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Electrophoretically deposited multiwalled carbon nanotube based amperometric genosensor for *E.coli* detection

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Abstract. This work reports on a sensitive and selective genosensor fabrication method for *Escherichia coli* (*E.coli*) detection. The functionalized multiwalled carbon nanotubes (MWCNT) synthesized via chemical vapour deposition have been deposited electrophoretically onto indium tin oxide coated glass surface and have been utilized as matrices for the covalent immobilization of *E. coli* specific probe oligonucleotide that was identified from the 16s rRNA coding region of the *E. coli* genome. This fabricated functionalized MWCNT based platform sought to provide improved fundamental characteristics to electrode interface in terms of electro-active surface area and diffusion coefficient. Electrochemical cyclic voltammetry revealed that this genosensor exhibits a linear response to complementary DNA in the concentration range of 10^{-7} to 10^{-12} M with a detection limit of 1×10^{-12} M.

1. Introduction

The detection of bacterial pathogens in food and water is of extreme importance as food borne and water borne diseases have become a major public health problem worldwide due to increasing pollution and changing lifestyle [1]. The presence of *Escherichia coli* (*E. coli*) is used as a potential marker for diagnoses of water borne diseases affecting humans [7]. Most of the strains of *E. coli* are harmless but some strains are pathogenic and cause diarrhoea, urinary tract infection, inflammation and peritonitis. The traditional methods for *E. coli* detection include multiple-tube fermentation, membrane filter and plate counts that are accurate and sensitive, but these techniques have certain limitations such as operational complications, lack of specificity and are time consuming (24–48 hrs) [8]. Therefore, rapid and sensitive technique for *E. coli* detection is urgently needed.

Electrochemical DNA biosensors based on the conversion of molecular recognition reactions into useful response signals, offer considerable promise for DNA analysis. Different nanomaterials have been utilized for matrix formation of various biosensors. The biomedical applications of nanoscale materials are governed by the fact that they exhibit size compatibility with bio-receptors such as cells, viruses, proteins and nucleic acids. There has been an explosion of interest in the use of carbon based nanomaterials for biosensor development owing to their properties like large surface area, conductivity, biocompatibility and ease of functionalization. Different carbon materials which have been studied for biosensing application include graphite powder, carbon nanotubes (CNTs), fullerenes, carbon nanofibers and graphene. Of these



CNTs are of special interest as they possess all the properties like large surface area, high conductivity, biocompatibility and their special geometry allows suitable biomolecule immobilization [4]. Two types of carbon nanotubes are known, single walled (SWCNTs) and multiwalled (MWCNTs). SWCNTs possess a singular graphite sheet rolled into a tube to create a cylindrical nanostructure, whereas MWCNTs consist of several shells of cylindrical tubes. Both MWCNTs and SWCNTs have been used to fabricate sensitive and selective enzyme based biosensors, immunosensors as well as genosensors [11, 12].

For biosensing application, a precise control over the spatial arrangement and distribution of the nanoparticles onto the substrate is utmost important. Among the various techniques, electrophoretic deposition (EPD) offers rapid deposition of desired materials and their composites with a simple design setup, thereby presenting a scope as an automated and continuous process on an industrial scale [2]. It provides tunable control over the degree of deposition of the charged colloidal particles onto an electrode surface under the influence of an applied electric field. In the present work, we have utilized electrophoretically deposited MWCNTs platform for the fabrication of genosensor for *E. coli* detection. In order to increase the biomolecules immobilization as well as dispersability of MWCNTs to render it suitable for EPD they have been functionalized with $-COOH$ groups. Then a specific probe oligonucleotide that is identified from the 16s rRNA coding region of the *E. coli* genome has been immobilized covalently onto the fabricated matrix to construct a label free electrochemical genosensor for sensitive and selective detection of *E.coli*.

2. Materials and methods

2.1 Chemicals

Toluene, Ferrocene, Bovine Serum Albumin (BSA), N-hydroxysuccinimide (NHS), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium ferricyanide ($K_3[Fe(CN)_6]$), sodium dihydrogen phosphate (NaH_2PO_4), sodium monohydrogen phosphate (Na_2HPO_4) and all other chemicals were of analytical grade and were procured from Sigma Aldrich (www.sigmaaldrich.com). Aqueous solutions and buffers were prepared in Milli-Q water (18 M Ω cm). Probe sequence specific to *E. coli* that identified from the 16s rRNA coding region of the *E. coli* genome and complementary target sequences were procured from Sigma Aldrich, Milwaukee, USA and are as follows: 13 DNA probe (pDNA): NH_2 -5'-GGT CCG CTT GCT CTC GC-3' complementary (cDNA): 5'-GCG AGA GCA AGC GGA CC-3'. The solutions of oligonucleotides were prepared in Tris-EDTA buffer (1 M Tris-HCl, 0.5 M EDTA) at pH 8.0 and stored at $-20^\circ C$ prior to use.

2.2 Synthesis and Functionalization of carbon nanotubes

Multiwalled Carbon nanotubes have been synthesized using chemical vapour deposition (CVD). Toluene and ferrocene have been used as precursors that act as catalyst and hydrocarbon source respectively [6]. Further, synthesized carbon nanotubes have been functionalized by using a refluxing process. In a 100 mL of round bottom flask, MWCNTs have been dispersed in 25 ml of concentrated nitric acid by continuous magnetic stirring for 12 hours at $80^\circ C$, resulting in generation of $-COOH$ group on the MWCNT surface [3]. Finally, functionalized MWCNTs have been washed with distilled water to remove the impurities from the solution by maintaining upto neutral pH and the sample is dried at room temperature.

2.3 Electrophoretic Deposition

Firstly, indium tin oxide (ITO) coated glass substrates have been hydrolysed by using $H_2O_2/NH_4OH/H_2O$ solution in the ratio 1:1:5 v/v for 45 min at $80^\circ C$. The solution of CNTs has been prepared in distilled water then sonicated for 2 hrs. For electrophoretic deposition technique, a constant DC voltage source for

electrophoretic deposition having two electrodes is used. A surface charge on MWCNT has been generated by mixing a magnesium nitrate solution into CNTs solution which acts as an electrolyte. ITO coated glass electrode and platinum electrode act as cathode and anode respectively by separation 1 cm is immersed into the suspension solution of CNTs. Electrophoretically films have been generated onto ITO electrode by applying 40V for 60 seconds. The fabricated MWCNT/ITO electrode was then removed from the suspension and washed with distilled water followed by the drying at room temperature. The procedure for EPD is shown in Figure 1(A).

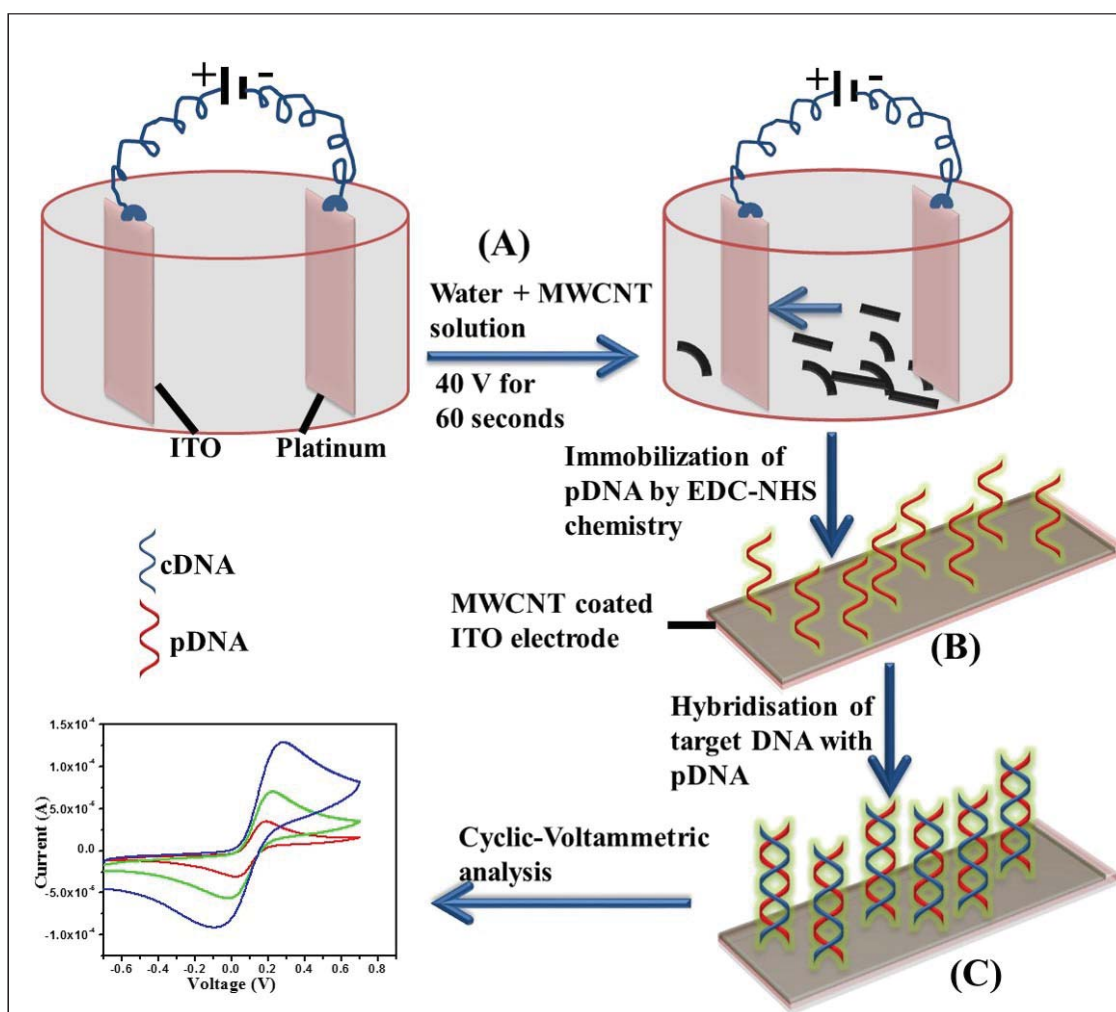


Figure 1. Schematic showing (A) electrophoretic deposition of MWCNTs onto ITO coated glass substrate, (B) covalent immobilization of pDNA onto the MWCNTs electrode, and (C) hybridisation of target DNA with pDNA on bioelectrode.

2.4 Immobilization of biomolecules onto fabricated electrode surface

The fabricated MWCNT/ITO electrode have been activated using EDC (2 mM) and NHS (5 mM) and kept for 1 hr in the dark. Subsequently, 40 ml of pDNA (10^{-7} M) have been immobilized onto the

modified electrode at 100% humidity at room temperature ($\sim 27^\circ\text{C}$) for about 6 hrs, followed by rinsing with Tris-HCl. The hybridization studies have been performed as a function of target DNA concentration with complementary sequences in a humid chamber for about 20 min at 35°C . The proposed mechanism for the synthesis of MWCNT/ITO and the fabrication of the nucleic acid sensor for *E. coli* detection is shown in Figure 1(B and C).

3. Characterization

Spectroscopic and morphological characterization has been carried out for the structural analysis using UV-Visible spectrophotometer (Lambda 950, Perkin Elmer), Fourier transform infrared (FT-IR) spectroscopy (Spectrum BX, Perkin- Elmer) and transmission electron microscopy (HR-TEM, Tecnaii-G2F30 STWIN). Contact angle (CA) measurements have been obtained using a contact angle meter (Data Physics OCA15EC). Zeta potential measurements have been done using Malvern zetasizer instrument (Zetasizer Nano, ZS90). Electrochemical characterization has been carried out by using Autolab Potentiostat/Galvanostat (Eco Chemie, The Netherlands) in phosphate buffer saline (PBS, 50 Mm, 0.9% NaCl, pH -7.4) containing 5 mM $(\text{Fe}(\text{CN})_6)^{3-/4-}$ using three electrode cell having Ag/AgCl as reference electrode and platinum as the counter electrode.

4. Results and discussion

4.1 Spectroscopic and morphological analysis of material synthesized

UV-visible spectra of MWCNTs have recorded and shown in Figure 2(A). The absorbance peak of the absorbance spectra was observed at 280 nm. The absorbance peaks observed at 280 nm is mainly due to π - π^* electronic transition of C-C bonds in MWCNTs by dispersion of sample in distilled water.

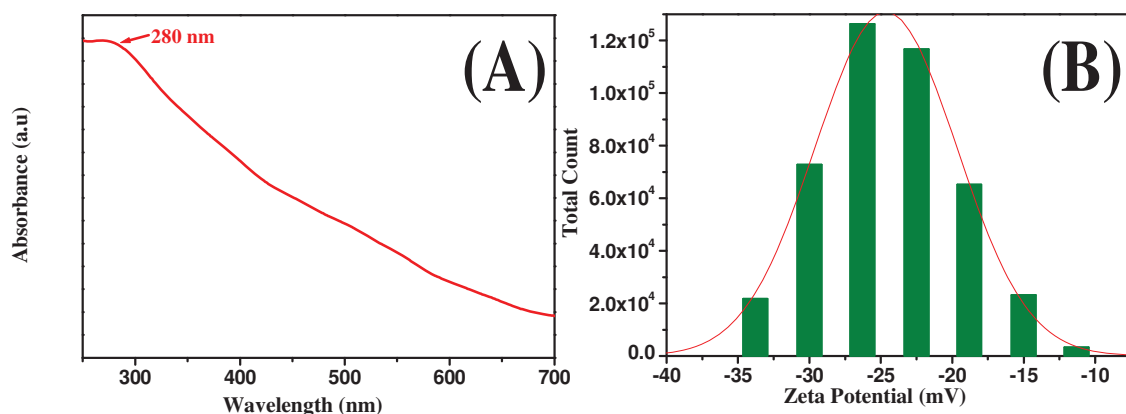


Figure 2. (A) UV-visible spectra of MWCNT and (B) Zeta potential analysis of the MWCNT colloid dispersed in water.

Figure 3 shows the Fourier transform infrared spectra (FT-IR) of (A) MWCNT/ITO and (B) pDNA/MWCNT/ITO electrodes. The FT-IR spectrum of MWCNT/ITO exhibits broad peak at 3000 cm^{-1} corresponding to -OH symmetric stretching in carboxylic acids showing MWCNTs have been functionalized by -COOH groups. The other peak has been observed at 1342 cm^{-1} mainly due to the C=C, which shows that synthesized MWCNTs have been functionalized by carboxylic group. Further, pDNA have been immobilized onto MWCNT/ITO electrode surface which shows characteristic peak at 1600

cm^{-1} which corresponds to N-H stretch of amide-I as shown in curve (Fig. 3B). The additional sharp peak observed at 3600 cm^{-1} which corresponds to N-H stretch of amide bond (1° amine) indicating the formation of amide linkage by immobilization of pDNA onto carboxylated MWCNT/ITO coated electrode surface which confirms the covalent attachment of DNA with MWCNTs surface.

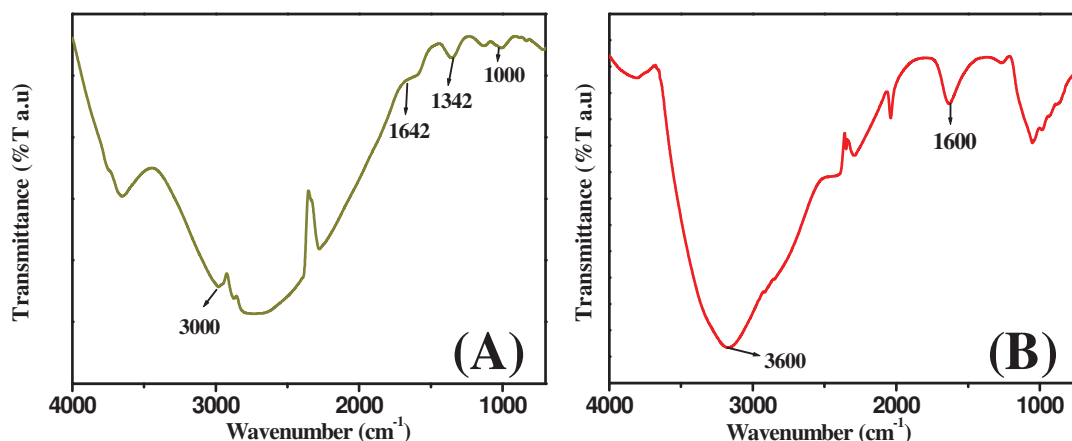


Figure 3. FT-IR spectra of (A) MWCNT /ITO electrode and (B) pDNA/MWCNT/ITO bioelectrode.

Figure 4 shows high resolution transmission electron microscopy (HR-TEM) studies has been carried for the structural investigation of synthesized MWCNTs. The uniformly distributed long tube like structures have been obtained having uniform diameter without any splintering in a long nanotube. The Fig. 4(A) shows distinct formation of long tubes of multiwalled CNTs which easily distinguish by the TEM images that confirmed the formation of multiwalled carbon nanotube. The Fig. 4(B) shows that multiwalled carbon nanotube have been formed and marked by red line which shows multiwalled formation take place. The Fig. 4(C) shows the lattice fringes of MWCNTs with an interplanar spacing 1.0\AA indicating high crystallinity in carboxylated carbon nanotubes. The diameter of MWCNTs varies from 50 to 80 nm with length.

4.2 Zeta potential analysis

To determine the particle stability of functionalized MWCNTs zeta potential measurements have been conducted at 25°C . All the experiments have been repeated three times and standard deviation has been calculated. It was observed that MWCNTs dispersed in Milli Q water at pH 7 after sonication for 30 minutes show an average zeta potential of -24.7 mV (Fig. 2 (B)) having a standard deviation of 0.23 indicating that the colloid solution formed is quite stable and suitable for EPD.

4.3 Contact angle analysis of electrodes

To investigate the film formation and DNA immobilization onto the ITO surface, contact angle measurements have been carried out using sessile drop method (Fig. 5). The decrease in value from 59.87° for the MWCNT/ITO electrode (Fig. 4B) to 35.37° for pDNA/MWCNT/ITO (Fig. 5C) electrode indicates the increase in the number of hydrophilic functional groups ($-\text{COOH}$, NH_2 and $-\text{SH}$) on the surface and thereby confirming the DNA immobilization.

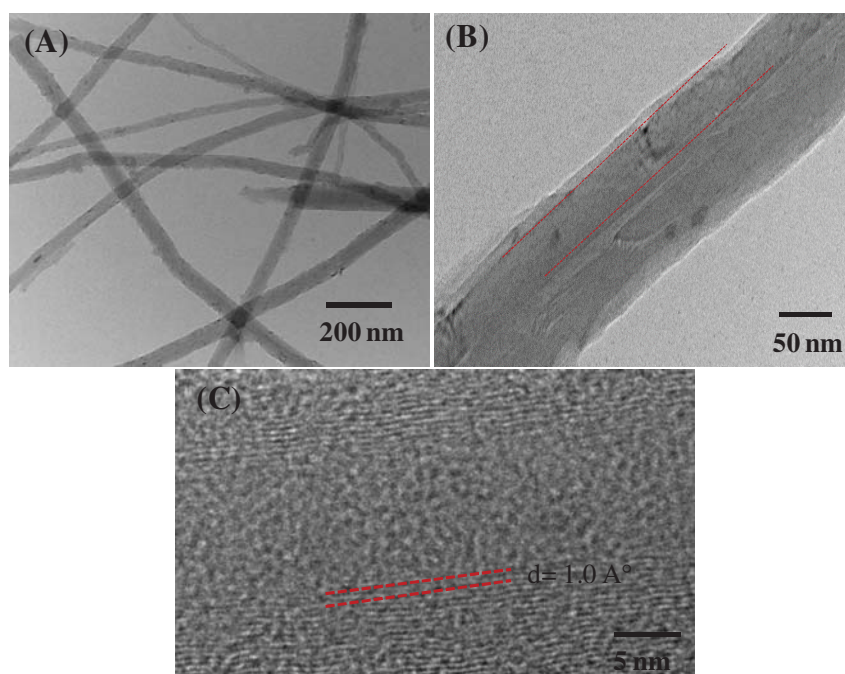


Figure 4. TEM images of (A) Carbon nanotubes, (B) High resolution TEM image of Single carbon nanotube showing different walls, and (C) High resolution image showing crystalline fringes of the synthesized multiwalled carbon nanotubes.

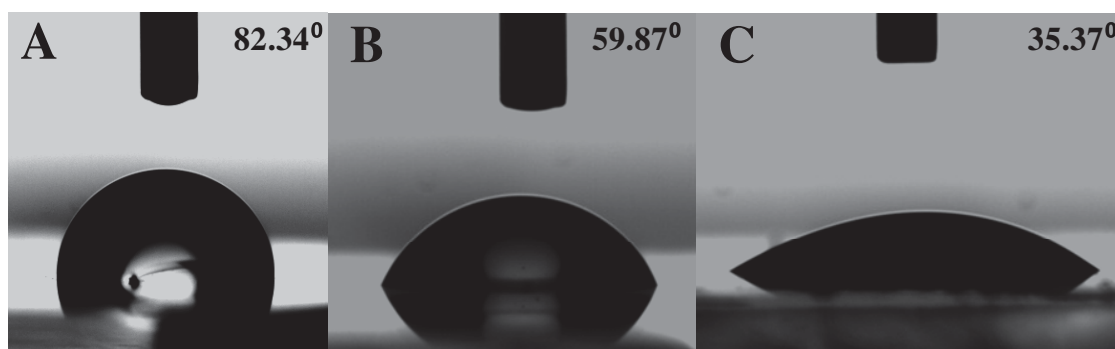


Figure 5. Contact angle images of (A) ITO coated glass surface (B) MWCNT coated ITO electrode (C) pDNA immobilized electrode.

4.4 Electrochemical characterization of electrodes

The electrochemical studies of different electrodes have been carried out by dipping the electrodes in 13 mL of phosphate buffer saline (PBS) (100 mM, pH 7.4, 0.9% NaCl) containing 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ using cyclic voltammetry (CV). CV analysis of bare ITO electrode, MWCNT/ITO electrode, and pDNA/MWCNT/ITO bioelectrode has been carried out at scan rate (v) of 50 mV/s. Figure 6(B) shows anodic peaks at 0.3078 mA, 0.1435 mA and 0.0704 mA for ITO, MWCNT/ITO and

pDNA/MWCNT/ITO electrodes, respectively. The decrease of anodic peak current of MWCNT/ITO electrode as compared to ITO indicates successful formation of film and further decrease in the current after pDNA immobilization is due to the hindrance in the diffusion of electrons caused due to immobilization of large biomolecule.

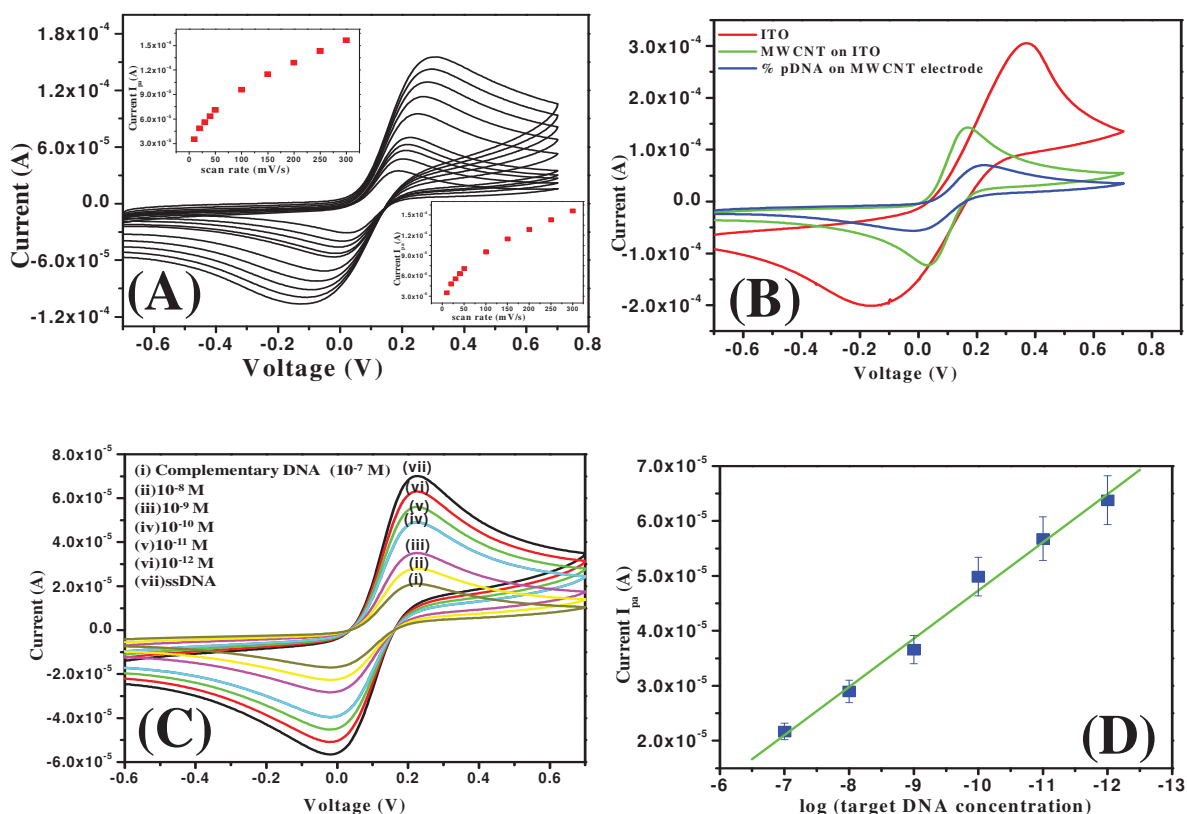


Figure 6. (A) Cyclic voltammetry response studies of pDNA/MWCNT/ITO bioelectrode at different scan rates (10-300 mV/s) in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ PBS solution at pH 7 (inset (i) variation of anodic peak current I_{pa} (A) with scan rate v (mV/s) and (ii) variation of anodic peak potential E_{pa} with log of scan rate v (mV/s), (B) CV studies of different substrates, (C) Biosensing studies of the pDNA/MWCNT/ITO bioelectrodes at different cDNA concentrations, (D) Variation of anodic peak current I_{pa} with cDNA concentration peak current (I_{pa}) concentration.

The CV studies of pDNA/MWCNT/ITO bioelectrode at different scan rates v (10–300 mV/s) indicate that as we move towards higher scan rate, anodic peak current (I_{pa}) shifts towards more positive value and cathodic peak current (I_{pc}) shifts in the reverse direction respectively. Besides this, the redox peak currents show linear behaviour as a function of $v^{1/2}$ (Fig. 6A inset (i) and (ii)) revealing it as a diffusion controlled electron-transfer process and is given by the following equations.

$$I_{pa} \text{ (A) (pDNA/MWCNT/ITO)} = 0.0045 + 0.4009 (\mu\text{As/mV}) \times \text{scan rate (mV/s);}$$

$$R = 0.9823 \quad (1)$$

$$\begin{aligned} E_{pa} \text{ (V) (pDNA/MWCNT/ITO)} &= 0.1008 + 0.0764 \text{ (s)} \times \log \text{ of scan rate (mV/s);} \\ R &= 0.9883 \end{aligned} \quad (2)$$

The diffusion coefficient of the pDNA/MWCNT/ITO bioelectrode has been determined using the Randle Sevcik equation:

$$I_{pa} = (2.69 \times 10^5) n^{3/2} A D^{1/2} C v^{1/2} \quad (3)$$

Where, I_{pa} is the peak current (I_{pa} anodic and I_{pc} cathodic), n is the number of electrons, A is the area of electrode (0.5 cm^2), D is the diffusion coefficient, C is the surface concentration, and v is the scan rate (50 mV/s). The D value has been obtained as $1.24 \times 10^{-7} \text{ cm}^2/\text{s}$. The total surface concentration of probe DNA is calculated using Laviron's theory and is found to be $1.65 \times 10^{-10} \text{ mol cm}^{-2}$, indicating high surface coverage of DNA onto MWCNT/ITO electrode [5]. The activity of the pDNA/MWCNT/ITO bioelectrode has been estimated as a function of pH varying from 6.0 to 8.0 at $25 \text{ }^\circ\text{C}$. The high magnitude of response current obtained at pH 7.0 (data not shown) indicates that pDNA/MWCNT/ITO bioelectrode is more active at pH 7.0, at which DNA molecules retain their natural structure and do not get denatured [9]. Thus, all experiments have been conducted at pH 7.0 at $25 \text{ }^\circ\text{C}$.

4.5 Biosensing studies

Figure 6(C) represents the CV studies of pDNA/MWCNT/ITO bioelectrode carried out at different concentrations of complementary DNA (10^{-12} M to 10^{-7} M) by immersing the electrode in PBS containing $5 \text{ mM } [\text{Fe}(\text{CN})_6]^{3-/4-}$. It has been observed that there is linear decrease in the anodic peak current with increase in analyte concentration indicating that decrease in the charge transfer to the working electrode. This mainly due to the increase in the concentration of cDNA results in more hybridization of heavy biomolecules with the immobilized probe take place and the hindrance in the charge transfer to the electrode increases. This causes decrease in the resulting current. Figure 6(D) shows variation in anodic

The equation for line of regression for linear region is given by:

$$\begin{aligned} I_{pa} \text{ (A)} &= 0.0165 + 8.778 \text{ (}\mu\text{A)} \times \log \text{ of conc. of cDNA (M);} \\ R &= 0.9987 \end{aligned} \quad (4)$$

The limit of detection (LOD) calculated for linear region using the expression $3\sigma/\text{sensitivity}$ [10], where σ is the standard deviation ($n = 10$) of the CV signals obtained in the absence of cDNA is $1 \times 10^{-12} \text{ M}$.

5. Conclusions

In summary, we demonstrate the studies of electrochemical genosensor for *E. coli* detection based on the functionalized multiwalled carbon nanotubes. Uniformly formed functionalized multiwalled CNTs have been synthesized using chemical vapour deposition technique and deposited onto indium tin oxide (ITO) coated glass substrates using electrophoretic deposition (EPD) method. The fabricated MWCNT/ITO electrode surfaces have been utilized for the immobilization of pDNA of *E. coli* through covalent bonding. The detection using an electrochemical cyclic voltammetry analysis reveals that the fabricated biosensor with an appropriately optimized protocol can accurately detect *E. coli* in the range of 10^{-7} to 10^{-12} M . Our sensing strategy could be extended for the detection of other micro-organisms and open up new avenues for the designing of electrochemical nucleic acid sensors.

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