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Development of an Antibody Functionalized Carbon Nanotube Biosensor for Foodborne Bacterial Pathogens

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Abstract

With increasing reports on bioterrorism and other bio-threats, rapid and real time detection methods for various pathogens are warranted. Attempts have been made to improve electrochemical biosensor performance by incorporating carbon nanotubes (CNTs). The high surface area of CNTs allows both immobilization of antibodies and electrochemical measurements. Salmonella monoclonal antibodies were covalently attached onto CNTs by using diimide activated imidation coupling. CNTs functionalized with antibodies were immobilized onto a glassy carbon electrode and the presence of pathogen was detected by studying the changes in charge transfer resistance and impedance, before and after the formation of antigen-antibody complex. CNTs behave as molecular wires allowing electrical communication between the underlying electrode and the conjugated antigen-antibody complex. Nyquist plots and cyclic voltammograms were studied and comparisons were made between glassy carbon electrodes as working-electrode by itself, electrodes immobilized with antibodies and after the formation of antigen-antibody complex. Cyclic voltammeter experiments had a potential scan rate of 100 mVs^{-1}, step height of 1.0 mV and applied potential from -1.0 V to 1.0 V. The electrochemical impedance experiments applied a frequency range of 100 kHz -100 mHz with an AC sine wave amplitude of 10 mV. Amplification in the current density was observed for CNTs immobilized on the electrode surface and decrease in current density and increased impedance was observed after the antigens bound specific antibodies. Enzyme-Linked Immune Sorbent Assay (ELISA) was done to determine the titer of the antibodies and their sensitivity at different dilutions for antigen detection. This technique could be an effective way to sense the formation of antigen-antibody complexes, with the potential to make the detection process rapid as compared to conventional pathogen detection methods.

Keywords: Biosensor; Antibody functionalized CNTs; Electrochemical impedance spectroscopy; Cyclic voltammetry

Introduction

Some foodborne diseases are well recognized, whereas some are considered emerging as they have recently become more common [1]. Various foodborne bacterial pathogens have been identified for foodborne illnesses such as *Campylobacter, Escherichia coli, Listeria monocytogenes* and *Salmonella* and they generally are found responsible for foodborne outbreaks [2-6]. Conventional methods for the detection and identification of microbial pathogens mainly rely on biochemical and microbiological identification methods such as Polymerase Chain Reaction (PCR) methods which involve DNA analysis [7-9], and immunology-based methods which involve antigen-antibody binding [10,11]. Electrochemical based detection methods are another possible means for identification and quantification of foodborne pathogens. Electrochemical biosensors can be classified as impedimetric, potentiometric, amperometric or conductometric sensors based on parameters such as impedance, potential, current and conductance respectively. These methods have several advantages like low cost, high selectivity and sensitivity and ability for easy miniaturization [1,12-17]. It is desired to devise a technique that is able to detect lower amounts of analytes, with comparable sensitivities to that of the conventional ELISA-based immunoassays. Hence, along with miniaturization, the optimization of the sensitivity and detection limits is desirable for the new methods of detection in order to compete with the conventional methods. One of the major ways of improving the sensitivity of the immunosensors is by incorporating nanoparticles. Several attempts have been made to improve the performance of electrochemical based biosensors by introducing nanomaterials like carbon nanotubes (CNTs) and [18,19] gold nanoparticles [20,21]. Recent studies demonstrated that CNTs can enhance the electrochemical reactivity of important biomolecules, however CNTs themselves do not provide any measurable signal for sensing biomolecules. In order to make an immunosensor using CNTs, a molecular recognization function, as well as signal transducer needs to be introduced. To endow CNTs with a molecular recognition function, functionalization is done to immobilize proteins, antibodies, enzymes or antigens onto the tip, inner or outer sidewalls of the CNTs [22-24]. The unique electronic and structural properties make CNTs a promising functional material for the development of advanced electrochemical biosensors with novel characteristics that could promote electron-transfer with redox active biomolecules. In this study, single-walled carbon nanotubes (SWCNTs) were functionalized with *Salmonella* specific antibodies and used to modify the electrode surface used in an impedance biosensor for bacterial cell detection. The sensor probes the formation of antigen-antibody complex by measuring the

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change in electrochemical properties of the sensor due to the insulating properties of the bacterial cell membrane. This technique is an effective, non-destructive, low cost characterization technique for determining the electrical properties in biological interfaces and moreover, the established method for *Salmonella* detection could provide an approach for the design of Electrochemical Impedance Spectroscopy (EIS) based immunosensors for analysis of other bacterial pathogen detection in food in the future.

Experimental

Materials and methods

All the chemicals were commercially available and used as received. SWCNT were purchased from Nanolab Inc. Waltham, MA. N-Ethyl-N'-(3-dimethyl-Aminopropyl) Carbodiimide Hydrochloride (EDAC), N-hydroxysulfosuccinimide (Sulfo-NHS), Potassium Ferricyanide (III) were purchased from Sigma Aldrich. Mouse anti *Salmonella* monoclonal antibodies, goat anti *Salmonella*, rabbit anti *salmonella* were purchased from Fitzgerald Industries International. Goat anti mouse Ig (H+L) HRP, rabbit anti goat IgG (H+L) HRP, goat anti rabbit Ig-HRP were purchased from SouthernBiotech. The electrolyte used in all electrochemical measurements was 0.5 M KCl, 0.1 M Phosphate Buffered Saline (PBS) and 0.5 mM $K_3Fe(CN)_6$, purchased from Sigma-Aldrich. *Salmonella enterica* serovar Typhimurium LT2 was purchased from ATCC (13311).

Apparatus

All the cyclic voltammetry (CV) and EIS measurements were carried out using the potentiostat, PGZ-301 Voltalab, Radiometer analytical instrument and the data was analyzed with the corresponding Voltamaster PGZ-301 software and IgorPro software. The standard three-electrode system consisted of a cylindrical shaped glassy carbon electrode (GC) with a surface area of 7.07 mm^2 and diameter 3 mm, an Ag/AgCl (3 M KCl) reference electrode, and a platinum wire (CH Instruments) as the counter electrode. The electrochemical measurements were carried out in a 10-mL cell at room temperature.

Fourier-transform-infrared spectrometer: Nicolet iS10 Fourier-Transform-infrared spectrometer (FTIR) (Waltham, MA) was used for determination of evidence of carboxylation and presence of antibodies. Attenuated total reflectance-FTIR (ATR-FTIR) was performed on dried and liquid samples on a germanium plate. Each sample run consisted of 64 scans from 500 cm^{-1} to 3700 cm^{-1}.

Thermogravimetric analysis: TA Instruments (Waltham, MA) TGA Q50 was used for thermo gravimetric analysis (TGA). Dispersions were each prepared by removing water from the sample through non-heated, vacuum drying. After removal of the water, the remaining sample was placed in a sealed scintillation vial to prevent absorption of moisture. For testing, approximately 20 mg of sample was placed in a titanium sample pan to ensure adequate signal. Each dispersion was run in argon using the following test method (i) Isothermal hold for 30 min. (ii) Temperature ramp to 120°C at 10°C/min. (iii) Isothermal hold at 120°C for 30 min. (iii) Temperature ramp to 800°C at 10°C/min. (iv) Isothermal hold at 800°C for 45 min.

Scanning electron microscopy: JEOL (Tokyo, Japan) 7000F FE-SEM with energy dispersive X-ray spectroscopy was used for performing Scanning Electron Microscopy (SEM). Samples were prepared on an aluminum SEM sample stub. The sample was then vacuum dried at 30 mmHg and 80°C for 24 hrs. After the sample was dried, it was gold-coated using a PELCO (Redding, CA) SC-6 Sputter Coater to increase the electrical conductance of the surface, which enhances the optical resolution.

Development of ELISA method, determination of the titer and sensitivity of antibodies

Antibody titer measurement of three different antibodies, *Salmonella* monoclonal antibody, goat anti mouse IgG and two polyclonal antibodies, goat anti *Salmonella* and rabbit anti *Salmonella* was done to determine quantitatively, how much antibody an organism has produced that recognizes a particular epitope, expressed as the greatest dilution that still gives a positive result. Indirect ELISA was conducted according to the procedure of Xu et al. [25], where *Salmonella* overnight culture broth was diluted within a range of 10^{-1} to 10^{-8} with 1x PBS (phosphate buffered saline) and plated 100 µl/well in polystyrene microtiter plate and incubated at 4°C over night. Plates were washed with washing buffer three times and blocked with 300 µl of a non-reacting protein or a blocking buffer (3% nonfat dry milk powder in1x PBS containing 0.05% Tween®) which was added to each antigen coated well and incubated at 37°C for 1 hour. Next, 100 µl of primary antibody (at different dilution ranging from 1/10,1/100 and 1/1000) was added to each well and incubated for 1 hour at room temperature and washed three times with wash buffer. The primary antibody binds specifically to the test antigen (*Salmonella*) that is coating the well. Secondary antibodies, Goat anti mouse Ig (H+L) HRP, rabbit anti goat IgG (H+L) HRP, goat anti rabbit Ig-HRP were diluted in antibody buffer (2% dry milk powder in 1x PBS containing 0.05% Tween®). Next, 100 µl of the secondary antibody was added, which binds to the primary antibody. This secondary antibody has an enzyme attached to it. A substrate for this enzyme is then added. The enzymatic reaction was stopped by adding the stopping solution (50 µl/well) and then absorbance at 450 nm was measured. This substrate changes color upon reaction with the enzyme. The color change shows that secondary antibody has bound to primary antibody. The higher the concentration of primary antibody present, the stronger the color change (or higher OD 450 nm) observed.

Activation

Salmonella monoclonal antibody was covalently linked to carboxylated SWCNTs according to the method as described by Jiang group [26]. The carboxylic acid group was added to sidewall or the tips of SWCNTs by oxidation treatment by the solution containing 3:1 H_2SO_4 and HNO_3 under ultra-sonication for 8 hours. These groups were further activated using 0.4 mM EDAC as a coupling agent. The EDAC treatment leads to the formation of highly reactive and unstable acyl urea derivative. It then forms a more stable active ester in the presence of 0.1 mM sulfo-NHS. As a result, an antibody was cross-linked to the SWCNTs through a nucleophilic substitution reaction between the active ester and the amine group of the antibody, which forms amide bond between CNTs and the antibody (Figure 1).

Immobilization

The antibody immobilization procedure was adopted from the method described by Tlili and group [27]. After rinsing the GC with deionized water and drying, antibodies conjugated with SWCNTs at different concentration (w/w) 1:10, 1:100 and 1:1000 were dropped on the GC surface and incubated for 1 hour at 4°C. The excess and weekly adsorbed antibodies were removed by rinsing with 1x PBS. Then, the antibody-modified GC surfaces were treated with 0.1% BSA for 30 min, to block the unreacted and non-specific sites. This approach forces primary antibodies to out-compete the blocking protein for binding

to cognate ligands while reducing nonspecific binding because the antibodies have no greater binding affinity for nonspecific epitopes than do the buffer proteins. After rinsing with 1x PBS, the electrodes were dried, and finally the sensors were ready for use. Thereafter, a self assembled monolayer was formed on dropping various dilutions of SWCNT-antibody on the electrode surface.

Detection of *Salmonella* by EIS and CV

A certain amount of carboxylated SWCNTs and antibody functionalized SWCNTs (1-20 µl) was pipetted onto the surface of the polished GC electrode and allowed to dry overnight at 4°C. EIS and CV were measured on the modified GC with carboxylated SWCNTs (SWCNT-COOH) and antibody functionalized SWCNTs in 0.5 mM $K_3Fe(CN)_6$ in 0.5 M KCl before and after modification of the GC. Next, different concentrations (10^{-1} to 10^{-8}) of *Salmonella* overnight culture was dropped on to the antibody functionalized SWCNTs and incubated for 4 hours at 4°C to facilitate the formation of antigen-antibody complex. The electrochemical measurements were carried out in a 10 ml cell at room temperature. The CV experiments had a potential scan rate of 100 mVs^{-1}, step height of 1.0 mV and applied potential from +1 V to -1 V. The EIS experiments applied frequency range from 100 kHz -100 MHz with an AC sine wave amplitude of 10 mV.

Results and Discussion

Characterization of functionalized SWCNTs

Antibodies were covalently linked to the SWCNTs by the formation of an amide bond between the carboxylic groups on SWCNTs and amine groups on the antibodies using EADC and sulfo-NHS chemistry to cross link antibodies to the CNTs through a nucleophilic substitution reaction. Several characterization studies were conducted to verify the functionalization of CNTs, such as, ATR-FTIR, TGA, SEM, transmission electron microscopy (TEM) and zeta potential analysis.

The FT-IR has shown limited ability to probe the structure of SWCNTs and distinguish it from the back ground noise because of their black character and strong absorbance. Therefore, ATR-FTIR was performed on germanium plate. In Figure 2, the spectrum of the SWCNT-COOH shows a sharp absorbance at 1715 cm^{-1} ascribing to C=O stretching of –COOH group. After antibody attachment onto the SWCNT-COOH, a new strong absorbance 1642 cm^{-1} indicates that the –COOH group on SWCNTs reacted with the amine group on the antibodies to form an amide bond (-CONH-).The band around 1550 cm^{-1} in SWCNT-COOH corresponds to the stretching mode of C=C double bond that forms the framework of the CNTs sidewall. Thus ATR-FTIR data confirmed that carboxylation as well as antibody

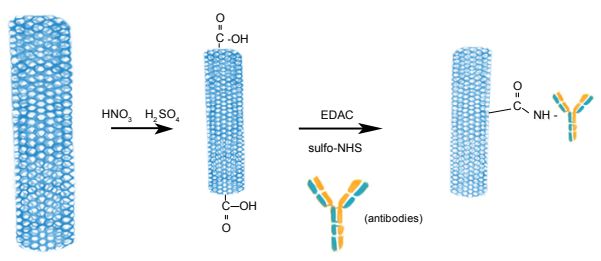

Figure 1: The schematic illustration of the process of functionalization of antibody with SWCNTs.

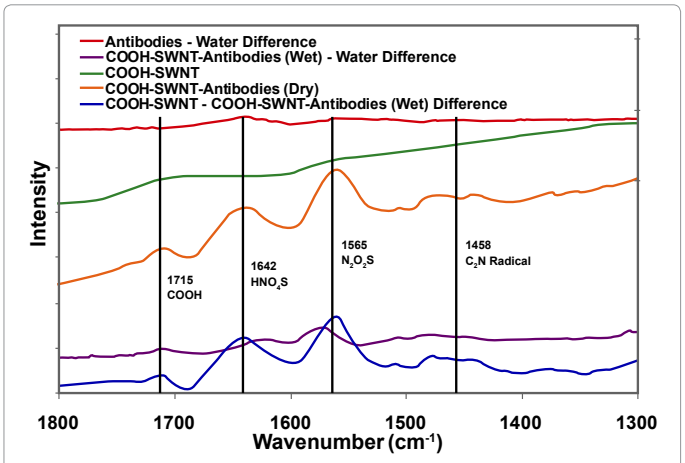

Figure 2: FT-IR spectra of SWCNT-antibody. Strong peak at 1642 cm^{-1}, confirms the amide bond formation between the –COOH group on SWCNT and $–NH_2$ on antibodies.

conjugation with SWCNTs has been successful. Zeta potential was measured for SWCNTs and SWCNT-COOH using Malvern Zetasizer NanoZ (Figure 3). The zeta potential of SWCNTs and SWCNT-COOH at 1:1000 dilutions in double distilled water is -3.2 and -34.9 respectively. The high magnitude of the measured zeta potential is an indication of the repulsive force that is present and can be used to predict the long term stability of the SWCNT-COOH as against SWCNTs. The high zeta potential of SWCNT-COOH is thus an indication that the particles will tend to repel each other and there is relatively less tendency of the particles to agglomerate, which is in agreement with the previous reports of increased dispersion of CNTs with oxidation [28-30].

More direct evidence for the functionalization of SWCNTs comes from the field emission SEM images (FESEM). The changes in morphology are remarkable. The SWCNTs appear more aggregated as against distinctly visible strands of SWCNT-COOH (Figure 4). The SWCNT-antibody surface is relatively rough and is quite different in morphology from the starting material.

Similarly, TEM images show less aggregated bundle of SWCNT-COOH and gives some evidence about functionalization (Figure 5).

TGA analysis measures changes in weight of the functionalized and non-functionalized CNTs samples with increasing temperature. Figure 6 provides quantitative information on SWCNTs functionalization from TGA analysis. This allows for measurements of mass change and associated phase transformation energetic of SWCNTs and functionalized SWCNTs.

Antibody titer analysis

As evident from the titer results (Figure 7 A,B&C), *Salmonella* monoclonal antibodies are more sensitive towards the lower concentrations of bacterial culture. Even at increased dilutions (1/1000), *Salmonella* monoclonal antibodies are more effective as against the other two polyclonal antibodies. This could be because of higher affinity of monoclonal antibodies towards the specific *Salmonella* proteins. Therefore, for functionalization of carbon nanotubes with antibodies, EIS and CV experiments, *Salmonella* monoclonal antibodies were used.

EIS and CV analysis

EIS analyses the resistivity as well as capacitive properties of materials, based on the perturbation of a system at equilibrium with

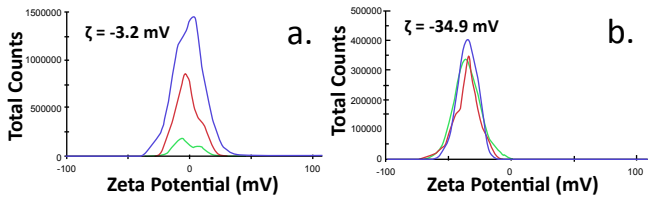

Figure 3: The zeta potential of (a) SWCNTs and (b) SWCNT-COOH at -3.2 and -34.9 respectively. The high zeta potential of SWCNT-COOH indicates low aggregation tendencies.

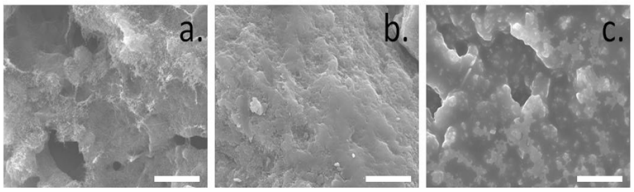

Figure 4: SEM images of (a) SWCNT by itself (b) SWCNT-COOH (c) SWCNT-antibody showing clear difference in their surface morphology.

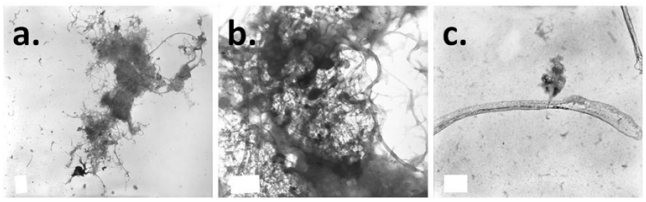

Figure 5: TEM images of (a) SWCNT by itself (b) SWCNT-COOH (c) SWCNT-antibody. Increased diameter observed in (c) is because of conjugation of antibody onto the surface/side walls of SWCNTs.

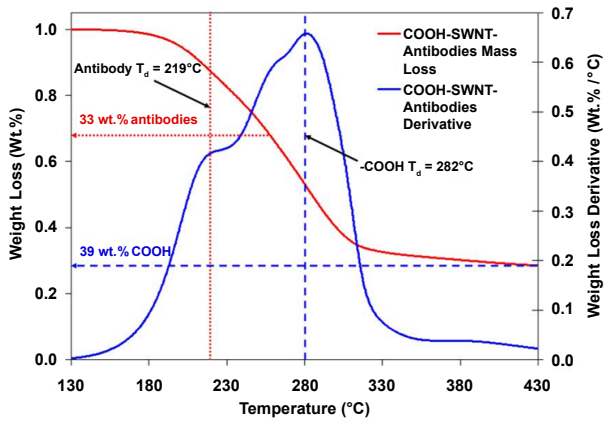

Figure 6: TGA analysis showing weight loss and derivative curves of the antibody functionalized SWCNTs.

a small sinusoidal excitation signal. The impedance thus obtained can be used to model with an equivalent circuit which is useful for the interpretation of the electric properties of self assembled layer formed by antigen–antibody interaction and charge transfer reactions.

GC electrode/ electrolyte interface

Cyclic voltammograms of the GC electrode were measured before and after the formation of the self assembled monolayer in the presence of 0.5mM Fe $(CN)_6^{-3}$/ $Fe(CN)_6^{-4}$ and the results are presented in the Figure 8A. As shown in the figure, a reversible cyclic voltammogram is observed on the GC electrode, indicating a clean gold surface. Figure 9A shows Nyquist plot evaluation of the GC electrode/electrolyte interface in 0.5 M KCl. The EIS is a label free detection method based on the application of a sinusoidal signal excitation to an electrochemical cell and the analysis of the current produced. The impedance is the cell resistance to an alternating current, is a parameter used to describe relationships between voltage and the current in an electrochemical cell. As a result of impedance measurements, two types of impedance are produced, real portion (Z′) and the imaginary part (Z′′), which is an indicative of characteristic capacitance and resistance elements of the electrode-electrolyte interface. As the formation of antibody-antigen complex at the electrode surface will bring about a change in the charge distribution in the electrical double layer as well as the resistance of the surface layer, it is expected that the impedance data would provide useful information about association-dissociation kinetics of biological complex at antibody modified electrode surfaces. The correlation between the impedance change and affinity complex formation has been reported by several authors [31-33].

GC electrode/CNT-COOH/electrolyte interface

SWCNT-COOH modified GC resulted in increased faradic currents as the carboxylic group on CNTs being an electron withdrawing group (-Inductive effect) facilitates the movement of holes and thereby increasing the flow of current in the p-type semiconducting SWCNTs (Figure 8B).

GC electrode/antibody/electrolyte

SWCNT-antibody modified GC resulted in faradic current block (Figure 8C) because of the insulating properties of the antibodies and also the amine groups present in the antibodies donate electrons to the p-type nano tubes, thereby combining the holes in the nano tubes and leading to an increase in the resistance at positive bias voltage [34,35]. The increase in the charge transfer resistance and increase impedance has been observed at the GC/electrolyte surface (Figure 9C).

GC/antibody-antigen complex/electrolyte

The increased diameter of the semicircle in the nyquist plot in (Figure 9D) represents increased impedance or the blocking behavior of the self assembled layer of antibodies (conjugated with SWCNTs) and antigen complex on the GC surface. A highly organized layer of the insulating monolayer is formed on the electrode surface that poses charge transfer resistance and decrease the flow of current through the redox active species (Figure 8D). The change observed in the electrical properties of the sensor is due to highly insulating properties of the cell membrane. Where the cell membrane is highly insulating, the interior of the cell is highly conductive because of dissolved charged molecules inside the cell sap. The conductivity of the cell membrane is around 10^{-7} S/m, whereas the conductivity of the interior of a cell could be as high as 1 S/m [36]. Thus electron transfer resistance is the parameter that s measured in the biosensors. The attachment of the bacterial cells retards the interfacial electron-transfer kinetics and increases the electron-transfer resistance. The biosensor has a linear response range of the bacterial concentration from 10^{-1} to 10^{-6} (dilution values refer to serial dilution of the bacterial overnight culture). The detection limit of this electrochemical sensor is 1.6×10^4 cfu/ml.

Conclusions

In this paper, an electrochemical impedance immuno sensor for the detection of low concentration (1.6×10^4 cfu/ml.) of *Salmonella* was developed by modifying GC by covalently conjugated antibodies

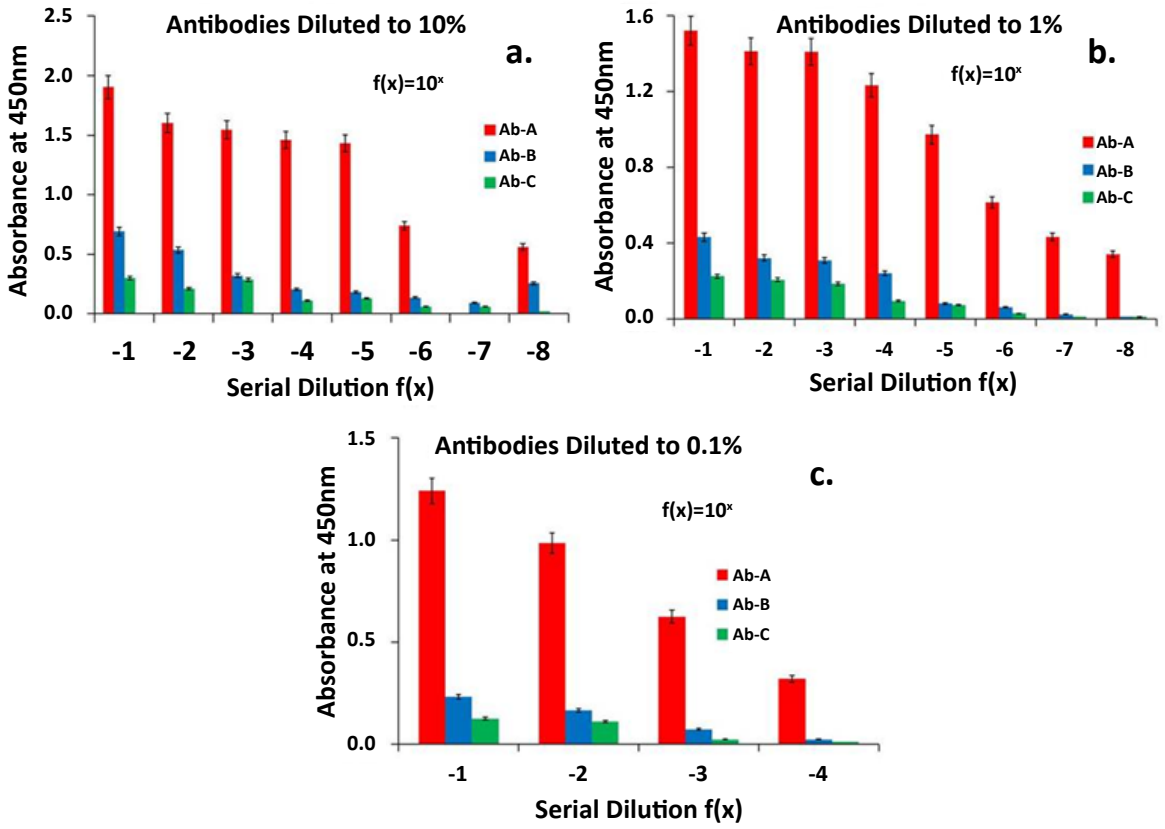

Figure 7: Ab-A is Salmonella monoclonal antibody; Ab-B is goat anti Salmonella; Ab-C is rabbit anti Salmonella] (a),(b) and (c) compares the OD 450nm of the three different antibodies at different concentration of bacteria and at three different dilutions, 1/10, 1/100 and 1/1000 of the antibodies.

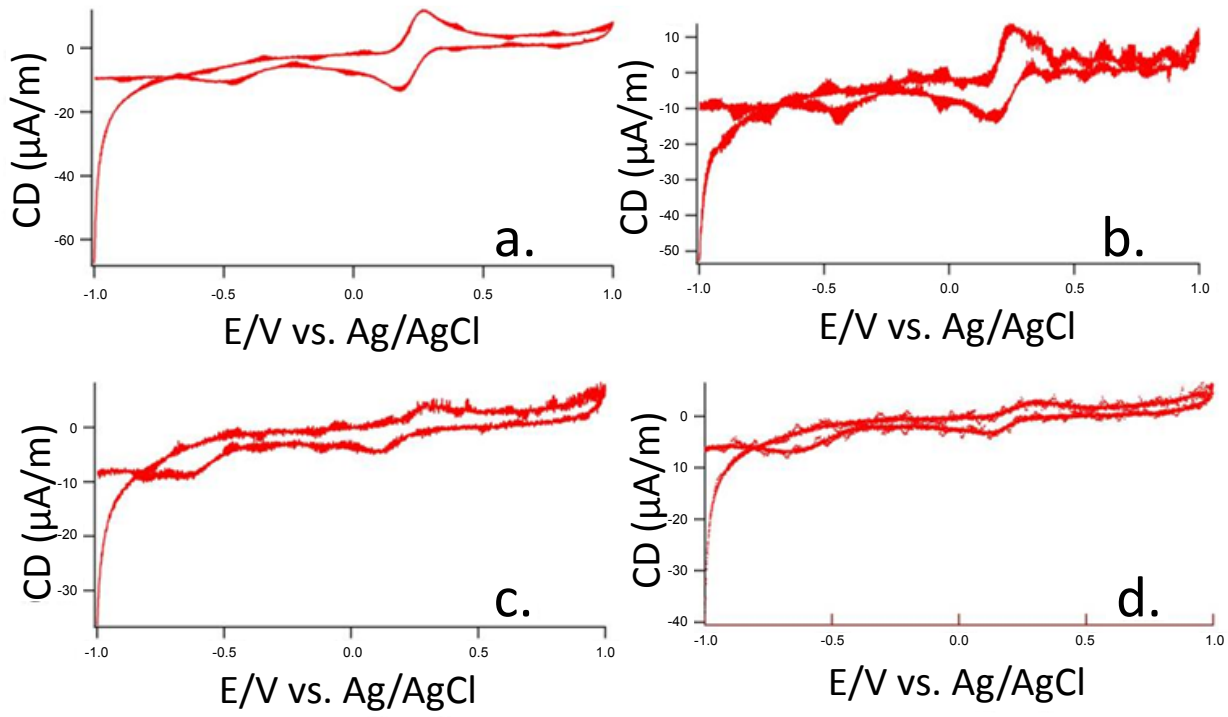

Figure 8: Cyclic Voltammetry the GC electrode in the pressence of 0.5mM Fe (CN) 6 -3/ Fe (CN)6 -4 (scan rate 100.0 mVs-1) ;(a) bare GC electrode (b) after functionalization with SWCNT-COOH (c) after immobilization of SWCNT-antibody (d) after the formation of antigen-antibody complex.

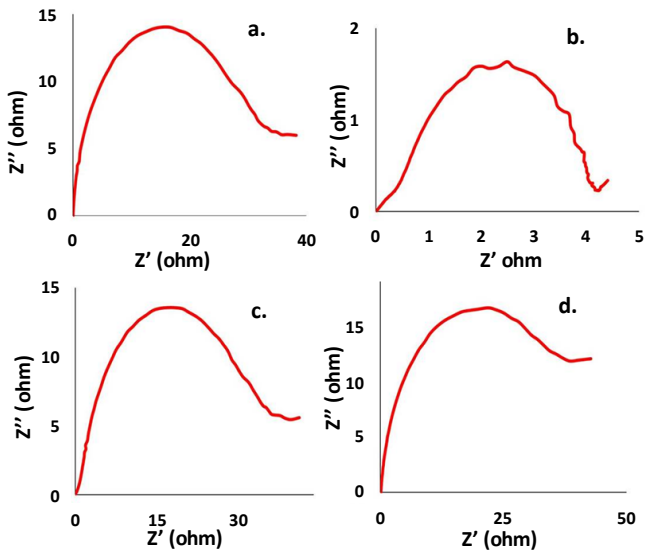

Figure 9: Impedance spectra in the presence of Fe $(CN)_6^{-3}$/Fe$(CN)_6^{-4}$ as redox probe (a) bare GC electrode (b) after functionalization with SWCNT-COOH (c) after immobilization of SWCNT-antibody (d) after the formation of antigen-antibody complex.

to SWCNTs. Covalent attachment of antibodies to SWCNTs was characterized using FT-IR, TGA, and microscopic techniques. Decreased current density and increased impedance was observed on the formation of self assembled monolayer of antibody-antigen complexes on the electrode surface, because of the insulating properties of the cell walls of the bacteria. EIS involves measurement of electrical properties of the sample involved and does not require chemical/fluorescent labels. Low voltages (+1.0 V to -1.0 V) and currents were used, making this technique non-destructive, which is especially useful in measurement of biological molecules. The proposed method could prove to be a feasible quantitative method for the analysis of any bacterial pathogen with the properties of stability, high sensitivity and selectivity.

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