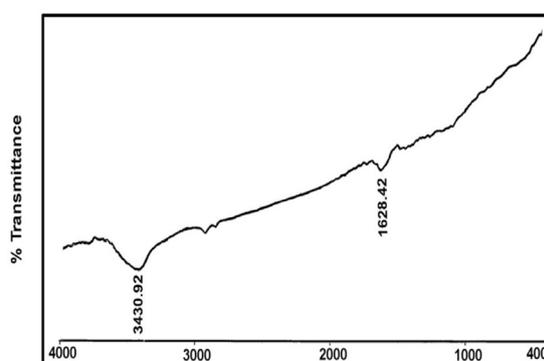

Fig. 1. Infrared spectra of MWCNTs treated with nitric acid.
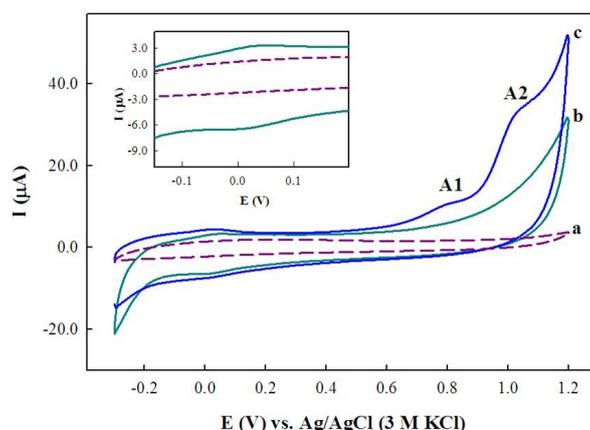
Fig. 2. Cyclic voltammograms of the modified GCE recorded in PBS (pH 7.0) at a scan rate of 0.1 Vs⁻¹, in the potential range between -0.3 to +1.2 V (a) bare GCE, (b) MWCNT-COOH/GCE, and (c) ss-DNA/MWCNT-COOH/GCE.
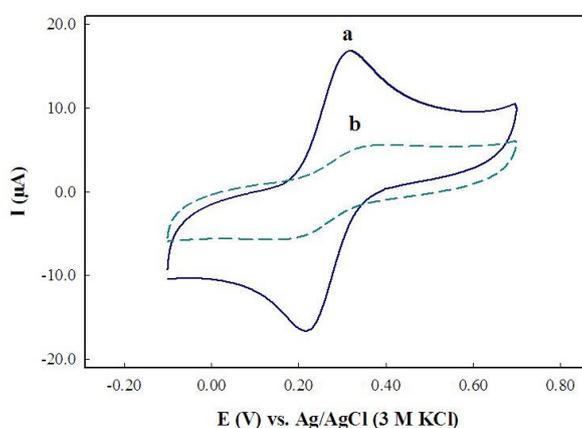

Fig. 3. Cyclic voltammograms of (a) MWCNT-COOH/GCE and (b) ss-DNA/MWCNT-COOH/GCE in presence of 0.01 M [Fe(CN)6]$^{3-/4-}$ in 0.1 M KCl. Scan rate: 0.1 Vs⁻¹.

can been seen from curve b, cyclic voltammograms of MWCNT-COOH/GCE displays one pair anodic and cathodic peak, corresponding to the redox of the carboxylic acid group [29]. As illustrated in curve c, two irreversible anodic peaks at +0.78 and +1.03 V (A1 and A2, respectively) were appeared in the first anodic scan after that MWCNT-COOH/GCE was immersed in 1 µM ss-DNA probe solution (pH 5.4) for 120 min. These irreversible anodic peaks assign to the oxidation of guanine (A1) and adenine (A2) residues in immobilized ss-DNA molecules [30,31]. This result illustrated that the ss-DNA sequences were successfully immobilized on the MWCNT-COOH/GCE.

To further confirm the ss-DNA immobilization process, change the electrochemical signal of an electro-active redox probe can be used for this purpose. Cyclic voltammograms of modified electrodes were carried out in 0.01 M [Fe(CN)$_6$]$^{3-/4-}$ (1:1) solution containing 0.1 M KCl in the potential

range -0.1 to +0.7 V at a scan rate of 0.1 Vs⁻¹ (Fig. 3). As shown in curve b, a significant reduce in peak current and increase in the ΔEp value appeared for ss-DNA/MWCNT-COOH/GCE. This could be attributed to modification of electrode surface with ss-DNA through amide bond formation between amine groups of guanine bases and carboxylic acid groups on MWCNTs [13]. The ss-DNA immobilized on MWCNT-COOH/GCE could act as blocking electron transfer between redox probe and the electrode surface and so decreases conductivity and peak currents.

Electrochemical impedance studies on DNA immobilization

EIS as a powerful technique which provides detailed information to describe the behavior of electrode–solution interface [32]. This technique now gained popularity for development of biosensor as well as for diagnostic applications due to useful information that can be attained from EIS
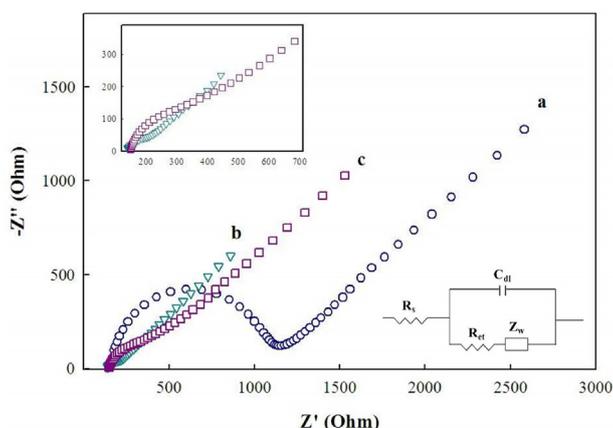
Fig. 4. Nyquist diagrams recorded for (a) GCE, (b) MWCNT-COOH/GCE and (c) ss-DNA/MWCNT-COOH/GCE. The inset displays the Randles circuit.

[33–35]. The Nyquist plot is a typical impedance spectrum which displays a semicircular portion at higher frequencies and a linear portion at lower frequencies. The diameter of semicircular portion corresponds to the electron transfer resistance (R_{et}), whereas the linear part corresponds to the lower frequency regimes where the electrochemical process is under mass transfer control [36]. Fig. 4 gives the Nyquist plot of a bare GCE (a), MWCNT-COOH/GCE (b) and ss-DNA/MWCNT-COOH/GCE (c) in 10 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl. The inset of Fig. 4 shows Randle's equivalent circuit that used to fit the experimental EIS data. As shown in curve a, the bare GCE demonstrated a larger semicircle with a R_{et} of 899.3 Ω, showing resistance towards the electron transfer process on the GCE. When MWCNT-COOH was deposited on the surface of the GCE, the semicircle portion significantly decreased relative to the bare GCE (curve b). The R_{et} value was estimated to be 61.4 Ω. The low R_{et} is due to excellent electrical conductivity of carbon nanotubes and thus accelerated electron transfer [37]. The R_{et} value was further increased to 196.8 Ω after immobilization of ss-DNA sequences on the MWCNT-COOH (curve c). This phenomenon can be explained by the fact that immobilized DNA molecules reduce effective area to transfer of electron due to increase in the thickness of interface. Also, electrostatic repulsions between the negatively charged DNA backbone and the negatively charged redox probe $[Fe(CN)_6]^{3-/4-}$ caused a high resistance of electron transfer between redox probe and electrode surface [38].

The difference in electron transfer resistance before and after ss-DNA adsorption confirmed that the DNA sequences were immobilized on the surface of modified electrode.

The standard electron transfer rate constant, k_s (cm s^{-1}), for the electrochemical reaction of $[Fe(CN)_6]^{3-/4-}$ at the surface of each electrode was calculated according to the following equation [36]:

$$k_s = R \times T / n^2 \times F^2 \times R_{et} \times A \times C \qquad (1)$$

where R_{et} is electron transfer resistance obtained from the EIS (Ω), A is the electrode surface area (cm^2) and C is the bulk concentration of the redox species (mol cm^{-3}). It should be noted that this equation is valid when the oxidized and reduced forms of the redox species have the same concentration ($C_{[Fe(CN)_6]^{3-}} = C_{[Fe(CN)_6]^{4-}} = 10 \times 10^{-3}$ mol cm^{-3}).

The value of k_s was 9.4×10^{-8}, 1.4×10^{-6}, and 4.3×10^{-7} cm s^{-1} for the bare GCE, MWCNT-COOH/GCE and ss-DNA/MWCNT-COOH/GCE, respectively. Thus, the most value of k_s for MWCNT-COOH/GCE shows that the diffusion of the redox probes to the electrode surface was facilitated in the presence of MWCNT-COOH. The electron transfer rate decreased after immobilization of ss-DNA due to the electrostatic repulsion between negatively charged ss-DNA and $[Fe(CN)_6]^{3-/4-}$.

Optimization of analytical parameters for DNA immobilization

The pH and ionic strength of DNA probe solution as well as incubation time play special role on the DNA immobilization process. Thus, optimal

experimental parameters were investigated by CV technique to achieve better condition for DNA immobilized on the sensor surface. Fig. 5A displays obtained cyclic voltammograms for immobilization of ss-DNA at different pH values. The redox peak current for $[Fe(CN)_6]^{3-/4-}$ reduced with decrease of pH value. This decrease peak currents in cyclic voltammogram correspond to the increasing number of ss-DNA immobilized onto the modified electrode due to barrier properties of ss-DNA. At pH 5.4, the lowest current is attributed to maximum amount of ss-DNA immobilized on MWCNT-COOH surface. To obtain the maximum immobilized ss-DNA, the effect of ionic strength conditions was investigated in certain range of NaCl concentration (0.0 to 2.0 M). Fig. 5B represents the gradually enhancement in amount of ss-DNA adsorption with increasing NaCl concentrations, that confirms with reduce in catodic and anodic peak current of redox probe. When NaCl concentration increases more than 1.0 M, no significant change in the peak current was observed. One possible explanation is that the positively charged ions existences in a low pH (5.4) and high salt concentration were electrostatically interact with negative charges

of phosphate groups on nucleotide chains. For example, sodium ions prefer major binding to oxygen atoms of the phosphate group and bind directly with DNA [39]. Consequently, anionic phosphate backbone of DNA can be neutralized and the electrostatic repulsive interaction between DNA molecules can be screened [25]. Also, the positively charged ions reduces the effective DNA diameter thickness which availed the compactness of ss-DNA attachment on surface [40,41]. Subsequently, pH 5.4 of buffer contain 1.0 M NaCl is chosen as the optimized conditions for ss-DNA immobilization.

The effect of incubation time of MWCNT-COOH/GCE with ss-DNA solution was also investigated. As can be seen from Fig. 5C, the redox peak currents were decreased with extending incubation time to 120 min. This result indicates that the carboxylic acid groups on MWCNTs are presumably fully attached with DNA probes. So the optimal incubation time was chosen 120 min for immobilization of ss-DNA onto the surface of modified electrode.

Thermodynamic study of adsorption

The area percentage (Θ) of DNA onto the modified electrode surface was estimated by using followed equation [25].
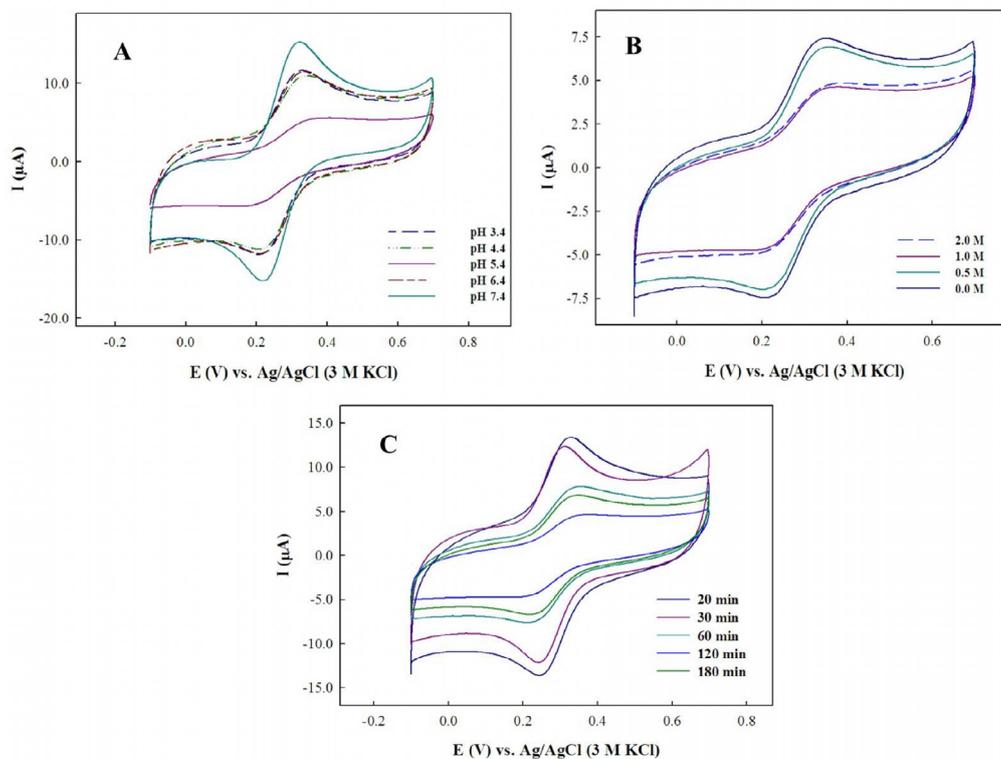

Fig. 5. Cyclic voltammograms recorded in presence of 0.01 M [Fe(CN)6] $^{3-/4-}$ containing 0.1 M KCl for ss-DNA immobilization on MWCNT-COOH/GCE as a function of (A) pH, (B) NaCl concentration and (C) incubation time.
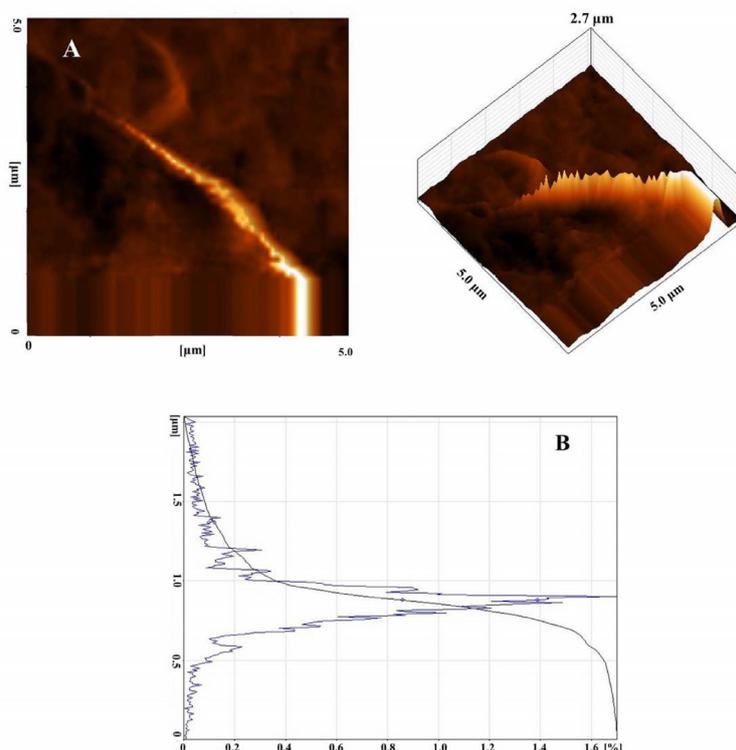
Fig. 6. (A) Typically AFM image of MWCNT-COOH (image size: 5μm × 5μm × 2.7μm), (B) Image height distribution.

$$\Theta = 1 - (R^{bare}/R^{ss\text{-}DNA}) \tag{2}$$

where R^{bare} and $R^{ss\text{-}DNA}$ are R_{et} values for two modified electrodes; MWCNT-COOH/GCE and ss-DNA/MWCNT-COOH/GCE, respectively.

The relation between ss-DNA concentration in buffer solution (C) and surface coverage obeys Langmuir adsorption isotherm and gives a straight line with a slope of K with correlation coefficient 0.9900, where K_{ads} is adsorption equilibrium constant

$$K_{ads}C = \Theta/(1-\Theta) \tag{3}$$

The relation between the standard state adsorption free energy (ΔG°_{ads}) and K_{ads} are given by following equation [42]:

$$K_{ads} = 1/55.5 \exp(-\Delta G^{\circ}_{ads}/RT) \tag{4}$$

where, 55.5 is water molar concentration, R and T are gas constant and absolute temperature, respectively. It is well known that the values of ΔG°_{ads} more positive than -20 kJ mol^{-1} are attributed to physical adsorption, whereas those more negative than -40 kJ mol^{-1} show chemically adsorption [43]. Thus according to calculated value

of ΔG°_{ads} (-47.75 kJ mol^{-1}) for ss-DNA immobilized onto the electrode surface at pH 5.4, ss-DNA sequences were immobilized via a chemisorption process and involves the formation of amid bonds.

Topographic AFM characterization of ss-DNA immobilization

In the last decade, AFM has become one of the most valuable techniques for imaging and study of biological samples. AFM is also an excellent tool for characterization of immobilized biomolecules on the different surface, especially for DNA molecules [44–48]. In our experiment, AFM images of MWCNT-COOH were taken before and after incubation in ss-DNA solution. Fig. 6A displays AFM topographic image of MWCNT-COOH on silica substrate. The AFM image shows average roughness of 215 nm for MWCNT-COOH that this value was obtained through DME software. AFM image after immobilization of ss-DNA onto the surface demonstrates topography changes (Fig. 7A) and the average surface roughness increased to 260 nm. Comparisons of height distribution show that average height was increased after ss-DNA immobilization (Fig. 6B and 7B). The
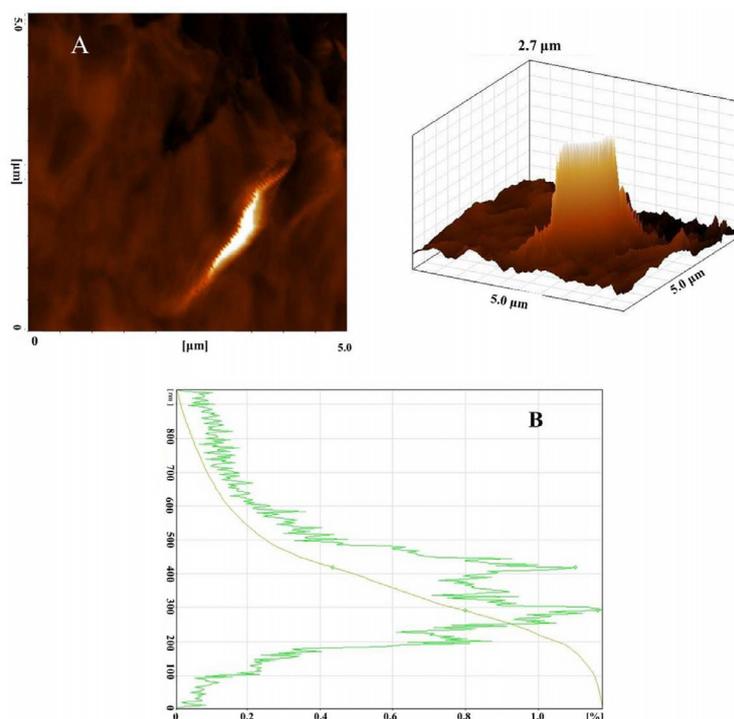
Fig. 7. (A) Typically AFM image of ss-DNA immobilization on MWCNT-COOH (image size: 5 μm × 5 μm × 2.7μm), (B) Image height distribution.

three-dimensional surface plots clearly indicate immobilization of DNA probe onto MWCNT-COOH.

CONCLUSION

The present study demonstrated a direct and simple technique for ss-DNA immobilization on MWCNT-COOH. Without the need for ss-DNA labeling and using additional reagent, covalent attachment of DNA probe will considerably facilitate with low pH and high ionic strength. The immobilization of ss-DNA was confirmed with increasing charge transfer resistance. The negative value of $\Delta G°_{ads}$ more than -40 kJ mol^{-1} shows covalent binding of ss-DNA. A comparison of AFM results suggests the formation of DNA layer on surface of MWCNTs-COOH. This simplistic and low-cost approach very promising for fabrication of wide biosensor based on oligonucleotides segment. The knowledge gained from this report offer new opportunities to future design of novel nucleic acid biosensor.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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