Double stranded DNA templates for the electrochemical determination of benzene derivatives

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Abstract— Multi-walled carbon nanotubes (MWCNT) modified DNA based biosensors were prepared by immobilizing dsDNA over MWCNT coated graphite electrode. The film forming abilities of MWCNT coated graphite electrodes were studied using Electrochemical Impedance Spectroscopy (EIS) and Cyclic Voltammetry (CV) by placing the electrodes in electrolyte containing redox couple. Differential Pulse Voltammetry (DPV) and EIS were performed to identify the relative change in the oxidation peak and charge transfer resistance respectively after its interaction with benzene derivatives (aniline, toluene, phenol, anisole and mesitylene). Under optimized conditions, calibration curves were obtained for the modified electrodes over various analytes using DPV and EIS. The proposed ds-DNA based MWCNT biosensor exhibited stability, high reproducibility, selectivity and regeneration, making it a potential tool for electrochemical detection of benzene derivatives in water based samples.

Index Terms— Benzene derivatives, dsDNA based Biosensor, CV, DNA, DPV and EIS.

I. INTRODUCTION

The occupational exposure limit of benzene in the United Kingdom (UK) and the United States (US) was 10 ppm based on the association of benzene exposure with anemia. Recently, it was lowered to 5 ppm and 1 ppm in these countries respectively as a reflection for the risk of neoplasia (the process of tumor formation). The American Conference of Governmental Industrial Hygienists (ACGIH) has considered benzene as A1 carcinogen and decreased the threshold limit value (TLV) to be less than 0.1 ppm [1]. Benzene structure represents a six carbon ring with three double bonds, represented by hexagon, proposed by Friedrich August Kekule in 1872. Each carbon atom ($1s^2 2s^2 2p_x^1 2p_y^1$) has to join with one hydrogen atom (1s1) and two carbon atoms. In such process, it does not have enough unpaired electrons to form the required number of bonds and hence it promotes one of the $2s^2$ pair into the empty $2p_z$ orbital forming sp^2 hybrids. This sp^2 hybrid forms sigma bonds with two other carbon and one hydrogen atom. The p electron of each carbon atom is overlapping with those on both sides of it. This extensive sideways overlap produces a system of pi bonds which are spread out over the whole carbon ring and termed to be 'delocalized'. The 6 delocalized electrons go in three molecular orbitals- two in each forming a stable molecule [2].Now, having understood the structural stability of benzene, it is important to understand the reason behind benzene toxicity in living organisms. Any living organisms eliminate an uptaken chemical compound by breaking down into soluble compounds. In human body, this happens in the liver by introducing functional groups on to the benzene ring to make it more soluble. It is for the same reason, benzene is classified as a non-mutagen in the Ames test. The metabolism of benzene has been reported to yield glucuronide and ,jsulphate conjugates of phenol, quinol, catecol, L-phenylmercapturic acid, mucoaldehyde and trans-muconic acid by ring scission [1]. In addition, the metabolic mechanisms of benzene involve the formation of phenol metabolites by peroxidase in bone marrow which again generates reactive quinols [3]. Quinol is oxidized to p-benzoquinone, which binds to vital cellular components or undergoes redox cycling to generate oxygen radicals [1, 4]. Further, there is a report on the relationship between the chemical constitution of organic compounds and their toxicity to insects [4]. It was shown that (a) the substitution of certain radicals in the benzene ring affects toxicity, (b) the toxic action depends not only upon the radicals but also on the substituted numbers and (c) in certain cases toxicity depends on the substituted radical's relative position. Quantitative structure-toxicity relationships (QSTR) were derived for an extensive series of halogenated benzene, anilines, phenols, nitrobenzenes, toluenes and other substituted benzenes. A combination of molar refractivity and nucleophilic susceptibility of one of the meta ring carbons was found to predict the toxicity [5]. Hence, it can be inferred that structurally, the aromatic compounds increase the toxicity within the living organism. These compounds undergo the formation of metabolites which causes phenotoxic and genotoxic effects on the living organisms. ssDNA based biosensors for the determination of benzene substituted aromatic compounds have been discussed earlier by the same authors [6]. In this report, the authors report the

application of dsDNA based biosensor for the determination of benzene derivatives.

The electrochemical determination of five commonly used benzene derivatives namely aniline, toluene, phenol, anisole and mesitylene, using double stranded DNA template structures has been performed. DNA exists in two forms, single stranded (ss) and double stranded (ds) DNA structures. Double stranded DNA is the most stable form of DNA found in biological system. The structure of dsDNA consists of two helical single stranded DNA coiled around each other with a pitch of 3.4 nanometers and radius of 1.0 nanometer. The two long strands entwine like vines, are in the shape of a double helix. The nucleotide repeats contain both the segments of the backbone of the molecule which holds the chain together and a nucleobase, which interacts with other DNA strand in the helix. Although each individual nucleotide is very small, DNA as a polymer can be very large macromolecule containing millions of nucleotides. Each strand of the dsDNA has a direction namely parallel and antiparallel direction. The direction of the polynucleotides in one strand is opposite to the direction of other strand and hence the name "antiparallel".

There are two forces which play a major role in the structural stability of DNA: hydrogen bonds between the nucleotides and base-stacking interactions among aromatic nucleobases. Hydrogen bonding is done between the purines and the pyramidines. In the aqueous environment, the pi bonds of nucleotide bases align perpendicular to the axis of the DNA molecule minimizing their interaction with the solvent shell [7].

As, and when DNA come across a molecule which damages its structure, a change in oxidation signal of purine bases in the DNA was observed. This property of DNA was used for determination studies, provided the molecule damages DNA. The interactions of analytes with the biosensor were evaluated from the change in electrochemical response before and after its interaction with benzene derivatives using DPV and EIS.

II. MATERIALS AND METHODS

A. Reagents

Graphite rods were purchased from Home Science Tools, Montana, USA. The procured rods were cut into 5 equal sizes and rubbed over micro alumina powder for several minutes until a smooth surface of diameter 0.636cm was obtained. In order to make electrical contact, conducting wires of equal length were pasted at the side of the sliced graphite rods using silver paste. It was then coated with Teflon leaving the bottom surface for it to act as sensor after modifications. MWCNT of (30±15) nm diameter and length of several microns were obtained from Applied Science Innovation Pvt. Ltd, Maharashtra, India. MWCNTs were oxidized using concentrated nitric acid by sonicating it for 30 minutes, in order to remove impurities. After which, the suspension was washed several times with water to remove trace amount of nitric acid in the nanotubes. Mono sodium phosphate and di-sodium phosphate were obtained from Merck, NJ, USA. Double distilled water was used throughout the experiment. All other chemicals were obtained from Sisco Research Laborotaties and were used without any further purification. DNA templates of 50bp length were manually designed and purchased from Sigma Aldrich, Mumbai, India:

P_1:5'TCTGAGTCTGTATGGAGTGACATGCTTTCTG GGTGGACTCAAGTTGAAGA3';

P_2:5'TCTTCAACTTGAGTCCACCCAGAAAGCATGT CACTCCATACTGACTCAGA3'

DPV measurements were carried out in 0.1M Phosphate buffer. CV and EIS measurements were made in 0.1M NaCl solution containing 10/10mM $K_3Fe(CN)_6/K_4Fe(CN)_6$. dsDNA templates were prepared by the addition of P_2 strands to the P_1 strands. The DNA mixture was heated to its annealing temperature (90 ºC) and slowly cooled there after using thermal cycler where the two complementary strands bind to each other via hydrogen bonds. The dsDNA/GTA/CS-MWCNT/G electrode was developed by immobilizing 70 mg/l of dsDNA templates at a fixed potential (+0.3 V versus Calomel/Platinum electrode for 180 s). During immobilization step, the electrode was immersed in 0.1 M Phosphate buffer (pH 5) containing desired quantity of dsDNA templates. After immobilization step, the electrode was washed with water to remove unbounded dsDNA structures and preserved at 4 °C for further use. Solutions of benzene derivatives were prepared immediately before each experiment.

B. Apparatus

All the electrochemical measurements were recorded using BioLogic Science SP-300 Instrument (France) running on EC-Lab software (Version 10.18) and with standard calomel electrode as reference electrode, platinum wire as counter electrode and graphite electrode (surface area = 0.318cm2) as working electrode. Calomel electrode used in this experiment has 0.241V (electrode surface area= 0.001cm2) as offset potential against normal hydrogen electrode. All the potentials were measured with reference to reference electrode. All the electrochemical measurements were made using 20ml cell containing 15ml of supporting electrolyte.

C. Preparation of modified electrodes

Prior to surface modification, graphite electrode was cleaned by polishing with 0.05µm alumina powder for 1 minute and sonicated in water for 30s. 1.25gm of oxidized MWCNTs was dispersed in 1ml of 1% V/V acetic acid

solution by sonication for 30 minutes. The modified electrode was prepared by casting desired quantity of MWCNT paste over graphite electrode. The resulting electrode was named as MWCNT/G, which can be stored at 4°C for further use. These electrodes can be reused by rubbing it over 0.05µm alumina powder until a smooth polished surface is obtained.

D. Immobilization of dsDNA structures

The electrode was pretreated by applying a potential of +1.5V for 30s in 0.1M phosphate buffer (pH 5) to remove electrochemical impurities. DNA based biosensor was developed by immobilizing dsDNA structures at a fixed potential (+0.3V versus Calomel/Platinum electrode for 180s). During immobilization step, the electrode was immersed in 0.1M Phosphate buffer (pH 7) containing desired quantity of dsDNAs. After immobilization step, the electrode was washed with water to remove unbounded DNAs and preserved at 4°C for further use.

E. Voltammetric Measurements

Electrochemical properties of modified electrode were studied by cyclic voltammetry (CV conditions: Potential from -0.7 to +0.7V at scan rate of 50mV/s) and electrochemical Impedence spectroscopy (EIS conditions: Frequency scan range from 0.1Hz to 1MHz and sinusoidal potential amplitude at 10mV in 51 frequency steps). 10/10mM solution of $K_3Fe(CN)_6/K_4Fe(CN)_6$ in 0.1M NaCl solution was used as redox probe to study the interfacial properties of the modified electrode immobilized with dsDNA.

Electrochemical detection of benzene derivatives was done from the change in the signals obtained before and after the biosensor reaction with these compounds using Differential Pulse Voltammetry (DPV conditions: potential increase of 0.04V, pulse amplitude of 0.05V, pulse width of 0.017s and pulse period 0.2s) and EIS. The anodic current at around 0.7 and 1.0 V were used as analytical signal for guanine and adenine oxidation respectively.

F. Electrochemical determination of benzene derivatives

The dsDNA immobilized modified electrode was immersed in the solution containing benzene derivatives for 5 minutes for the dsDNA in the electrode to react with the analyte. DPV and EIS measurements before and after the interaction with benzene derivatives were made. The relative percentage of survived dsDNA after the analyte's interaction was calculated from the change of signals obtained at electrode with and without dsDNA. This has been related to the difference of signals corresponding to that of original purines as follows:

$$\Delta I_{surv\ DNA\ (rel)}\% = [(I_{surv\ DNA} - I_{MWCNT})/(I_{DNA} - I_{MWCNT})]*100 \quad [1]$$

$$\Delta R_{ct(rel)}\% = [(R_{ct(surv\ DNA)} - R_{ct(MWCNT)})/(R_{ct(DNA)} - R_{ct(MWCNT)})]* 100 \quad [2]$$

where I is the anodic peak current measured during DPV measurement in 0.1M phosphate buffer at the modified electrode without dsDNA and R_{ct} is the electron transfer resistance measured at the peak potential obtained for 10/10mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ in 0.1M NaCl solutions at the modified electrodes without dsDNA. The indexes used, characterize the chemical modifiers of graphite electrode [8].

III. RESULTS AND DISCUSSION

DNA based biosensors with the layers of dsDNA immobilized over MWCNT at the graphite surface have been investigated. Amount and concentration of MWCNT were optimized from the obtained electrochemical responses. Figure 1 displays the schematic representation of the preparation of DNA based biosensor. Electrochemical pretreatment was performed by anodization and cathodization at ±1.5V for 30s (versus standard calomel/platinum reference electrode) in order to electrochemically activate the working electrode and to remove electrochemical impurities at the electrode surface [9]. However, the responses of the electrochemically activated working electrode depend on the experimental parameters such as the potential limits, redox reaction time, composition, concentration and pH of the supporting electrolyte. This pretreatment procedure was found to improve the hydrophilic character of the electrode surface [10].

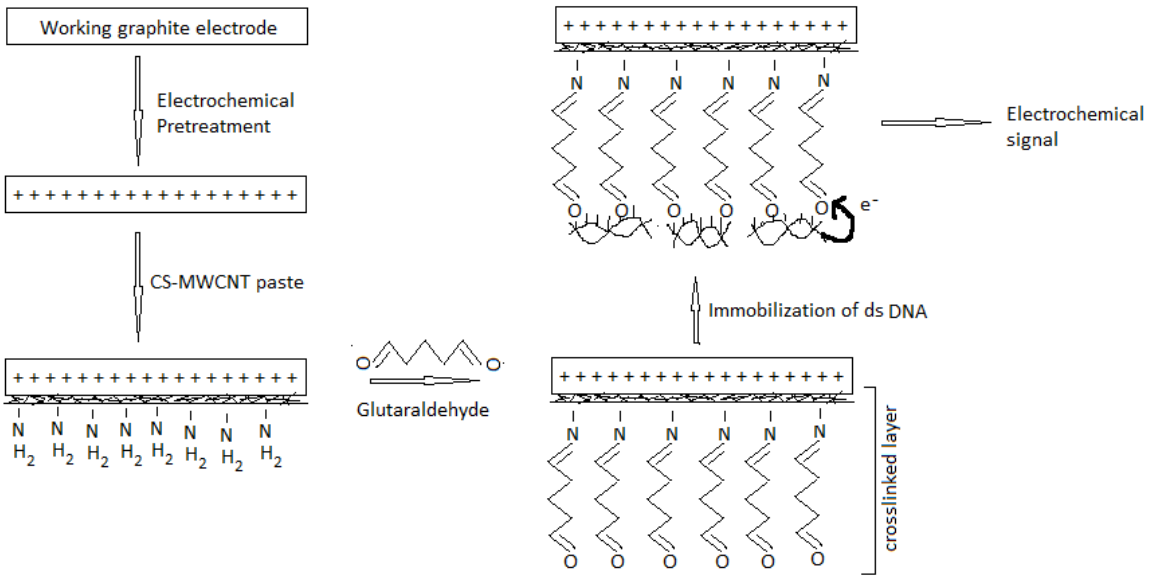

Figure 1: Schematic representation of biosensor preparation

A. Effect of MWCNT concentration

The increase in the quantity of MWCNT provided a greater surface area for DNA to immobilize over the electrode surface. This enhances the direct electrochemical response of dsDNA and is in consistant with the reported work [6,11]. Hence, it is necessary to optimize the minimum quantity of MWCNT needed to immobilize the known minimal concentration of DNA templates for a particular electrode surface area. Figure 2 displays the DPV response of the 30µl of MWCNT at different concentrations varying from 0.25 to 2mg dispersed in 1ml of 1% acetic acid solution. The reponses were recorded for MWCNT in 70mg/l of dsDNA template mixtures for 180s in 0.1M phosphate buffer (pH 7). Saturation peak was obtained for 1 mg/ml of MWCNT concentration.

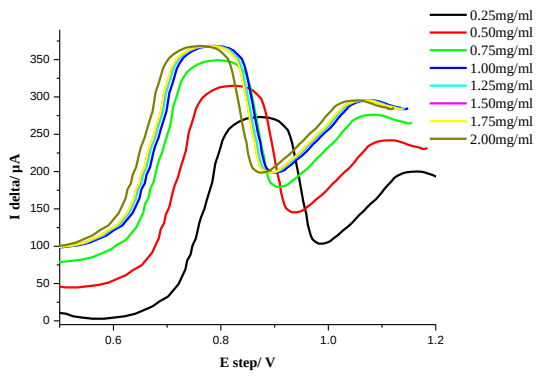

Figure 2: DPV response at different concentrations of MWCNT paste

B. Effect of immobilization time

The amount of dsDNA adsorbed over the working electrode is directly proportional to the sensitivity of benzene derivatives. Immobilization step was performed by applying a potential of +0.3V in 0.1M phosphate buffer (pH 5) for varying time upto 300s. As the immobilization time increases, the corresponding sensor signals for guanine and adenine bases increased as expected. Figure 3 shows the DPV response of 70mg/l dsDNA structures varying from 30 to 300s for the working electrode containing 1 mg/ml of MWCNT paste in 0.1M phosphate buffer, pH 7. Longer the immobilization time, greater the quantity of purine bases adsorbed and hence larger the DPV response. It was found that after a certain immobilization time (180s), the peak current almost remained to be stable, as the DNA occupied the entire working electrode surface area leaving no space for the remaining purines in the buffer to get adsorbed. This is consistent with the earlier findings [6,11].

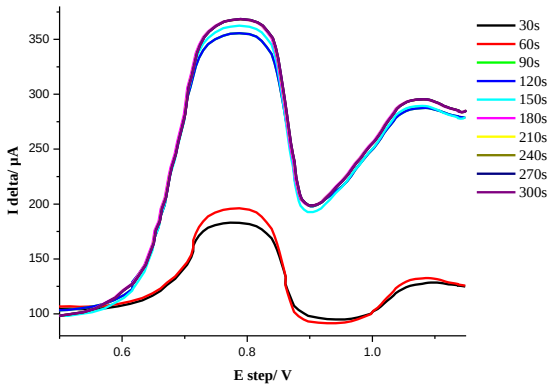

Figure 3: DPV response of 70mg/l of dsDNA at various immobilization time

C. Effect of immobilization concentration

The amount of dsDNA concentration immobilized over working electrode containing 1 mg/ml MWCNT was

varied from 10 to 100 mg/l for 180s at a potential difference of +0.3V. The oxidation peak for guanine and adenine almost remained stable for the immobilization concentration from 60 to 100 mg/l (Figure 4).

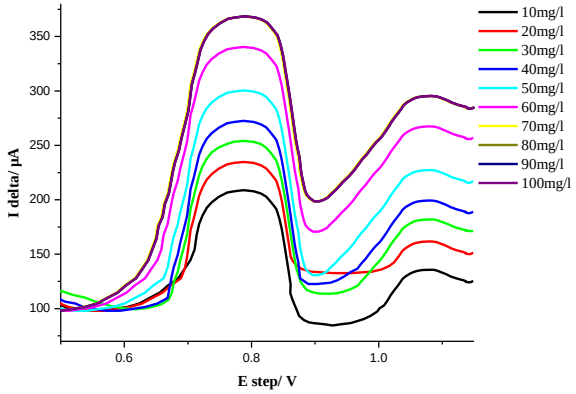

Figure 4: DPV response of 10 to 100mg/l of dsDNA over working electrode

D. Electron transfer characteristics of the working electrode

In order to study the interfacial electron transfer properties of the modified electrode immobilized with DNA templates, EIS and CV were performed using the electroactive ferrocyanide/ferricyanide redox couple in 0.1M NaCl solution. Nyquist plot of the working electrodes displays a semicircle at high frequencies and it is linear at low frequencies. The semicircle portion and the linear portion of the Nyquist plots represent electron transfer- limited process and diffusion limited process respectively. MWCNT coated graphite electrode shows a small semicircle diameter indicating excellent conductivity of MWCNT. However, on the addition of purine bases, the electron transfer resistance increases but not greater than the electron transfer resistance of bare graphite electrode.

Nyquist plot (dependence of an imaginary part of the impedence Z" vs a real part of the impedence Z') of the modified electrodes represent a semicircle at high frequencies illustrating an electron transfer limiting process. For bare graphite, a short linear part of low frequencies are observed resulting from the diffusion of limiting step of the electrochemical process is obtained [12]. It is important to consider the fact that this part of the spectrum represents the properties of the electrolyte solution and the diffusion of the redox couple in the supporting electrolyte and thus not affected by the modification of the electrode surface [13]. The impedence data were simulated using the Randles equivalent circuit consisting of a parallel combination of the capacitance (C_{dl}) and the charge transfer resistance (R_{ct}) redox reactions in series with the supporting electrolyte resistance (R_{sol}).

It was found that the R_{sol} shows a negative resistance in the Nyquist plot. Numerous examples of negative resistance have been reported and in all the cases, the condition, Re[Zω], ω→0<0, is associated with a passivation event in which the steady state current decreases with increasing voltage[14]. In other words, the electrochemical adsorption of a blockage intermediate from the electrolyte over the working electrode is a passive event ultimately represented as a negative resistance. However, the charge transfer resistance is at the positive portion representing the active transport of the redox ions.

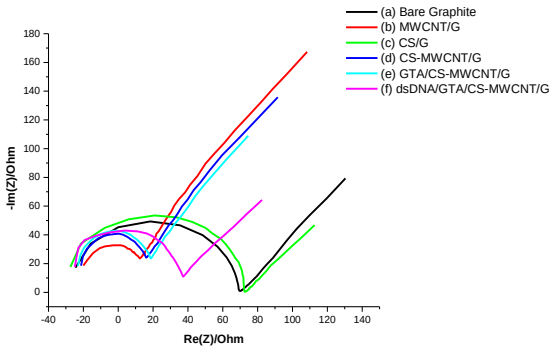

Figure 5: Nyquist plot of working electrodes in 0.1M NaCl containing 10/10mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ ions

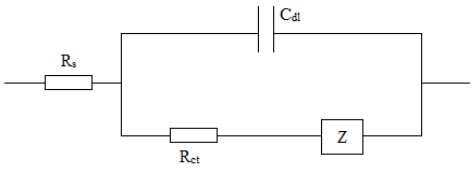

Figure 6: The scheme of equivalent circuit simulating the impedance spectra.

Table 1: Parameters of the equivalent circuit simulating the complex impedance spectra of the electrodes in the presence of 0.1M NaCl solution containing 10/10mM $K_3Fe(CN)^6/K_4Fe(CN)^6$.

Working electrode	R_{sol} Ω	R_{ct} Ω	C_{dl} µF
Bare Graphite	-24.53	70.38	0.77
MWCNT/G	-20.01	13.39	1873.6
CS/G	-27.63	72.77	64.39516
CS-MWCNT/G	-22.06	16.28	26138.32
GTA/CS-MWCNT/G	-23.05	18.62	124897
dsDNA/GTA/CS-MWCNT/G	-24.69	37.40	902.21

The increase or decrease in R_{ct} reflecting the increase or decrease in the diameter of the semicircle is directly associated with the blockage behavior of the electrode surface for the charge transfer to the redox couple in the supporting electrolyte [8]. For bare graphite, the value of R_{ct} is 70.3±0.5 Ohm and it reflects the semicircle part with greater diameter. As MWCNTs are added to the graphite surface, the diameter of the semicircle decreases and hence decreasing the R_{ct} value till 13.39±0.5 ohm. However, when chitosan, glutaraldehyde and dsDNA were introduced, the R_{ct} value increases as tabulated in Table 1. MWCNT immobilized on the graphite surface plays an important role similar to an electron conducting tunnel making electron transfer to the electrode surface

easier. The increase in the R_{ct} value for MWCNT electrode containing dsDNA is due to the formation of highly organized layer of the dsDNA formed over the modified electrode, resulting in the blockage of electron transfer to the redox couple, in other words, restricting the redox species to penetrate the MWCNT layer [8].

To confirm EIS, CV was performed in the same supporting electrolyte. The mechanism of DNA detection using $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ resides in the barrier effect of the DNA towards the redox couple [8], resulting in the reduction in redox couple signal (Figure 7) after the addition of dsDNA to the modified electrode.

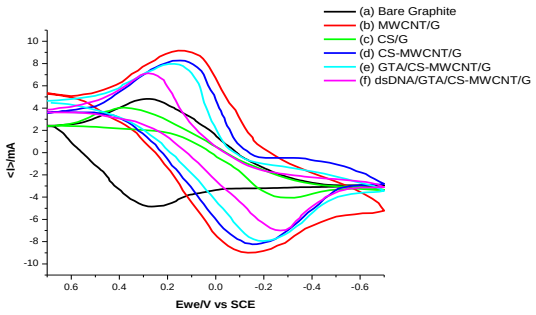

Figure 7: CV of the working electrodes in 0.1M NaCl containing 10/10mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ ions

E. Electrochemical Determination of Benzene Derivatives

DNA bases were attacked by exposing the modified electrodes to benzene derivatives. Survived purine bases were calculated from the DPV peaks and Rct values obtained from EIS before and after the exposure. Figure 8, 9 and 10 display the calibration curves obtained from the average relative portion of survived DNA bases.

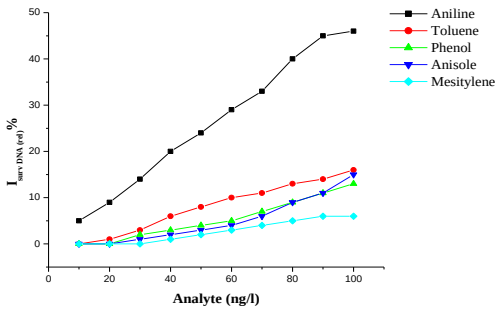

Figure 8: Calibration plot obtained from benzene derivatives using adenine signal of DPV

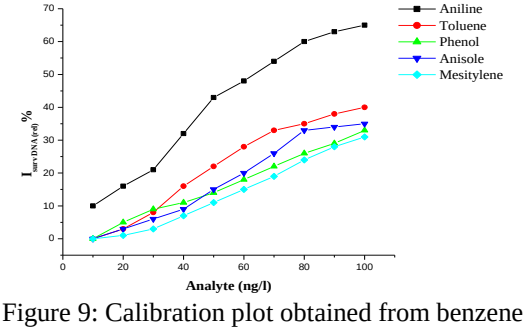

Figure 9: Calibration plot obtained from benzene derivatives using guanine peak signal of DPV

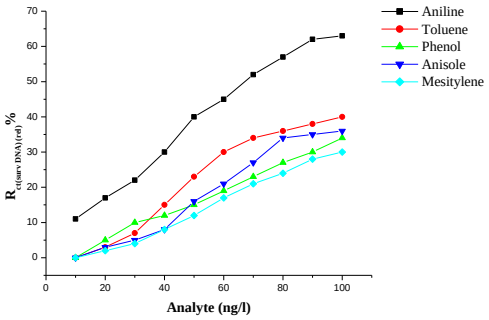

Figure 10: Calibration plot obtained from benzene derivatives using Rct values of EIS

It can be noticed that as the analyte concentration increases, the relative percent DNA damage also increases, consistant with the DNA damaging property of benzene derivatives. These values may be used for the design of biosensor for the determination of benzene derivatives present in the sample.

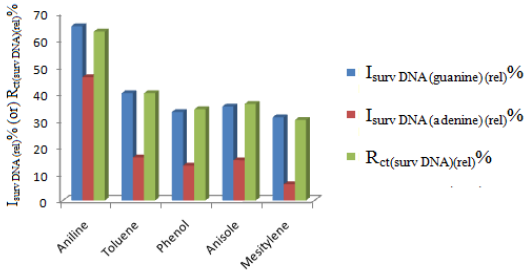

Figure 11: Relative percentage of the charge transfer resistance and current value obtained for sdDNA/GTA/CS-MWCNT/G for 100 ng/l of benzene derivatives

It was observed from figures that the relative percentage values obtained for guanine oxidation signal and charge transfer resistance values have significant correlation. Hence, it can be inferred that the changes in the oxidation of guanine base at the electrode surface is directly associated with the film forming abilities and electron transfer characteristics of the redox probe. It may be noticed from the available literature [15] that guanine structure is most commonly affected after DNA interaction with many compounds. This information supports the association of relative percentage of guanine oxidation values and the relative percentage of charge transfer values.

IV. CONCLUSIONS

MWCNT paste over the graphite electrode has ensured a good detection window for the voltammetric and impedimetric evaluation of the presence of dsDNA structures. Addition of chitosan as binding agent, further dipping the CS-MWCNT/G electrode in glutaraldehyde solution forms a protecting film inside the electrolyte during analysis. dsDNA based biosensor was made to

react with the analytes in the solution. Calibration curves were obtained for each of the benzene derivatives. It was demonstrated that the proposed EIS and DPV procedures can be used for the detection of benzene derivatives.

V. ACKNOWLEDGEMENTS

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