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Journal of Virological Methods

Investigating the potential of multiwalled carbon nanotubes based zinc nanocomposite as a recognition interface towards plant pathogen detection

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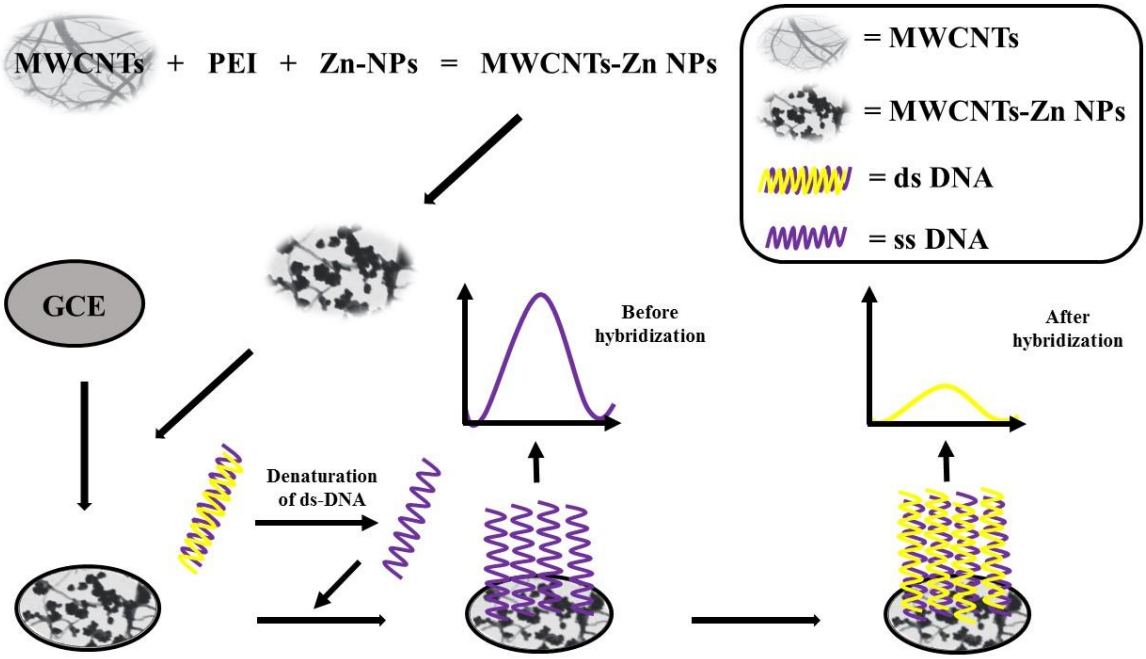
Graphical Abstract



Research Highlights

- A bio-recognition interface is constructed based on carbon nanotubes and zinc nnaocomposite.
- The morphology consists of bunches of Zn NPs (25-500 nm) anchored along carbon nanotubes.
- The combined affinity and synergistic effects produce sensing platform with excellent three fold selectivity for the detection of viruses.

ABSTRACT

The emergence of nanotechnology has opened new horizons for constructing efficient recognition interfaces. This is the first report where the potential of a multiwalled carbon nanotube based zinc nanocomposite (MWCNTs-Zn NPs) investigated for the detection of an agricultural pathogen i.e. Chili leaf curl betasatellite (ChLCB). Atomic force microscope analyses revealed the presence of multiwalled carbon nanotubes (MWCNTs) having a diameter of 50-100 nm with zinc nanoparticles (Zn-NPs) of 25-500 nm. In this system, these bunches of Zn-NPs anchored along the whole lengths of MWCNTs were used for the immobilization of probe DNA strands. The electrochemical performance of DNA biosensor was assessed in the absence and presence of the complementary DNA during cyclic and differential pulse voltammetry scans. Target binding events occurring on the interface surface patterned with single-stranded DNA was quantitatively translated into electrochemical signals due to hybridization process. In the presence of complementary target DNA, as the result of duplex formation, there was a decrease in the peak current from 1.89×10^{-04} to 5.84×10^{-05} Ampere. The specificity of this electrochemical DNA biosensor was found to be three times as compared to non-complementary DNA. This material structuring technique can be extended to design interfaces for the recognition of

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the other plant viruses and biomolecules.

Keywords: Multiwalled carbon nanotubes; Zinc nanocomposite; DNA biosensor; Chili leaf curl betasatellite; Hybridization

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1. Introduction

Nanotechnology has emerged as one of the most exciting tools for the creation of efficient materials, devices, and systems through control of size at the 1–100 nm scale. Now a plethora of nanomaterials with different sizes, shapes, and compositions for a range of application are available (Khademhosseini et al., 2016). The exceptional properties of nanomaterials offer remarkable prospects for designing novel sensing systems, especially for improving the bioanalytical methods. Further, their properties can be tailored with respect to their size and structure which make them ideal candidates for devising innovative products.

During recent years, rapid and highly selective detection of DNA has gained much importance in the field of diagnostics, tissue matching, gene analysis, and forensic (Watson and Crick, 1953). On the basis of unique identity between the nucleic acid sequences of different organisms, diagnosis of several diseases is possible (Kogan et al., 1987). For the detection of nucleic acid based sequences, many techniques have been employed, however, polymerase chain reaction (PCR), gel electrophoresis, and membrane blot is among the most widely used techniques (Angelis et al., 1999; Beaudet and Belmont, 2008; Ding et al., 2009; Mullis and Faloona, 1987; Roberts et al., 2009; Southern, 1975). These conventional methods are accurate, but for routine analysis, these are slow and laborious, especially for the timely and rapid detection. To achieve this goal, nanotechnology based interfaces could offer favorable methods for DNA detection. Bio-affinity between nanomaterials and DNA molecules can be manipulated to design innovative biosensor platforms (Anzai, 2016; Bajwa et al., 2014; Dong et al., 2015; Juan-Colás et al., 2016). For the detection of DNA sequences, different biosensors e.g. plasma resonance, lateral flow, fluorophore-quencher, G-quadruplex, fluorescence resonance energy transfer (FRET) based biosensors have been reported (Chakraborty et al., 2015; Jensen et al., 2013; Jia et al., 2016; Jiang et al., 2014; Qiu et al., 2015). Among them, electrochemical biosensors are popular choices due to their sensitivity and rapidness (Gong et al., 2017; Shen et al., 2016; Wang et al., 2016). These sensors offer a cost-efficient, but a sensitive platform for the detection of various diseases like α and β-thalassemia (Chen et al., 2016). A glassy carbon electrode (GCE) modified with poly(2,6-pyridinedicarboxylic acid) (P(PDCA) can detect gemcitabine (GEM) up to 0.276 mg L^{-1} (Tığ et al., 2016). Whereas, a pencil graphite electrode (PGE) fabricated with DNA and Au nanoparticles has been developed in order to determine contamination caused by bacteria especially *Bacillus cereus* in dairy products (Izadi et al., 2016). An electrochemical biosensor based on a gold nanotube array (AuNTsA) has been used for the DNA detection of *Mycobacterium tuberculosis* with a detection limit of 0.05 ng μL^{-1} (Torati et al., 2016). For the assessment of total antioxidant capacity (TAC), a biosensor modified with dA_{20} (adenine-rich oligonucleotide) was used in commercial samples of juices (Cruz et al., 2016). In an effort to develop electrochemical DNA biosensor, graphene oxide/gold nanoparticles were used for the detection of *Helicobacter pylori* with a detection limit of 27 pM (Hajihosseini et al., 2016). Another innovative use of electrochemical DNA biosensor was to determine epidermal growth factor receptor *(EGFR)* exon 19 mutation (Xu et al., 2016). A biosensor is reported for the screening of DNA level modification and damage caused by chemotherapeutic drugs and a screen printed gold electrode (SPGE) for the detection of *Cucumber-Mosaic virus* (CMV) (Ilkhani et al., 2016; Zulkifli et al., 2016). Despite the material advancements very scanty data is available where electrochemical biosensors have been applied for the detection of agricultural pathogens for their rapid and timely identification to prevent huge economic losses.

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Among nanomaterials, multiwalled carbon nanotubes (MWCNTs) are particularly attractive for bioelectronic detection. Their electronic conductance is strongly responsive to any small change in the surface chemistry e.g. attachment of any ligand or biomolecule to their surface. Various novel systems can be designed by coupling high surface-to-volume ratio, outstanding electron transport properties, ease of manipulating the rich chemistry of MWCNTs with other nanostructures, and biomolecules. The resultant tailored electronic conductivity of MWCNTs makes them extremely attractive material for constructing better electrochemical sensing devices. Purposely designed and loaded nanoparticles on MWCNTs can serve as an amplification source and thus can lead to sensitive detection of bioanalytes like DNA.

Timely identification of a pathogen plays a very important role in an agricultural production system. *Chili leaf curl virus* is one of the important viruses belongs to genera *Begomovirus* of the family *Geminiviridae* that causes chili leaf curl disease in chili pepper (*Capsicum annum* L.) (Briddon et al., 2003; Shih et al., 2003). The begomoviruses are either bipartite or monopartite. In bipartite begomovirus, there are two main genomic contents i.e. DNA-A and DNA-B. However, monopartite begomoviruses are those begomoviruses that comprises of only single genomic content which has homogeneity to bipartite *Begomovirus* DNA-A component (Stanley et al., 2005). The *Begomovirus* complex comprises of alphasatellites and betasatellites which are small satellite like molecules involve in self-replication and to determine the pathogenicity respectively (Jyothsna et al., 2013; Mansoor et al., 1999). In many areas of the world, Chili leaf curl betasatellite (ChLCB) is the dominant one and it causes infection in chili (Hussain et al., 2009).

Here in this study, we have introduced the designing of a microenvironment based on novel MWCNTs, zinc nanoparticles (Zn-NPs), and methylene blue (MB). This is used as a recognition interface for a DNA sequence with improved response characteristics. The electrochemical DNA biosensor developed thus represents a simple, straightforward approach for the detection of the sequence of interest. This protocol has the potential for establishing as a diagnostic tool for other biomolecules as well.

2. Experimental details

2.1 Reagents and chemicals

MWCNTs, phosphate buffered saline (PBS) pH=7.4, 0.1 M methanol solution of zinc chloride ($ZnCl_2$), polyethylene imine (PEI), 0.1 M sodium borohydride ($NaBH_4$) solution, sodium dodecyle sulfate (SDS), 1 M potassium nitrate (KNO_3), 50 mM PBS solution of potassium ferricyanide ($K_3Fe(CN)_6$), 5 mM methylene blue (MB), 1 M nitric acid (HNO_3), silver chloride (AgCl), PCR green mixture and short oligonucleotides were purchased from Shenzhen Nanotech. Port. Co. Ltd (China), Sigma-Aldrich, Eurofins MWG Operon, and Merck. The ultra pure water obtained from Barnstead™ Smart2Pure™ was used throughout the experiments (Thermo Scientific).

The primers used for the amplification of probe and complementary target DNA from the clone of ChLCB (Acc. No. FR751147). The set of primer sequences used in this study for the amplification of ChLCB is CbC1F (5'TAT-GAATCGATATGCACCACGTATATGAA 3') CbC1R **(**5'ATACTG-TCGACTCACACACACACATTCGTAC 3') (Tahir et al., 2011).

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Similarly, for the amplification of cotton leaf curl Multan betasatellite (non-complementary DNA) from clone LN870411, following set of primers are used, i.e. β 01 (5' GGTACC ACTACGCTACGCAGCAGCC 3') and β 02 (5' GGTACC TACCCTCCCAGGGGTACAC 3') (Briddon et al., 2002).

2.2 Instrumentation

Electrochemical investigations were carried using Autolab Potentiostat/Galvanostat (PGSTAT) with in-built the General Purpose Electrochemical System software (GPES version 4.9). For data treatment, a linear baseline correction was applied in the case of cyclic voltammetry and the data were smoothed by the Savitzky and Golay method (level 04).

For the amplification and quantification of DNA sequences, Thermal cycler C1000 Touch^{Tm} (BIO-RAD, USA) and NanoDrop^{TM} 1000 spectrophotometer (Thermo Scientific) were used, respectively. In order to characterize the nanocomposite, different techniques have been applied. The surface charge on the as-synthesized composite was determined by Zetasizer Nano ZS (Malvern) which works on the principle of dynamic light scattering. The morphology was studied with field emission scanning electron microscope (FE-SEM-JSM-7500F-JEOL, Japan) and atomic force microscope (AFM- SHIMADZU WET-SPM 9600, Kyoto, Japan).

2.3 Preparation of MWCNTs-Zn NPs composite

To create reaction centers for the loading of nanoparticles, amine functionalization of MWCNTs was carried out by following a protocol (Hameed et al., 2017). Briefly, 0.05 g of MWCNTs was dissolved in 5 mL PEI solution (1% v/v) prepared in methanol, followed by the incubation for 2 h at room temperature. Afterwards, this mixture was further dissolved in 40 mL of methanol till stable colloidal solution is formed. Then $ZnCl_2$ (10 mL; 0.1 M) was added into it, followed by the drop-wise addition of $NaBH_4$ solution (30 mL; 0.1 M) in the mixture. Finally, MWCNTs were washed with methanol and water (15 mL each), and then put in a vacuum concentrator for drying at 50 ^oC.

2.4 Fabrication of electrochemical DNA biosensor

For the preparation of electrochemical DNA biosensor, the surface of GCEs was polished by using 0.05, 0.3 and 1 µm alumina slurries. After that GCEs were cleaned by sonication for 5 min in ethanol, HNO_3 (1 M) and water (5 mL each, in batch mode). For the characterization of GCEs cyclic voltammetry were recorded between -1 to 1 V in $K_3Fe(CN)_6$ (50 mM) and KNO_3 (1 M) solution against AgCl (reference electrode) at a scan rate of 100 mVs^{-1} scan rate.

The amplification of ChLCB was done using the specific set of primers CbC1F/CbC1R by following PCR profile, 94^oC for 5 min (preheat treatment) followed by 34 cycles of 94 ^oC for 30 sec (denaturation), 52 ^oC for 30 sec (annealing), 72 ^oC for 45 sec (extension) and final extension at 72 ^oC for 10 min (Tahir et al., 2011). Amplified product (1.4 kb) was confirmed by running 4 µL of the reaction along with 1 kb ladder

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(Thermo Scientific) in agarose gel (1 %) stained with ethidium bromide. The fragment of the interest i.e. 1.4 kb was eluted from agarose gel using commercially available gel extraction kit of Axygen™. Finally, the gel eluted product was used as a positive control.

For non-complementary DNA, amplification was performed using specific set primers (β 01 and β 02) by following PCR profile, 94 °C for 5 min (preheat treatment) followed by 34 cycles of 94 °C for 30 sec (denaturation), 50 °C for 30 sec (annealing), 72 °C for 45 sec (extension) and final extension at 72 °C for 10 min.

2.5 Construction the interface

30 mg of MWCNTs-Zn NPs composite was dissolved in 1 mL ultrapure water by sonication for 5 min. GCE surface was drop coated with 10 µL of this dispersion and incubated for 1 min at 90 °C in an oven till solvent evaporation. Meanwhile, the amplification of DNA sequence was done using a specific set of primers CbC1F/CbC1R from ChLCB (Acc. No. FR751147). 2 µL of oligonucleotide probe after the snap cooling incubating at 35 °C for 3 h was immobilized along with MWCNTs-Zn NPs composite on GCE surface. For the removal of unbound DNA of the interface probe, the modified surface was soaked in SDS (0.1 %) for 10 min. The detailed procedure about the fabrication of the interface is shown in Fig. 1.

2.6 Hybridization studies

The DNA hybridization events between probe strands of the interface and target DNA were studied in $K_3Fe(CN)_6$ (50 mM) solution prepared in PBS (pH=7.4). In order to ensure that probe attached to the interface is single stranded, the temperature of the cell (Metrohm; 6.1415.210) containing $K_3Fe(CN)_6$ solution was maintained at 94 °C. Then target DNA (ChLCB) was added into the cell subsequently after the addition of MB (5 mM). After each reaction, the modified electrode was rinsed thoroughly with SDS in order to ensure that probe remains single stranded and free from the unbound DNA. To check the specificity of the probe, the designed electrochemical biosensor was also exposed to non-complementary DNA by keeping all the same constituents, in the cell. The change in the oxidation current was studied by cyclic voltammetry (CV) and differential pulse voltammetry (DPV) scans in GPES version 4.9 software and final data were compiled by using MS excel.

2.7 Sample preparation for microscopic analyses

MWCNTs and MWCNTs-Zn NPs composite (1 mg mL^{-1} each) were dispersed separately in methanol. From each dispersion, 10 µL were drop-coated on quartz wafers followed by their drying at room temperature overnight. For AFM analysis, 100 µm thickness cantilever attached to a silicon nitride probe (OMCL-TR800PSA-1) was used for scanning the surface of the sample with the 0.57 m^{-1} and 73 kHz force constant and resonance frequency, respectively.

For FESEM study, above prepared MWCNTs and MWCNTs-Zn NPs composite dispersions, 10 µL each, were dropped on carbon coated copper grids (200 mesh). In order to ensure the accuracy of both microscopic results, all the samples were prepared in triplicate and analyzed at at least three different areas.

3. Results and discussion

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3.1 Characterization of nanocomposite

The structure and morphologies of MWCNTs and MWCNTs-Zn NPs were investigated and Fig. 2 (A and B) shows their respective FESEM images. The unmodified MWCNTs can be seen in the form of dispersed strands with an average diameter of 20-25 nm (Fig. 2A). However, their morphology and dimension were changed after the formation of composite. Fig. 2B shows the presence of Zn-NPs, in the form of agglomerates, on the surface of MWCNTs (Fig. 2B). Further, these agglomerates were found anchored in different places along the whole strands of MWCNTs.

AFM analysis was carried out to gain further insight into the morphology of the prepared materials. AFM studies revealed that the diameter of MWCNTs is about 20-25 nm (Fig. 3A). However, after the formation of composite, the height of MWCNTs was increased to 50-100 nm (Fig. 3B). This increase in height can be attributed to the presence of Zn-NPs on the surface of MWCNTs. These Zn-NPs are about 30-50 nm in height and 25-500 nm in width. Fig. 3C, shows a histogram plot which depicts their average size fall in the range between 200 and 250 nm.

To support the mechanism of composite synthesis, surface charges were investigated. PEI is used to modify the surface chemistry of MWCNTs. It possesses a large number of imine groups in the polymer chain which coordinate with Zn^{2+} ions when its precursor solution is added to the PEI coated MWCNTs dispersion. Previous studies have reported the formation of similar types of metal complexes i.e. PEI-Zn^{2+}-NH_3 (Liu et al., 2013). When reducing agent is added, it results in the formation and later aggregates of Zn-NPs due to the chemical reduction of Zn^{2+} ions. Fig. 4 shows the zeta potential plot of MWCNTs and MWCNTs-Zn NPs composite. The values were found to be -18.52 mV and +30.85 mV, respectively. This change of surface charge from negative to positive is a strong evidence of the modification of MWCNTs and the accumulation of Zn-NPs. Further, the amount of charge on MWCNT-Zn NPs composite is positive enough to hold negatively charged probe DNA (Zheng et al., 2014). Based on previous studies, the zeta potential values of ZnO at pH=7.9 ranges in between +22 mV and +24 mV (Mohd Omar, Abdul Aziz, and Stoll, 2014). The surface charge analysis and data from previous studies corroborates the formation of Zn-NPs, decorated over MWCNTs.

3.2 Electrochemical characterization

The biorecognizability of the prepared nano interface was observed by recording CV scans in the absence and presence of complementary DNA. In this study, we have introduced first time, coupling of synergistic effects of MWCNTs and Zn-NPs with MB, and cyanide ions as the electrochemical probe for the detection of plant pathogens. When this interface was exposed to target DNA, Fig. 5 exhibits the resultant CV scans. Very well-defined redox peaks with a significant decrease in magnitude of oxidation current from 1.89×10^{-04} A to 5.84×10^{-05} A can be observed. The decrease in the peak current can be assigned to hybridization of complementary DNA with probe DNA strands immobilized on the nanomaterial interface. When an interface is immersed in the solution, the concentration of MB is greater near the electrode surface as positively charged MB binds to the negatively charged single-stranded DNA (ssDNA) strands attached to the composite surface, through attractive coulomb interactions. The electron flows from the electrode surface to reduce MB^+ attached with ssDNA near the electrode surface to leucomethylene blue, which in turn reduces ferricyanide ions. Therefore, the highest MB reduction signal was observed. On the other hand, in double-stranded duplexes the bases are arranged in a helical structure and MB cannot bind to DNA and consequently its concentration

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decreases near the interface. Therefore, an obvious decrease in the voltammetric peak was observed as the indicator of duplex formation (Ansari et al., 2009).

3.3 Specificity and selectivity of modified DNA biosensor

Specific detection is a very important characteristic, especially while dealing with bioanalytes. The specificity and selectivity of this biosensor were investigated by recording its DPV response to complementary and non-complementary DNA, 7 mM each. MB was used as the redox indicator and Fig. 6 depicts the results of DPV signals of electrochemical reactions taking place at the interface of MWCNTs-Zn NPs composite-probe DNA interface in the absence of target DNA (curve a); and in the presence of non-complementary DNA (curve b) and complementary DNA (curve c). The highest magnitude in peak current (Ip) 6.48 x 10^{-4} A was observed in the absence of target DNA of any kind. However, after the hybridization of probe interface with complementary DNA, a significant decrease in the amount of 1.91 x 10^{-4} A occurred. An obvious decrease in the voltammetric peak after duplex formation can be ascribed to the electrocatalytic cycle of MB that was prevented by hybrid formation on the interface. Thus the lowest MB reduction signal was observed from the full hybrid sequences on the electrode surface. However, in case of non-complementary DNA, a negligible decrease in the oxidation peak current (5.49 x 10^{-4} A) was observed i.e. only 15% as compared to background signal. Which means the developed nano-bio interface is almost three fold more specific towards the sequence of the interest as compared to non-complementary sequence. It is a direct evidence of no hybridization event and duplex formation (Torati et al., 2016). Thus, this interface can recognize a specific DNA sequence by a facile and straightforward method without performing laborious laboratory techniques.

3.4 Detection of different concentrations of complementary DNA

Fig. 7 shows the DPV response recorded when MWCNTs-Zn NPs composite-probe DNA

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was exposed to various concentrations of complementary target DNA from 4 mM to 7 mM. It can be observed that there is a gradual decrease in peak current with the increase in concentration of complementary target DNA from 1.91-5.49 x 10^{-4} A. The value of current at 4 mM is almost equal to the value found in case of non-complementary DNA i.e. 5.49 x 10^{-4} A. Therefore the detection limit of this modified electrochemical DNA biosensor can be considered as 5 mM (Zheng et al., 2014). It also corroborates that the developed interface can be applied for the DNA based detection of virus.

4. Conclusions

In this study, we have synthesized and investigated the electrocatalytic potential of Zn-NPs loaded MWCNTs for the recognition and discrimination of biomolecules as DNA. The Zn-NPs were decorated in the form of patches along the whole length of MWCNTs which acted as the catalytic centers. Dynamic light scattering confirmed the presence of positive charge on the composite that can effectively hold negatively charged probe DNA. CV and DPV scans showed that the developed composite can detect ChLCB sequence, selectively. Under optimum conditions, electrochemical signals decreases with the concentration of the target DNA. At one hand, three fold selectivity and specificity of this sensor with complementary and non-complementary DNA manifest that this work can be expanded for in-field detection of virus in the crops. On another, this protocol can be modified to assess the potential of other nanostructures to construct bio interfaces, for even more improved detection of biomolecules.

Competing interests

There are no competing financial interests declared by the authors.

Acknowledgement

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Figure Legends

Graphical Abstract

Fig. 1 Schematic illustration showing the preparation of MWCNTs-Zn NPs composite (A); target DNA extraction followed by preparation of reaction mixture (B) and construction of MWCNTs-Zn NPs composite interface (C).

Fig. 2 FESEM images of MWCNTs (A) and MWCNTs-Zn NPs composite (B).

Fig. 3 AFM images of MWCNTs (A), MWCNTs-Zn NPs composite (B) and histogram plot of MWCNTs-Zn NPs composite (C).

Fig. 4 Zeta potentials of MWCNTs and MWCNTs-Zn NPs composite, respectively.

Fig. 5 CV scans of MWCNTs-Zn NPs probe interface in the absence of target DNA (a) in presence of complementary DNA; PBS (pH=7.4) containing 50 mM $[Fe(CN)6]^{/4-}$, 5 mM MB, scan rate 100 mVs^{-1}.

Fig. 6 DPV scans of MWCNTs-Zn NPs probe interface in the absence of target DNA (a), non-complementary DNA (b) i.e. 7 mM and complementary DNA (c) i.e. 7 mM; PBS (pH=7.4) containing 50 mM $[Fe(CN)6]^{3-/4-}$, 5 mM MB, scan rate 100 mVs^{-1}.

Fig. 7 DPV scans of MWCNTs-Zn NPs probe interface towards different concentrations of complementary DNA i.e. 4, 5, 6 and 7 mM; PBS (pH=7.4) containing 50 mM $[Fe(CN)6]^{3-/4-}$, 5 mM MB, scan rate 100 mVs^{-1}.

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Fig. 1.

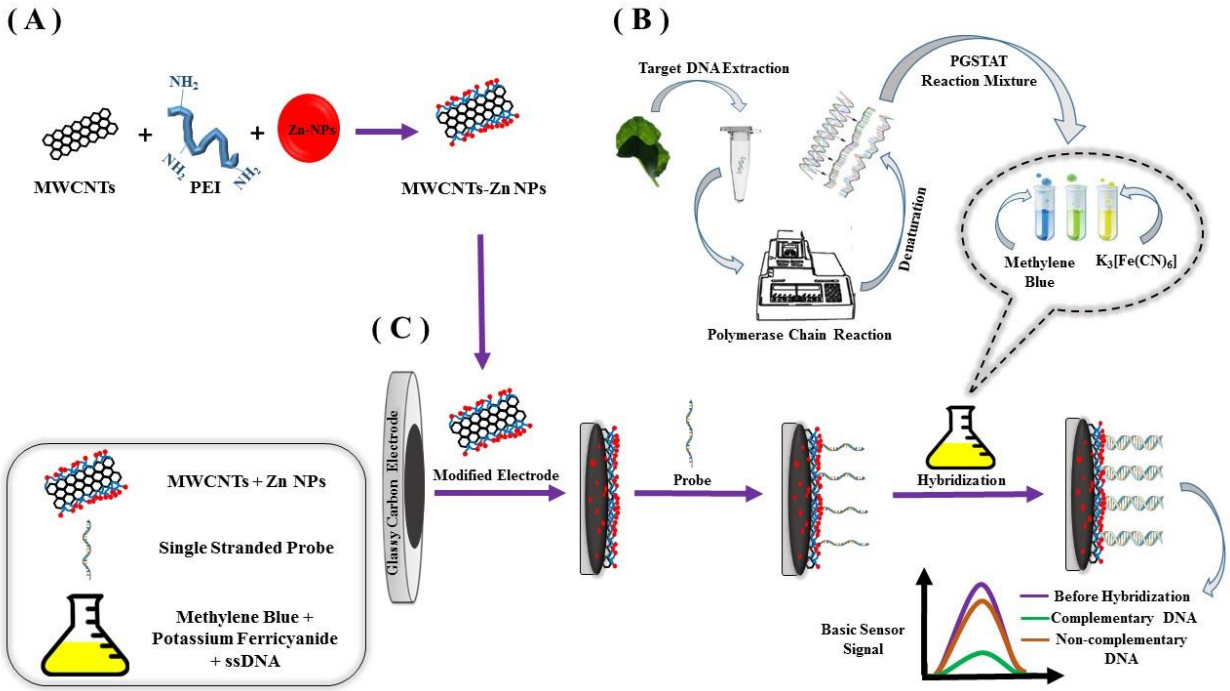

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Fig. 2.

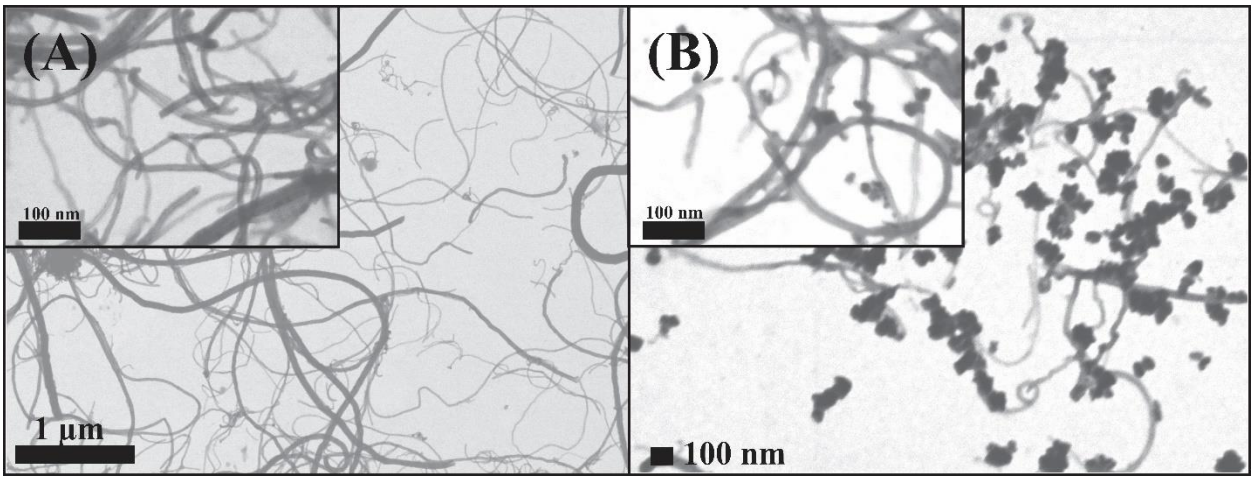

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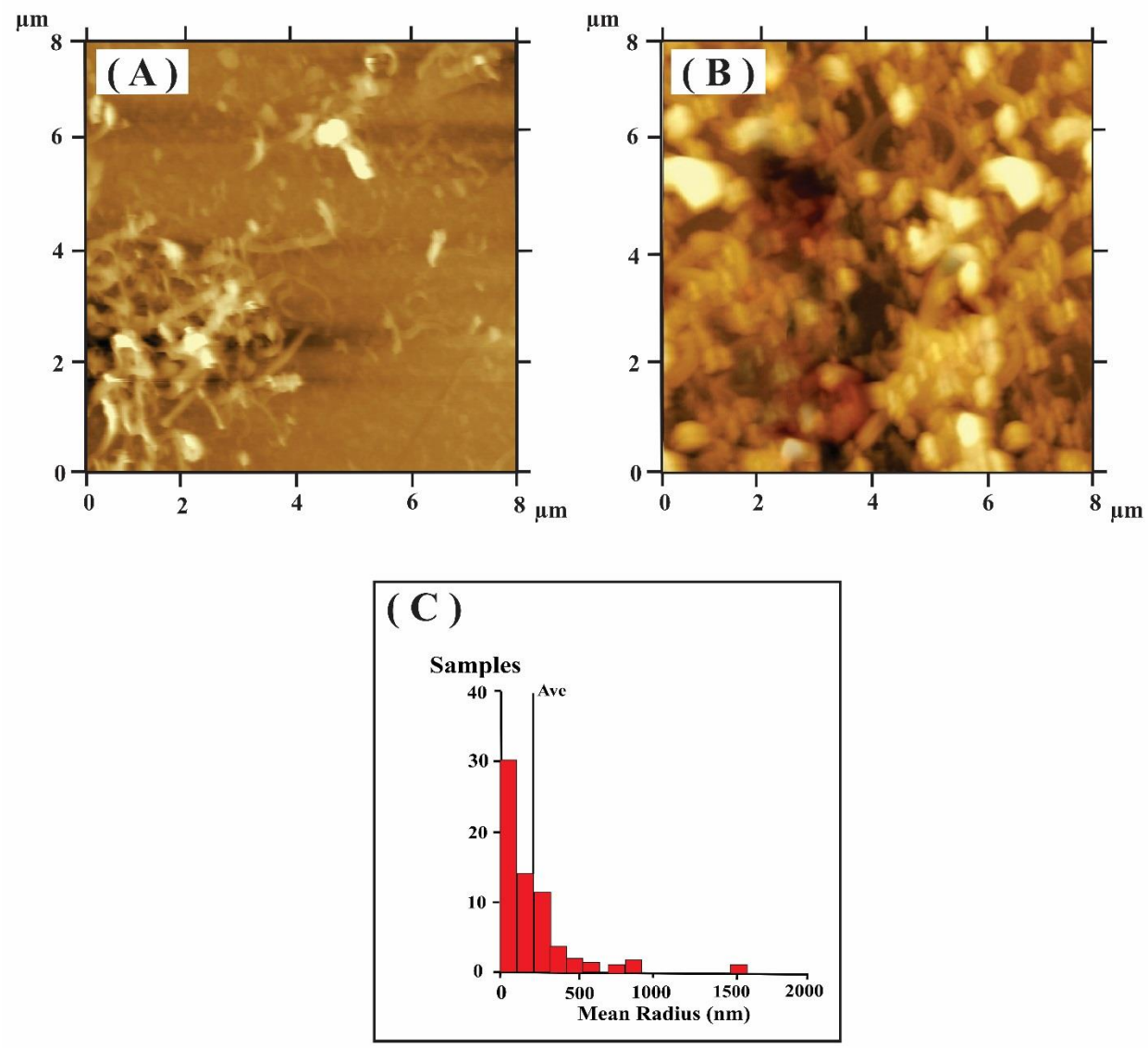
Fig. 3.



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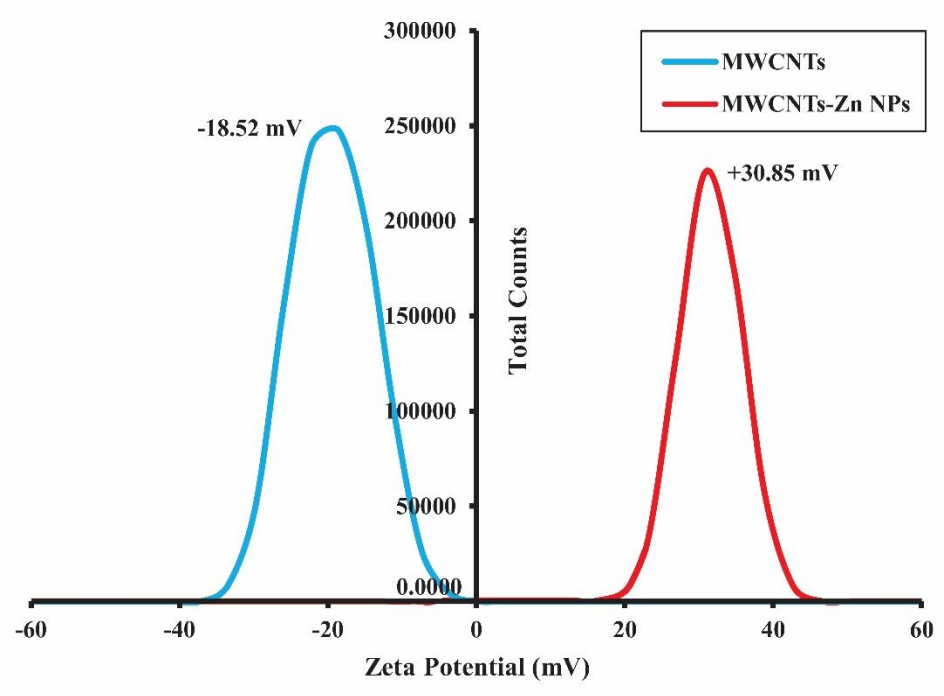

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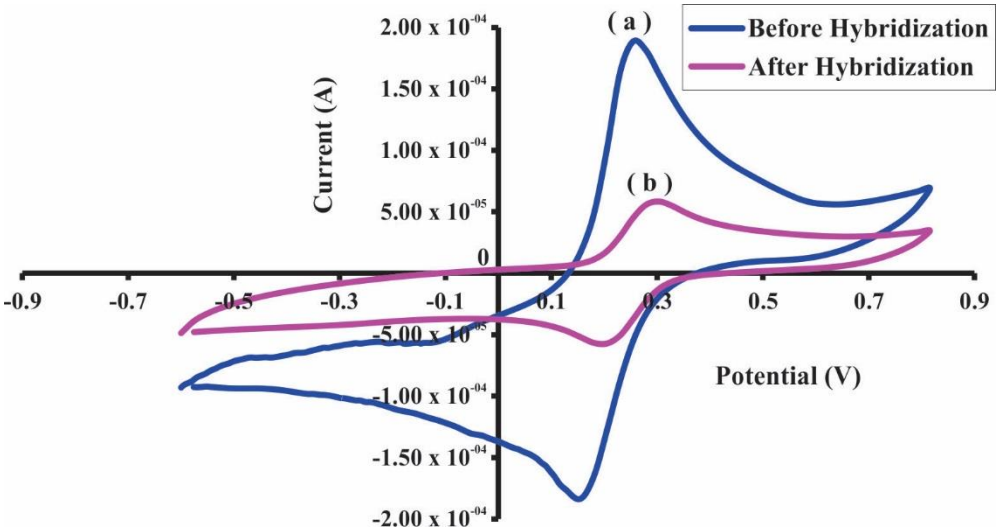

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Fig. 6.

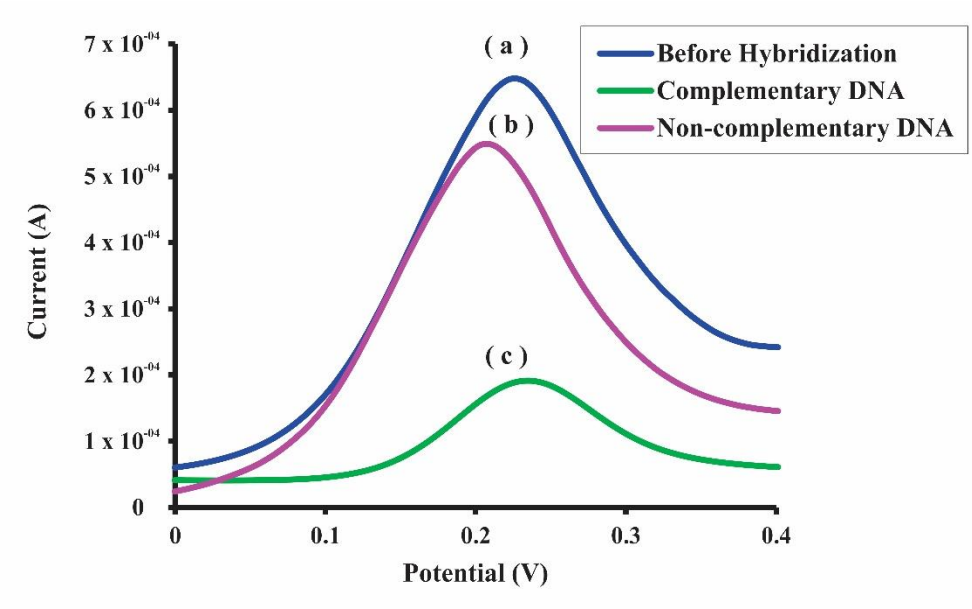

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Fig. 7.