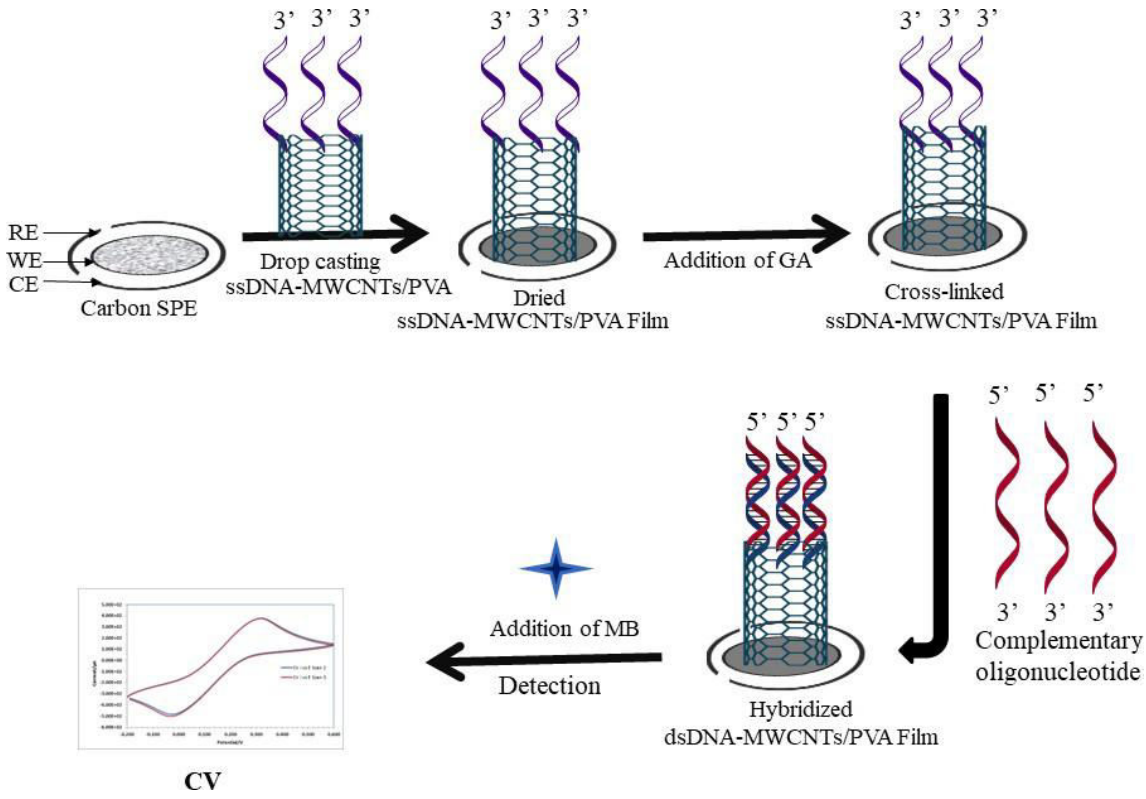
CHAPTER 7

CHAPTER 7. ELECTROCHEMICAL DETECTION OF BREAST CANCER RELATED BRCA1 GENE USING DNA CHIP

Research highlights:

- Label free DNA based biosensor was developed to detect BRCA1 gene.
- Detection limit and linear range were found 1×10^{-4} to 1×10^{-14} M and linear range were found 1×10^{-8} to 1×10^{-12} M.

Graphical abstract:



7.1 Introduction:

Breast cancer is a significant global health issue, being a leading cause of female mortality. It is also the most diagnosed cancer among women. Breast cancer constitutes 11.7% of the newly diagnosed cancer cases worldwide every year (Sung et al., 2021; Fu X. et al., 2020).

One of the most prevalent tumour suppressor genes associated in breast cancer is the breast cancer susceptibility gene 1 (BRCA1). The BRCA1 gene, located on human chromosome 17q12-21, is a key player in breast cancer susceptibility, being responsible for approximately 80% of breast cancer cases within families. The BRCA1 gene is linked to ovarian, pancreatic, and colon cancers as well (Wang et al., 2020; Fu X et al., 2020; Shahrokhian et al., 2018). This gene codes for a 220 kDa nuclear protein and mutations in this gene are identified in at least 5% of unselected patients with breast cancer (Hall et al., 1990; Chen et al., 1996).

BRCA1 encodes a tumour-suppressor protein essential for regulating the cell cycle, repairing damaged DNA and maintaining genomic stability. Mutations in BRCA1 increase the risk of breast cancer by affecting the function of the encoded protein. Moreover, alterations in the gene expression levels can impact biological processes, contributing to both sporadic and hereditary breast tumour progression (Ashwoth et al., 2006; Xia Ya Mu et al., 2020; Deniz et al., 2021).

BRCA1 mutations or deletions have a substantial effect, as individuals carrying them are much more likely to develop breast cancer, frequently of the aggressive triple-negative subtype, which tends to establish early and progress rapidly (Miki et al., 1994). Women with BRCA1 mutations face a higher likelihood of developing breast cancer and/or ovarian cancer, with probabilities ranging from 45% to 75% and 18% to 40%, respectively. The cumulative risk of breast cancer by age 80 among healthy female BRCA1 mutation carriers is approximately 80%, compared to one in eight women in the general population (King et al., 2003). It is crucial to understand the molecular roles of BRCA1, achieve accurate detection of its mutations and study strategies for prevention and treatment.

Various conventional methods, such as capillarity electrophoresis, sequencing, PCR-mediated techniques, high performance liquid chromatography (HPLC) and single strand conformation polymorphism assays, have been utilized for BRCA1 detection (Deniz et al., 2021; Wang et al., 2020). However, these technologies have limitations including low sensitivity, high cost, and complexity, hindering their widespread use. Consequently, there is a necessity to develop rapid, affordable and convenient detection systems for monitoring early disease progression. The detection of breast cancer, particularly related to the BRCA1 gene, poses challenges due to the time-consuming nature of clinical techniques and the high costs associated with the methods. Moreover, depending completely on surgical methods for diagnosis could lead to adverse outcomes and inaccurate assessments. Therefore, there is a critical need for the development of cost-effective and highly sensitive methods for the early detection of breast cancer (A. Benvidi et al., 2015).

Advancements in ultrasensitive DNA biosensors are revolutionizing early-stage cancer detection, aiming to increase cure rates. Traditional label-based techniques like in situ hybridization and microarrays, while effective, often require expensive fluorescent dyes or commercial kits and exhibit limited sensitivity and selectivity, along with time consuming protocols. Consequently, attention has shifted towards label-free procedures in DNA sensors due to their minimal sample preparation requirements, rapidity and cost effectiveness. A prominent label-free strategy involves the direct detection of redox-active nucleobases based on their electrochemical properties, such as guanine oxidation, utilizing redox markers like Ferrocene and Methylene blue (MB). An electrochemical DNA biosensor, utilizing this approach, emerges as an ideal technique for early cancer and mutation detection. Their remarkable features, including label-free operation, low cost, portability, high sensitivity, selectivity and simplicity in detecting biological fluids, underscore their significance in clinical diagnostics.

An electrochemical biosensor is prominent for its superior sensitivity, selectivity, reproducibility, and affordability, making it a popular choice in DNA biosensors. Electrochemical biosensors, significantly, facilitate the transition towards point-of-care diagnostic devices (Mansor et al., 2014).

Fundamentally, DNA biosensors rely on immobilizing single-stranded oligonucleotide probes on electrode surfaces to achieve specificity towards target DNA. The recognition outcome, based on DNA hybridization, is then translated into a readable signal by the transducer (Mansor et al., 2014). This integration of biological and physical principles highlights the effectiveness and versatility of DNA biosensors in various diagnostic applications, particularly in early cancer detection.

This work presents a DNA chip based electrochemical detection of the BRCA1 gene. The process involved attaching a single stranded DNA (ssDNA) oligonucleotide probe to multi-walled carbon nanotubes (MWCNTs) through strong covalent bonds. This complex was then mixed into a solution containing polyvinyl alcohol (PVA) polymer, resulting in a dispersed mixture. This mixture was drop cast on a screen-printed carbon electrode, forming a thin film. Then, glutaraldehyde (GA) was used to crosslink, to make sure the stability of the probe-MWCNTs complex within the PVA matrix on the electrode surface.

7.2 Materials and methods:

7.2.1 Reagents and Appratus

The ssDNA probes for BRCA 1 gene of was designed in NCBI/PRIMER BLAST and synthesized of HPSF purification grade. Raw MWCNTs were purchased from Ad Nanotechnologies, India. Potassium ferricyanide $K_3[Fe(CN)_6]$ and hydrochloric acid (HCl) were purchased from Qualigens, India. Sodium chloride (NaCl), sodium phosphate dibasic anhydrous (Na_2HPO_4), sodium phosphate monobasic anhydrous (NaH_2PO_4), ethylenediaminetetraacetic acid disodium salt disodium salt (EDTA), Tris (hydroxymethyl) aminomethane hydrochloride (Tris HCl), Sodium hydroxide pellets (NaOH) and Sodium lauryl sulphate (SDS) were purchased from SRL Pvt. Ltd., India. Polyvinyl alcohol (PVA) and Glutaraldehyde (GA) were purchased from Loba Chemie Pvt. Ltd. India. Methylene blue (MB) was purchased from Sigma Aldrich, India. Screen printed carbon electrode was bought from PalmSens BV, Netherlands.

All reagents utilized in this study were of analytical grade and molecular biology grade. They were used as received without further purification, except the multiwalled carbon nanotubes (MWCNTs).

Sequences of probe:

Immobilized (probe) sequence	ACTGAAGAGACTACTCATGTTGTTATGA
complimentary sequence	TGACTTCTCTGATGAGTACAACAATACT

7.2.2 Apparatus:

Cyclic voltammetry was performed using a portable EmStat3+ electrochemical workstation. A screen-printed carbon electrode immersed in a solution containing 50 mM $K_3[Fe(CN_6)]$ with 0.5 M NaCl in 50 mM phosphate buffer, pH 8.0 (PB) and scanning was carried out in the electrochemical potential range of -0.2 to +0.6 V at 0.1 V/s. Prior to coating the electrode, multi-walled carbon nanotubes were dispersed in polyvinyl alcohol using an ultrasonicator bath. The electrode was then coated and incubated for further processing. YORKO serological water bath was used to incubate the electrode.

7.2.3 Immobilization of ssDNA probe MWCNTs-COOH:

MWCNTs-COOH were previously prepared in our laboratory. The immobilization of ssDNA probe on MWCNT-COOH was started by mixing 1 mg of MWCNTs-COOH with 0.5 ml of 1 µM ssDNA probe, followed by the addition of 4.5 ml of Milli-Q water. To initiate the reaction, 1 ml of 0.1 N HCl was added to the mixture. The reaction proceeded under shaking condition at 60˚C and 250 rpm for 1 h. Subsequently, the solution was kept at -80˚C overnight before lyophilization (Jinal Thakkar's thesis 2022).

7.2.4 Fabrication of ssDNA-modified electrode (ssDNA-MWCNTs/PVA/GA):

To develop the DNA based biosensor, an integrated chip with a screen-printed carbon electrode (SPE) containing working, reference and counter electrodes, was used for the experiments. 1 mg of the ssDNA-MWCNTs complex was mixed with 0.5 ml of PVA (1 mg/ml). The suspension was subjected to ultrasonication for 75 h. To prevent DNA degradation during dispersion cooling packs were applied in the ultrasonicator bath. The SPE was rinsed with

Milli-Q water. The electrode surface was added with a 4 µl solution of the ssDNA-MWCNTs-PVA mixture and air-dried at room temperature. Following this, 2 µl of 2.5% glutaraldehyde was applied to crosslink the PVA polymer, and the film was left to air dry at room temperature. The crosslinked film on the electrode was washed three times with Milli-Q water to remove excess glutaraldehyde. This methodology was adapted from prior work on the development of a glucose biosensor (Gupta et al., 2016; Jinal Thakkar's thesis 2022). Subsequently, the electrode was treated with 10 µl of 1% BSA for 10 min., followed by multiple washes with Milli Q water (Ishikawa et. al., 2009).

7.2.5 Hybridization:

Throughout the hybridization process, a 10 mM Tris-EDTA (TE) buffer at pH 8.0 was consistently utilized. Different concentrations of complementary target single-stranded DNA (ssDNA) sequences were prepared in the 10 mM TE buffer. To initiate hybridization, 5 µl of each target ssDNA solution, ranging from 1×10^{-18} to 1×10^{-4} M concentration, was carefully applied on the electrode surface. Subsequently, the electrode was transferred into a sterile plastic box and incubated in a serological water bath maintained at 57°C for 10 min. After incubation, the electrode was thoroughly washed with water to eliminate any unbound oligonucleotides.

7.2.6 Electrochemical characterization of hybridization effects:

Before each reading Cs (Complementary strand) ssDNA, the SPE was incubated with 1% BSA (10 µl) for 10 min. then washed with water. The electrode was incubated with the Cs for 10 min at 57ºC temperature in sterile box using hot water bath. Then the electrode was incubated with MB (20 µM in 50 µl) prepared in 50 mM PB (pH 8.0) for another 10 min. the electrode washed with Milli-Q water and 0.05% SDS (sodium dodecyl sulfate) solution. Finally CV was recorded in a solution containing 50 mM $K_3[Fe(CN)_6]$, 0.5 M NaCl in 50 mM PB, pH 8.0.

7.3 Results and discussion:

7.3.1 Modification of MWCNTs-COOH with ssDNA probe:

Multi-walled carbon nanotubes (MWCNTs) offer both high surface area and mechanical strength, making them ideal for immobilizing with single stranded DNA (ssDNA) on their surface. The carboxylic groups present on multi-walled carbon nanotubes (MWCNTs) made to react with the free 3' hydroxyl group of single-stranded DNA (ssDNA) in the presence of hydrochloric acid, forming an ester linkage between them. This process established a covalent bond between the ssDNA molecules and the oxidized MWCNTs. Additionally, the negatively charged phosphate groups on the DNA molecules interacted with the positive end of the MWCNTs. Furthermore, other components of DNA exhibited slight interactions with the side walls of oxidized carbon nanotubes via weak van der Waals bonds **(Fig 7.1)**.

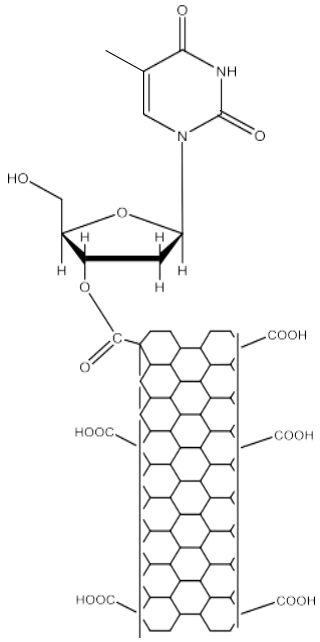


Figure 7.1 Formation of ester linkage between ssDNA probe 3' hydroxyl group (OH) and COOH-MWCNT (Jinal Thakkar's Thesis 2022).

7.3.2 Characterization of BRCA1 chip (GA/ssDNA-MWCNTs-PVA/SPE electrode):

In the present study, the responses of both the GA/ssDNA-MWCNTs-PVA/screen-printed electrode (SPE) and the bare SPE were compared by conducting cyclic voltammetry from -0.2 to 0.6 V in a solution containing 50 µl of 0.5 M NaCl and 50 mM [$K_3Fe(CN_6)$], prepared in 50 mM phosphate buffer at pH 8.0. The experimental results prove that the PVA-GA film forms a stable layer on the electrode surface, providing structural support for the ssDNA-MWCNTs complex. The inclusion of multi-walled carbon nanotubes (MWCNTs) enhances the surface area and electrical conductivity of the electrode, while also contributing to its mechanical strength, thereby securely anchoring the covalently attached ssDNA probes. The current observed on the electrode, after coating with the GA/ssDNA-MWCNTs-PVA film, exhibits an increase, indicating successful dispersion of the ssDNA-MWCNTs within the PVA matrix **(Fig.7.1).**

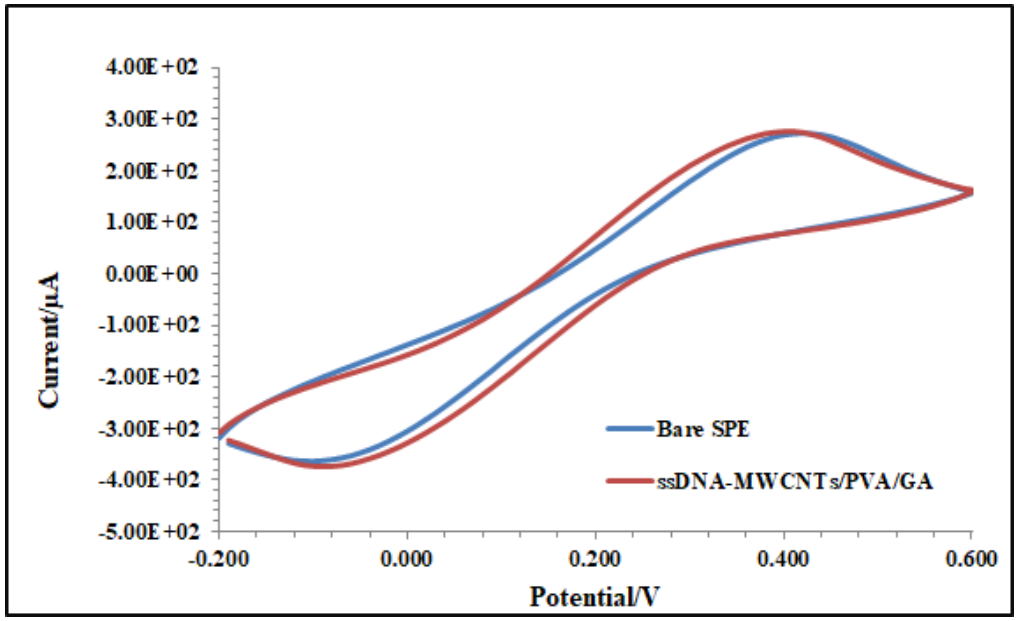


Figure 7.2. Cyclic voltametry scans of bare SPE and BRCA1 chip (ssDNA-MWCNTs/PVA/GA) in 50 mM phosphate buffer, pH 8.0 with 50mM $K_3[Fe(CN)_6]$ and 0.5 M NaCl at 100 mV/s scan rate.

Bang et al. highlighted the effectiveness of pre-treating the electrode surface with bovine serum albumin (BSA) in preventing nonspecific adsorption of methylene blue (MB). BSA acts by obstructing the interaction between methylene blue and the electrode surface, attributed to the strong repulsion between the negatively charged BSA and the methylene blue molecules (Bang et al., 2005; Bang & Jeon, 2001). Additionally, the use of both BSA and sodium dodecyl sulfate (SDS) treatments further reduced the likelihood of nonspecific adsorption of methylene blue.

7.3.3 Characterizing electrochemical reaction with different Cs concentrations of BRCA1:

The biosensor works on electrochemical transduction to detect the specific binding between a single stranded DNA (ssDNA) probe and its complementary (Cs) target, with methylene blue (MB) as the constant marker during incubation. MB exhibits distinctive interactions with both ssDNA and dsDNA due to its aromatic cationic nature. It binds to DNA through intercalation primarily at guanine bases, requiring a minimum of two GC base pairs for effective intercalation. In the presence of the target or Cs, the formation of a double-stranded DNA structure facilitates MB intercalation between successive guanine-cytosine (G-C) base pairs, leading to an increase in current. The biosensor demonstrates good detection abilities, with detection limits ranging from 1×10^{-4} M to 1×10^{-14} M and a linear range spanning from 1×10^{-8} M to 1×10^{-12} M, exhibiting a strong correlation **(R^2=0.9775)** with a logarithmic distribution.

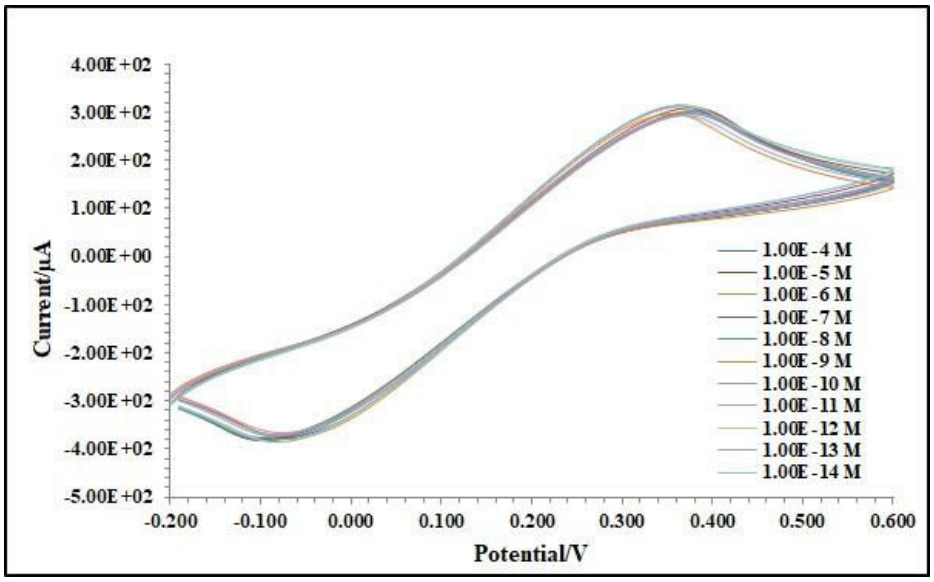


Figure 7.3. Cyclic voltammetry scans of BRCA1 chip in 50 mM phosphate buffer, pH 8.0 with 50mM $K_3[Fe(CN)_6]$ and 0.5 M NaCl after hybridization with various concentrations of target sequence of BRCA1 gene in solution (1×10^{-4} to 1×10^{-14} M) after incubation in MB to find the limit of detection.

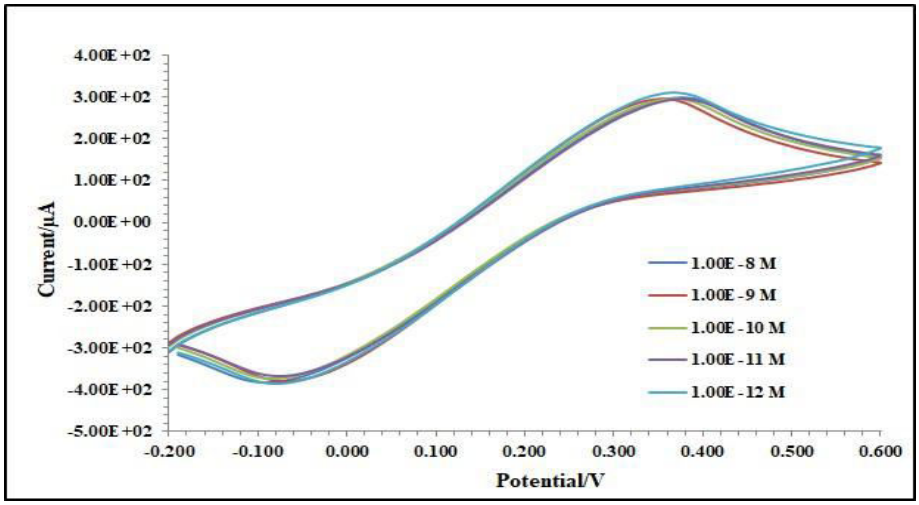


Figure 7.3 (a). Cyclic voltammetry scans of BRCA1 chip in 50 mM phosphate buffer, pH 8.0 with 50mM $K_3[Fe(CN)_6]$ and 0.5 M NaCl after hybridization with various concentration of target sequence of BRCA1 gene in solution (1×10^{-8} to 1×10^{-12} M) after incubation in MB to find the linearity range.

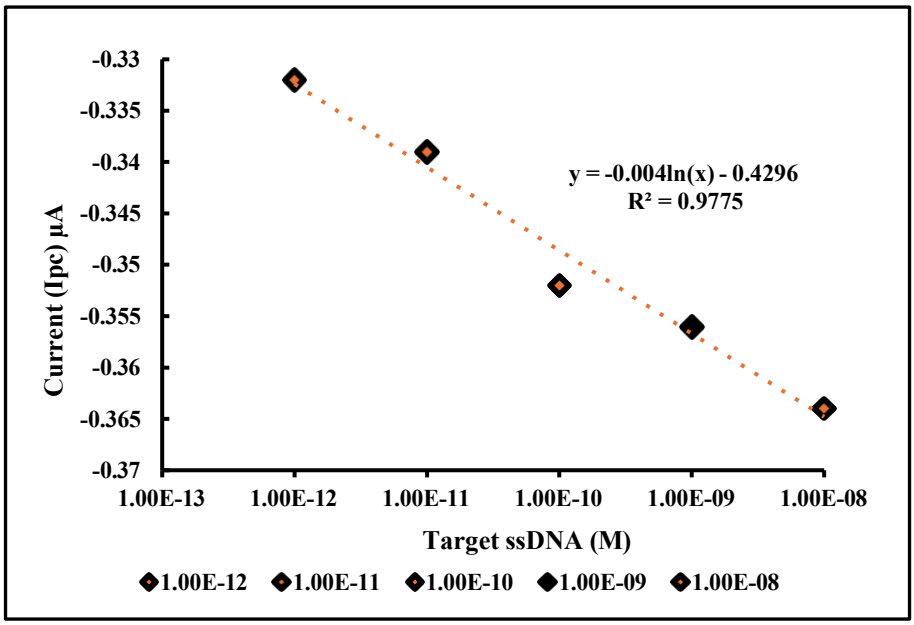


Figure 7.3 (b). Plot of different concentrations of target ssDNA (1×10^{-8} to 1×10^{-12} M) vs. I (µA) cathodic peak current showing linearity in logarithmic distribution with (R2=0.9775).

7.4 Conclusion:

A novel electrochemical DNA biosensor has been developed for investigating the presence of breast cancer based on the specificity of hybridization using the BRCA1 gene probe, with methylene blue (MB) serving as the hybridization indicator. Results indicate successful binding of the ssDNA probe to carboxylic acid group-modified multi-walled carbon nanotubes (MWCNTs) through esterification. These MWCNTs, when dispersed into a polyvinyl alcohol (PVA) matrix, form a stable film on the electrode surface, offering not only a platform for the ssDNA probe but also high surface area and electrical conduction. The reduction in the current signal of MB amplifies with increasing concentrations of the target ssDNA, displaying the extent of hybridization onto the electrode surface. Complementary target ssDNA of the BRCA1 gene was effectively detected by DNA biosensor with a linear range from 1×10^{-8} to 1×10^{-12} M. Due to unavailability of real sample analysis sensor couldn't checked for practical applicability. This part would perform by our laboratory in future.