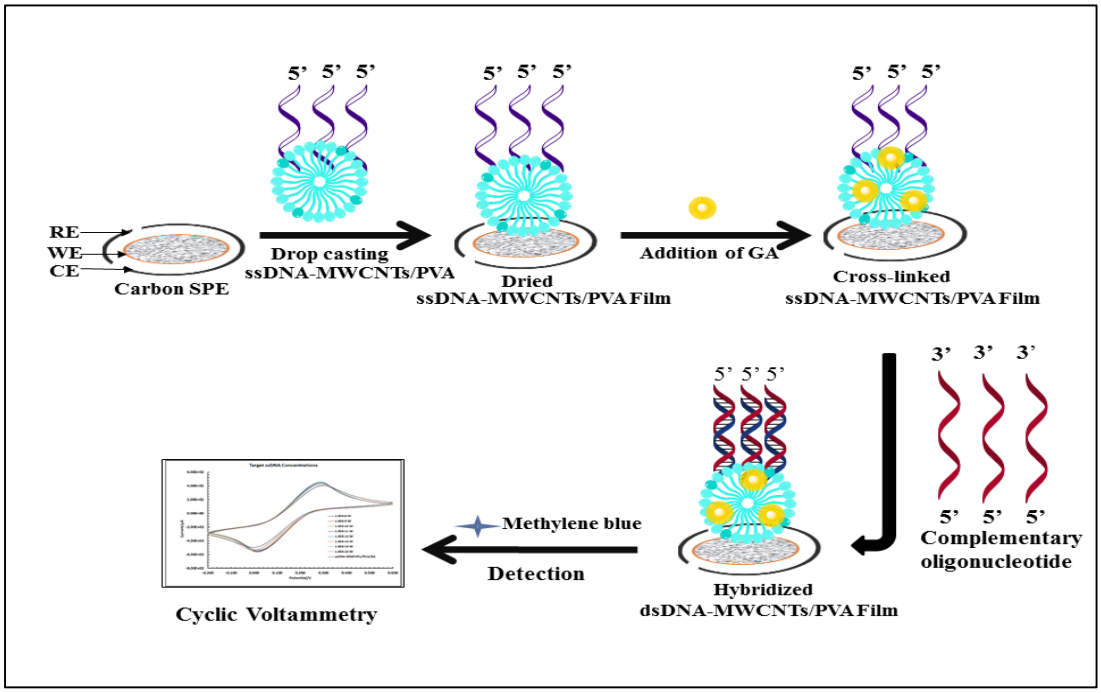
CHAPTER 5

ELECTROCHEMICAL BIOSENSOR FOR CYCLIC VOLTAMMETRIC DETECTION OF SARS-COV-2 USING DNA-CHIP

Chapter 5. Electrochemical biosensor for cyclic voltammetric detection of SARS-CoV-2 using DNA-Chip

Research highlights:

- Label free DNA chip based electrochemical biosensor was developed to detect a previously identified protein gene of Covid-19.
- The biosensor eliminates cDNA amplification step of RT-PCR and can directly detect reverse transcript cDNA product.
- The sensor takes only few minutes to detect cDNA of Covid-19.
- The detection limits of the DNA chip were found to be 1×10^{-8} M and 1×10^{-15} M. Linear range found is 1×10^{-10} M to 1×10^{-15} M (R^2=0.9622).
- The DNA chip showed high specificity for the identified protein gene of Covid-19 and has potential for point-of-care diagnostics.
- The DNA chip sensor can be replicated within the acceptable margins of error.



5.1. Introduction:

The coronavirus (Covid-19) outbreak caused by novel corona virus (SARS-CoV-2) has been declared as a global pandemic by WHO (Geneva, Switzerland) since January 9, 2020 and continues to be international health crisis (Mojsoska et al., 2021)(Falaschi et al., 2020). Total 0.277 billion people has been infected so far with 5.38 million deaths reported worldwide and the count still continues to rise. India alone has reported 51.5 million cases so far with 0.81 million deaths reported. Novel corona virus (SARS-CoV-2) has been known as an etiological agent. Early stage diagnosis of Covid-19 is very important to treat the patients and isolate them from healthy individuals to prevent its spread. The strategies of most of the countries to deal with the pandemic relies on early identification of infected subjects and their close contacts. This can help slower the transmission of the virus in the healthy population (Mojsoska et al., 2021)(Falaschi et al., 2020).

The worlds health organization (WHO) has made a global strategy and standards for testing Covid-19. Currently, total three strategies are available to detect Covid-19 including CT-scan, RT-PCR and ELISA. CT-scans have high sensitivity (86-98%) but offer low specificity (25%) as imaging features share similarity with viral pneumonia (Udugama et al., 2020). Though, CT-scan helps to diagnose and evaluate progress of the infection, decisive diagnosis can be achieved by real-time reverse transcriptase polymerase chain reaction (RT-PCR) (Falaschi et al., 2020; Mojsoska et al., 2021). However, very often false negative or false positive results can be achieved by both RT-PCR and ELISA. The debate of comparing these methods further gets complicated by the facts that RT-PCR takes several hours whereas ELISA and CT-scan give the results within few minutes. All these techniques require skilled technicians and specialized facilities for the detection of SARS-CoV-2 virus infections (Falaschi et al., 2020; Mojsoska et al., 2021). The strategy of isolating infected subjects to slow down the transmission depends on rapid testing. ELISA is used for rapid testing and is cost effective but its accuracy is still a question mark. Mostly, the PCR tests are carried out in labs with centralized facilities but still transportation of the samples and batch analysis may extend the time of analysis (Falaschi et al., 2020; Mojsoska et al., 2021). Developing or supplementing onsite point-of-care (POC) tests with some modifications could help to improve the bottlenecks of centralized testing labs. New improvements can offer speedy testing and open up a way for fast screening of the samples (Mojsoska et al., 2021). More than 140 commercial tests for detecting SARS-CoV-2 virus are launched in the market since 2020. Majority of these

test kits are RT-PCR based with 2 step procedures. The kits use reverse transcriptase to make cDNA from RNA in first step and then Real-Time PCR to amplify and detect cDNA. However, few companies have commercialized one step RT-PCR kits for SARS-CoV-2 combining both reverse transcription and cDNA amplification. But, accuracy of these kits is not that much appreciative due to low amplification rates (Falaschi et al., 2020; Mojsoska et al., 2021).

The delay involved with RT-PCR can be reduced by using electrochemical DNA-chip. Samples received for SARS-CoV-2 testing would go through reverse transcriptase polymerase chain reaction and give cDNA as a product. Presence of this cDNA could be checked and quantified directly using electrochemical biosensor instead of going for qPCR for amplification. Electrochemical biosensor could give response within minutes. Thus, replacing qPCR (cDNA amplification) step with electrochemical detection techniques to detect cDNA immediately after reverse transcriptase PCR can save the time of analysis.

Electrochemical biosensors offer promising methodology for rapid medical diagnosis to detect infectious disease biomarkers. This approach can potentially equip us with more timely and effective decision-making methods (Alatraktchi, Svendsen, & Molin, 2020; Kaushik et al., 2020; Mujawar et al., 2020; Ozer, Geiss, & Henry, 2020). Moreover, miniaturized potentiostats are already lunched and commercialized in the market for on-site or point-of care application. Earlier, various electrochemical sensors have been developed to detect infectious diseases, biomarkers and pollutants such as *Vibrio parahaemolyticus*, *Escherichia coli* O157:H7, influenza virus subtype H1, Hepatitis B, thrombin and paraoxon (Bang, Cho, & Kim, 2005; Lee et al., 2017; Sun et al., 2012; Thakkar, Gupta, & Prabha, 2019; Thuy et al., 2012; Zhou, Liu, Hu, Wang, & Hu, 2002). Ongoing Covid-19 pandemic has ramped up electrochemical biosensor research to detect and quantify the infection. Mojsoska and his team in 2021 has developed a graphene screen printed electrode strip to detect SARS-CoV-2. The electrode surface was functionalized with 1-pyrene butyric acid N-hydroxysuccinimide ester (PBASE) linker to bind anti-spike protein S1 monoclonal antibody. The sensor exhibited the linear range from 260×10^{-9} to 1040×10^{-9} M for spike protein S1 with incubation time of 45 min (Mojsoska et al., 2021). Zhao *et. al.*, in 2021 has developed nucleic acid based electrochemical chip for detection of ORF1ab gene of SARS-CoV-2. The team has used a combination of nanoparticles to functionalize ssDNA oligonucleotide probe for ORF1ab gene. The sensor showed linear range from 1×10^{-12} to 1×10^{-17} M with 3 hours of incubation time period (Zhao et al., 2021). Majority of the RT-PCR tests are designed to detect

ORF1ab gene (in open reading frame), RdRP (RNA dependent RNA polymerase gene), E gene (envelope protein gene), N gene (nucleocapsid protein gene) and S (Spike protein) gene (Udugama et al., 2020). SARS-CoV-2 is single stranded positive-sense RNA (+ssRNA) of 29.9 kb size. The virus contains four structural proteins S, E, M and N **(Figure 5.1a)** with 14 open reading frames. Amongst these 14 open reading frames, ORF1a and ORF1b comprise almost two thirds of the genome and encodes 16 non-structural proteins Nsp1-16 (Lavigne et al., 2021; Udugama et al., 2020; Wang et al., 2020). Nsp3, a multidomain non-structural protein is the largest protein encoded by the coronavirus genome. It is believed to be conserved and has crucial role in viral replication. The protein takes part in the formation and activity of viral transcription complex. Nsp3 protein is also believed to separate the translated proteins, increase virulence and enhances inflammatory responses in lungs (Lavigne et al., 2021; Wang et al., 2020).

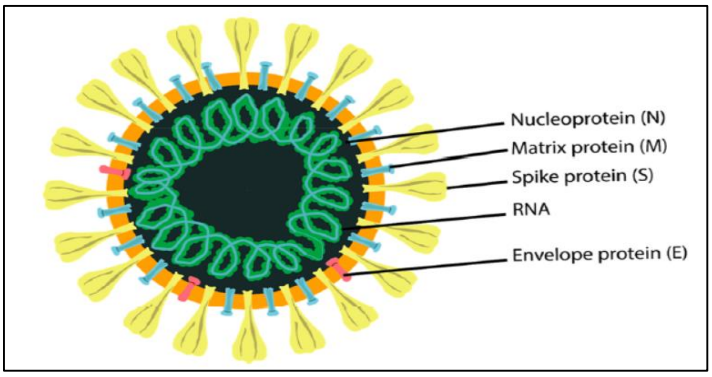


Figure 5.1a. Graphical representation of structural proteins of SARS-CoV-2 (Udugama et al., 2020)

This work presents a DNA-chip for electrochemical detection of one of the nonstructural protein gene of SARS-CoV-2. ssDNA oligonucleotide probe was covalently bound to functionalized MWCNTs. This ssDNA-MWCNTs complex was dispersed into PVA polymer and was coated onto screen printed carbon electrode. The film was crosslinked using glutaraldehyde. Thus prepared DNA-chip was checked to bind with its complementary ssDNA with the incubation time of 10 min. The chip can detect its complementary strands in the linear range from 1×10^{-10} to 1×10^{-15} M and showed no interference with other oligonucleotide sequence. The results obtained here are comparable with PCR. The sensor chip can be replicated with acceptable margin of error and can also be regenerated for its reuse with decreased sensitivity. The electrode developed showed a possibility to replace the cDNA amplification step of two step RT-PCR methodology

which can save the time during testing. With government's consent and biosafety measures, the DNA-chip can be used to scan the samples to confirm its on field performance.

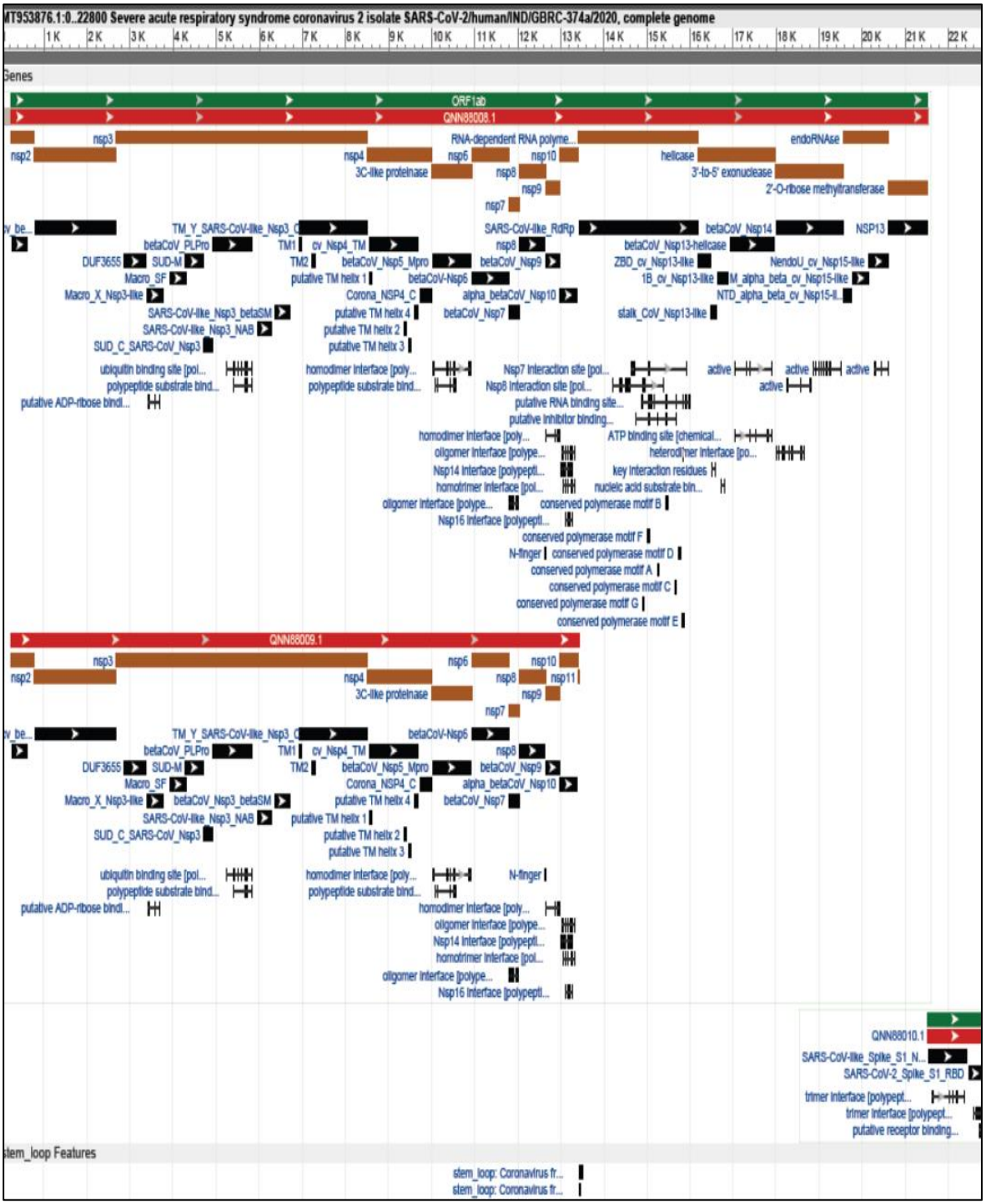


Figure 5.1b. Graphical representation of ORF1ab genome of SARS-CoV-2 (Accession no. MT953876.1; Isolate: "SARS-CoV-2/human/IND/GBRC-374a/2020") (Retrieved from: https://www.ncbi.nlm.nih.gov/nuccore/MT953876.1?report=graph)

5.2. Experimental:

5.2.1. Materials:

All the reagents purchased were of high purity and molecular biology grade and were used as received without further purification except MWCNTs. Potassium ferricyanide (26664) was purchased from Qualigens, India. Sodium chloride (33205), sodium phosphate dibasic anhydrous (1944143), sodium phosphate monobasic anhydrous (59443), ethylenediaminetetraacetic acid disodium salt (EDTA, disodium salt) (054448), Tris (hydroxymethyl) aminomethane hydrochloride (Tris HCl) (2044123), Sodium hydroxide pellets (NaOH) (1949181) and Sodium dodecyl sulphate (SDS) (54468) were purchased from SRL Pvt. Ltd., India. Hydrochloric acid (HCl) was purchased from Qualigens, India. Polyvinyl alcohol (PVA) (0531500500) and Glutaraldehyde (GA) (0396500500) were purchased from Loba Chemie Pvt. Ltd. India. Raw MWCNTs were purchased from Ad Nanotechnologies, India. Methylene blue (MB) (M4159) was purchased from Sigma Aldrich, India. ssDNA probe for Nsp3 gene of SARS-CoV-2 was designed in NCBI/PRIMER BLAST for the isolate "SARS-CoV-2/human/IND/GBRC-374a/2020" (Accession no. MT953876.1). ssDNA probe for pre-identified nonstructural protein gene with a 55% GC rich sequence of 20 bases and its complementary strand were synthesized and were of HPSF purification grade. Screen printed carbon electrode was purchased from PalmSens BV, Netherlands.

The target oligonucleotide stock was prepared in TE buffer (10 mM; pH=8.0) in the concentration of 1 mM and was stored at -20 ˚C. More dilute solutions of targets were prepared using TE buffer (pH=8.0) just before use. 20 μM methylene blue was prepared in 50 mM PB (pH=8.0). 50 mM $K_3[Fe(CN_6)]$ was used as an electrolyte and 0.5 M NaCl was used as a supporting electrolyte and were dissolved in 50 mM PB (pH=8.0). All the solutions were stored at 4 ˚C. All the reagents were prepared in autoclaved Milli Q water only.

5.2.2. Apparatus:

Cyclic voltammetry was carried out from -0.2 to +0.6 V at 0.1 V/s scan rate on screen printed carbon electrode in 50 mM $K_3[Fe(CN_6)]$ with 0.5 M NaCl in 50 mM PB(pH=8). Portable EmStat3$^+$ electrochemical workstation (PalmSens BV, Netherlands) was used to carry out CV measurements. Locally manufactured ultrasonicator bath was used to sonicate MWCNTs into PVA polymer. SHIMADZU AFINITY 1 instrument with ATR method (400-4000 cm^{-1}, resolution

4 cm^{-1}, 5 scans) was used to record FTIR spectra of ssDNA-MWCNTs complex. YORKO Serological water bath was used to incubate the electrode.

5.2.3. Purification of MWCNTs and Functionalization of MWCNTs-COOH with ssDNA probe:

Commercially available multiwalled carbon nanotubes (MWCNTs) were purchased from Ad nanotechnologies, India. These MWCNTs contain certain impurities such as amorphous carbon and metal impurities like Fe, Ni, Co, which were used as catalyst for the synthesis of MWCNTs via laser oven method or arc discharge evaporation method. To remove these metal impurities, MWCNTs were washed with 50 % Conc. HCl. Metal impurities react with hydrochloric acid and convert them into metal chlorides which were washed out by several washings in water. These purified MWCNTs were further processed for oxidation. For this, purified MWCNTs were treated with the mixture of (3:1) Conc. HNO_3 and Conc. H_2SO_4 for 24 hours at 33ºC. Thus, oxidized MWCNTs generated were washed several times with water to completely remove acid and dried at room temperature.

To functionalize MWCNTs with ssDNA probe, 0.5 ml of 1 μM ssDNA probe was added into 1 mg of MWCNTs-COOH followed by 4.5 ml of Milli Q water. Then 1 ml of 0.1 N HCl was added into the reaction mixture. The reaction mixture was kept on shaker at 250 rpm for 1h at 60 ˚C. After that the mixture was frozen at -80˚C for 12h and then it was lyophilized.

5.2.4. Preparation of ssDNA modified electrode chip (ssDNA-MWCNTs/PVA/GA):

Screen printed carbon electrode integrated chip having working, reference and counter electrode altogether was purchased from PalmSense BV, Netherlands to develop the genosensor. 1 mg of ssDNA-MWCNTs complex was first mixed into 0.5 ml (1mg/ml) of PVA. The suspension was ultrasonicated for 75 hours. Cooling packs were kept into the ultrasonicator bath to ensure DNA does not degrade while dispersing. Screen printed electrode was first washed with MQ water. 4 μl of ssDNA-MWCNTs-PVA mixture was drop casted onto the electrode surface and was allowed to air dry at room temperature. 2μl of 2.5% glutaraldehyde was then added to the dried film to crosslink the PVA polymer. The film was allowed to get air dried at room temperature. The crosslinked film on the electrode was then washed three times with water to remove excess glutaraldehyde. This procedure was adopted from our earlier work to develop glucose biosensor (Gupta et al., 2016). This was then followed by pretreating it with 10 μl of 1% BSA for 10 min. The electrode was then washed with Milli Q water several times.

5.2.5. Hybridization:

10 mM TE buffer (pH=8) was used throughout the hybridization process. Various concentrations of complementary target ssDNA sequence were made in 10 mM TE buffer (pH=8.0). For hybridization to take place, 5 µl of target ssDNA of different concentrations (1×10^{-15} to 1×10^{-8}) were pipetted onto the electrode surface. The electrode was then placed into a sterile plastic box. This box was then kept inside a serological water bath at 58.3 ˚C for 10 min. Then the electrode was washed with water to remove unbound oligonucleotides.

5.2.6. Electrochemical measurements to investigate hybridization:

Every time before measuring the electrochemical response against the complementary target ssDNA, the screen printed electrode was first incubated with 10 µl of 1% BSA and was washed with Milli Q water for 10 min. Then the electrode was incubated with target ssDNA for 10 min and again was washed with Milli Q water. Following it, the electrode was then incubated with 50 µl of 20 µM methylene blue (prepared in 50 mM PB; pH=8.0) for 10 min. Methylene blue was used here as a redox indicator. Following that, the electrode was washed several times with water. It was then followed by 0.05% SDS wash and water to remove adsorbed MB. Electrochemical measurements were recorded in 50 mM $K_3[Fe(CN_6)]$ with 0.5 M NaCl in 50 mM PB (pH=8.0).

5.2.7. Interference study:

Specificity of the DNA chip sensor was checked against PupE gene (195 bp) of *Mycobacterium smegmatis* which is 70-80 % GC rich. PupE gene was previously synthesized from Eurofins, India and was stored at -20˚C. PupE oligonucleotides (1×10^{-15} M) was first heated at 98˚C for 10 min. Then, 5 µl of it was drop casted onto the electrode surface. The electrode was then incubated at 58.3 ˚C in a sterile plastic box in serological water bath for 10 min. The electrode was then washed with Milli Q water to remove unbound oligonucleotides from the surface. It was then followed by incubation in 50 µl of 20 µM MB for 10 min and was given water washes. 0.05% SDS and water washes were given to the electrode to remove unbound MB. Changes in the cathodic peak current was observed and compared with controls (Coated electrode and complementary target incubated electrode).

5.2.8. Replicability and regeneration the electrode:

Total three screen printed carbon electrodes were coated simultaneously with the same fabrication chemistry ssDNA-MWCNT/PVA/GA. Current response of all the three electrodes were measured

in 50 mM PB (0.5 M NaCl, 50 mM $K_3[Fe(CN)_6]$; pH=8.0). Margin of error for the sensor replication procedure was calculated by comparing both anodic and cathodic peak responses. According to published reports (Zhou et al., 2002), the sensor probe can be regenerated by treating it with alkali and acidic solutions. Here, two methods were used to regenerate the used electrode probe. In one method, the used electrode was first treated with 1.2 M NaOH for 10 min following its wash in Milli Q water. Then the electrode was treated in 1.2 M HCl for 2 min following its wash in Milli Q water. Then, the electrode was washed with ethanol and water to clean it. In second method, the electrode was dipped in Milli Q water at 62 ˚C for 10 min following its wash with Milli Q water again. Performance of the regenerated electrode surfaces were checked in 50 mM PB (0.5 M NaCl, 50 mM $K_3[Fe(CN)_6]$) and compared.

5.3. Result and Discussion:

5.3.1. Functionalization of MWCNTs-COOH with ssDNA probe:

MWCNTs provide high surface area and mechanical strength to functionalize ssDNA onto its surface. As shown in FTIR spectra (**Figure 5.2(b)**), MWCNTs has carboxylic group which reacted with free 3' hydroxyl group of ssDNA and formed an ester linkage between them in the presence of hydrochloric acid. In other words, covalent linkage was established between the ssDNA molecules and oxidised MWCNTs. Also, there is phosphate group on the DNA molecule, which is negatively charged, interacts with the positive end of the MWCNTs. Other components of DNA would also slightly interact with the side walls of oxidised CNTs via weak van der Waals bonds **(Figure 5.2(a))**.

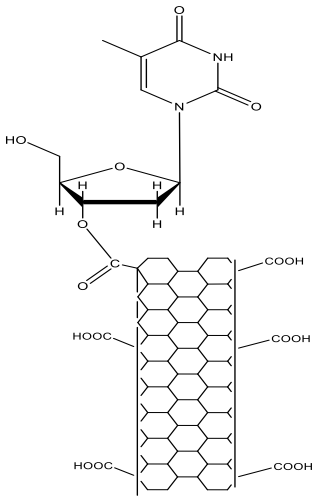


Figure 5.2(a). Ester linkage between 3' Hydroxyl group of ssDNA probe and oxidized MWCNT

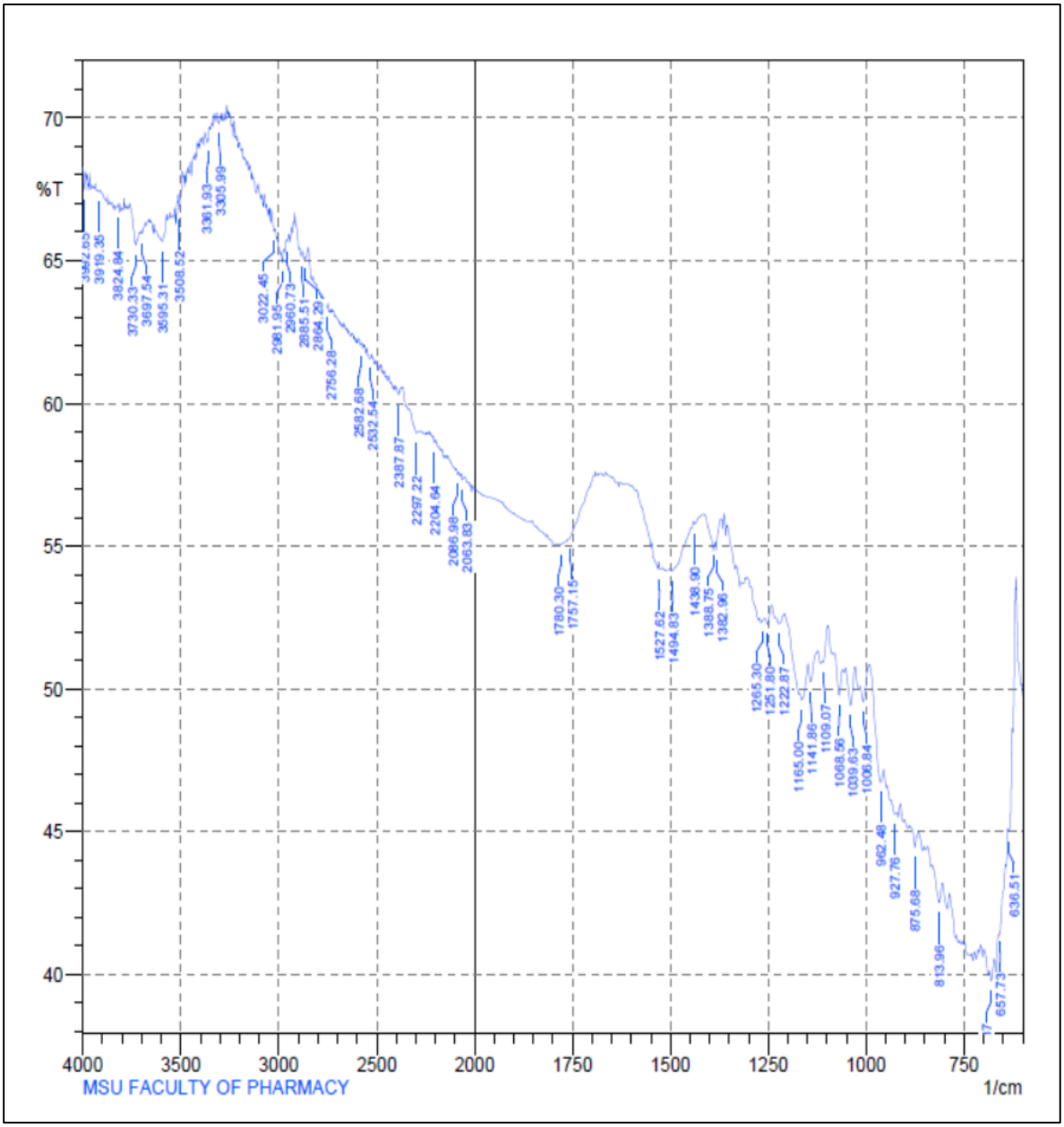


Figure 5.2(b). FT-IR spectra stating ester linkage between ssDNA-MWCNT complex

FT-IR spectra of DNA-Oxidised CNTs composite **(Figure 5.2(b))** showed 1780 cm^{-1} peak of C=O Stretching, which confirms the esterification between the hydroxyl group of the DNA and carboxylic group of the oxidised MWCNTs. Other peaks at 1165 cm^{-1} are associated with the C-C-O stretching. Some other peaks shown at 1109 cm^{-1} corresponds for symmetric stretching of PO_2 and 1222 cm^{-1} for asymmetric stretching for PO_2 due to the phosphate group in the DNA. Also because of the thymine unit in DNA, peak at 1527 cm^{-1} was observed. Guanine and cytosine peaks were also observed at 1382 cm^{-1} and 1438 cm^{-1} (Hembram & Rao, 2009).

5.3.2. Characterization and replication of GA/ssDNA-MWCNTs-PVA/SPE electrode:

Bang *et. al.*, in 2005 reported that pretreatment of BSA on the electrode surface removes the chances of MB getting adsorbed non-specifically. BSA blocks the interaction between methylene blue and electrode surface. A very high degree of repulsion between negatively charged BSA and neutral methylene blue was explained by Bang and his co-workers (Bang et al., 2005; Bang & Jeon, 2001). Use of BSA and SDS treatments removed the chances of non-specific adsorption of methylene blue. Here, response of the both GA/ssDNA-MWCNTs-PVA/SPE and the bare SPE was compared by running cyclic voltammetry from -0.2 to 0.6 V in 50 µl of 0.5 M NaCl, 50 mM $K_3[Fe(CN)_6]$ prepared in 50 mM PB (pH 8.0). The experiment showed that PVA-GA makes a fixed film onto the electrode surface and provide support to the ssDNA-MWCNTs complex to hold upon. MWCNTs offers high surface area and electric conductance. MWCNTs also offers high mechanical strength which hold covalently attached ssDNA probe onto its surface. Current of the electrode after coating with the film GA/ssDNA-MWCNTs-PVA can be seen to be increased due to properly dispersed ssDNA-MWCNTs into PVA matrix **(Figure 5.3(a))**. With the same fabrication chemistry, electrodes were fabricated in triplicate to check sensor's replicability. Every time, the newly fabricated sensor's performance was checked by running cyclic voltammetry from -0.2 to 0.6 V in 50 µl of 0.5 M NaCl, 50 mM $K_3[Fe(CN)_6]$ prepared in 50 mM PB (pH 8). While comparing their performance, it is evident that sensor can be replicated. The sensor showed margin of error **(Figure 5.3(b))** at anode was Ipa= 0.381±0.0187 (±4.92%) and at cathode was Ipc=-0.470±0.0123(±2.60%). The electrode is stable for 1 month when stored at 4 ˚C.

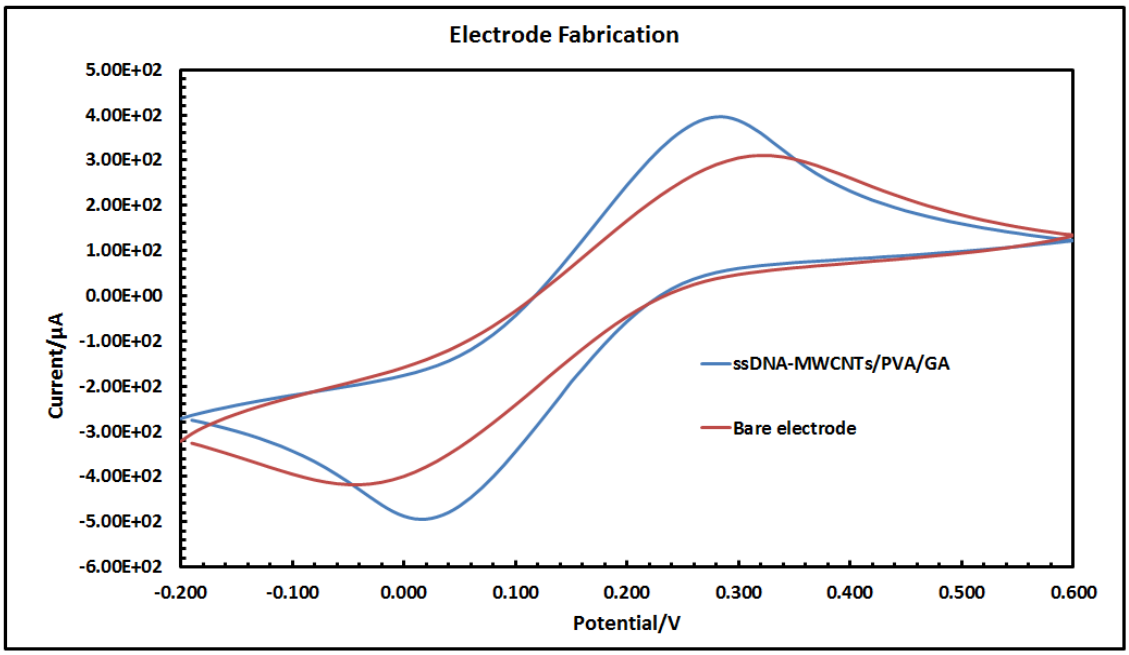


Figure 5.3(a). CV measurements of bare screen printed carbon electrode and ssDNA-MWCNTs dispersed PVA matrix coated electrode in 0.5 M NaCl, 50 mM $K_3[Fe(CN)_6]$ prepared in 50 mM PB (pH 8).

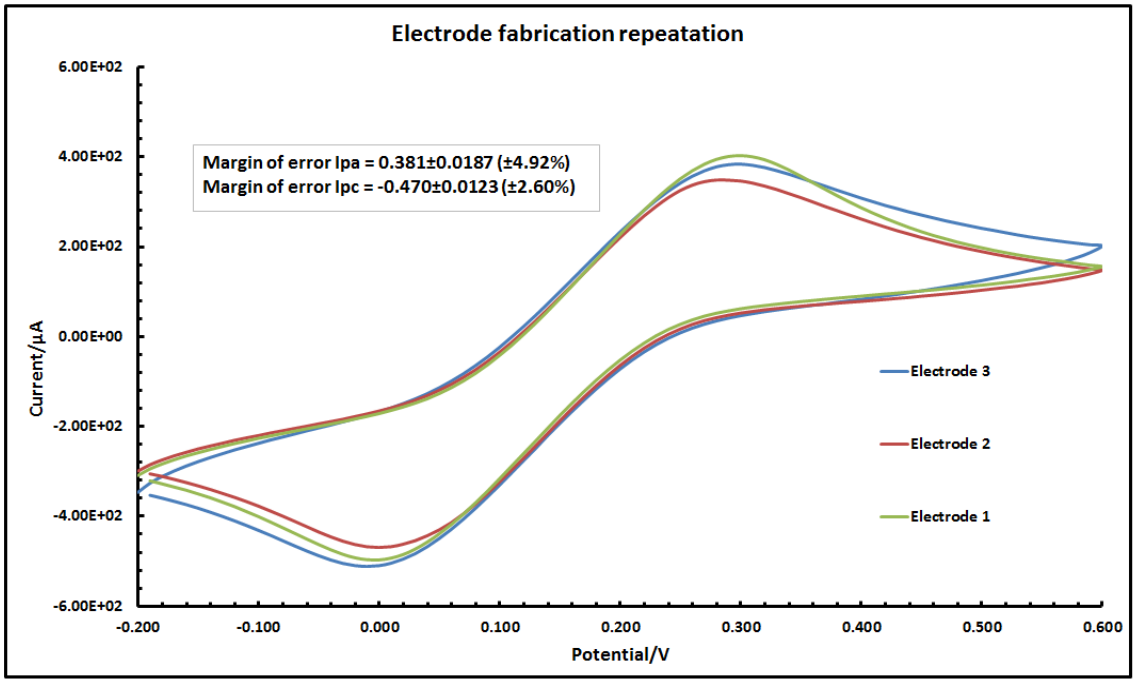


Figure 5.3(b). CV measurements of bare screen printed carbon electrode and ssDNA-MWCNTs dispersed PVA matrix coated electrode in 0.5 M NaCl, 50 mM $K_3[Fe(CN)_6]$ prepared in 50 mM PB (pH 8).

5.3.3. Electrochemical responses of complementary target with different concentration:

Methylene blue (MB) reacts differently with ssDNA and dsDNA. Methylene blue is aromatic cationic dye with electrochemical properties. It belongs to phenothiazine family and usually binds with DNA via intercalation at two guanine bases. Thus, atleast two G-C base pair is required to intercalate MB into DNA structure as an electrochemical marker (Bang et al., 2005). The proposed biosensor works on the same principle. The biosensor's performance relies on electrochemical transduction of the specific hybridization between ssDNA probe and its complementary target sequence. Under constant MB concentration incubation, various complementary targets can be qualitatively analyzed. **Figure 5.4** shows the electrochemical response of various target concentrations against the coated ssDNA probe under constant MB concentration incubation. It is evident from the **Figure 5.4** that the response for the reduction of MB increased with increased concentration of target sequence up to 1×10^{-8} M and then tended to keep constant. This suggest that all the ssDNA probes onto the electrode were occupied by its complementary strands and thus the electrode showed saturation in the electrochemical response after incubation with 1×10^{-8} M complementary strand. The formation of double helixed DNA caused MB to intercalate upon two G-C base pairs and hence increased current. The higher and lower detection limit found was 1×10^{-8} M and 1×10^{-15} M **(Figure 5.4)** with linear range of 1×10^{-10} M to 1×10^{-15} M (R^2=0.9622) with logarithmic distribution **(Figure 5.5, 5.6(a) & 5.6(b))**. The sensitivity of the chip is comparable to PCR and takes only 30 min. for the detection. Analytical characteristics such as linear range and sensitivity limits of the fabricated chip was compared with reported biosensors and is summarized in **Table 5.1.**

Electrode	Linear range (M)	Detection limit (M)	Incubation time	References
Graphene SPE /PBASE/MAb-Spike S1	260×10^{-9} to 1040×10^{-9} M	260×10^{-9} and 1040×10^{-9} M	45 min.	(Mojsoska et al., 2021)
Au@SCX8-TB-RGO-AP-LP-Target/HT/CP/Au@Fe_3O_4/SPE	1×10^{-12} to 1×10^{-17} M	1×10^{-17} M	3 hr.	(Zhao et al., 2021)
ssDNA-MWCNTs/PVA/GA	**1×10^{-10} to 1×10^{-15} M**	**1×10^{-8} and 1×10^{-15} M**	**30 min.**	**Present work**

Table 5.1. Comparison of the DNA chip with reported biosensors

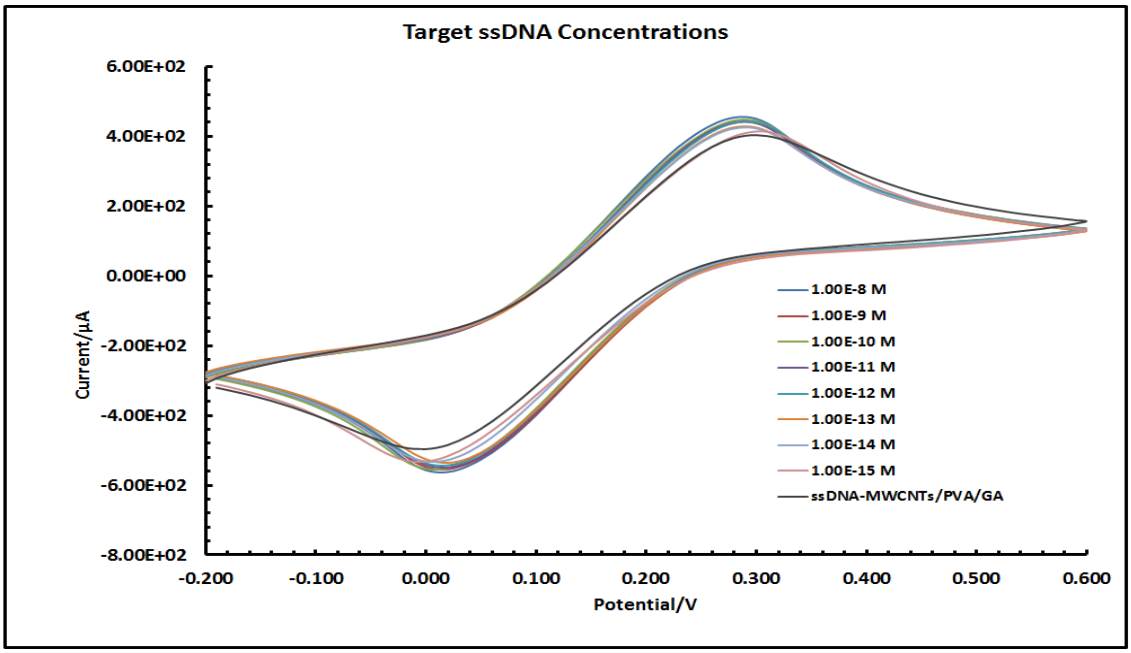


Figure 5.4. Cyclic voltammetry of ssDNA-MWCNTs/PVA/GA in 0.5 M NaCl, 50 mM $K_3[Fe(CN)_6]$ prepared in 50 mM PB (pH 8) after hybridization with different concentrations of target ssDNA sequence (1×10^{-8} M to 1×10^{-15} M) followed by MB incubation.

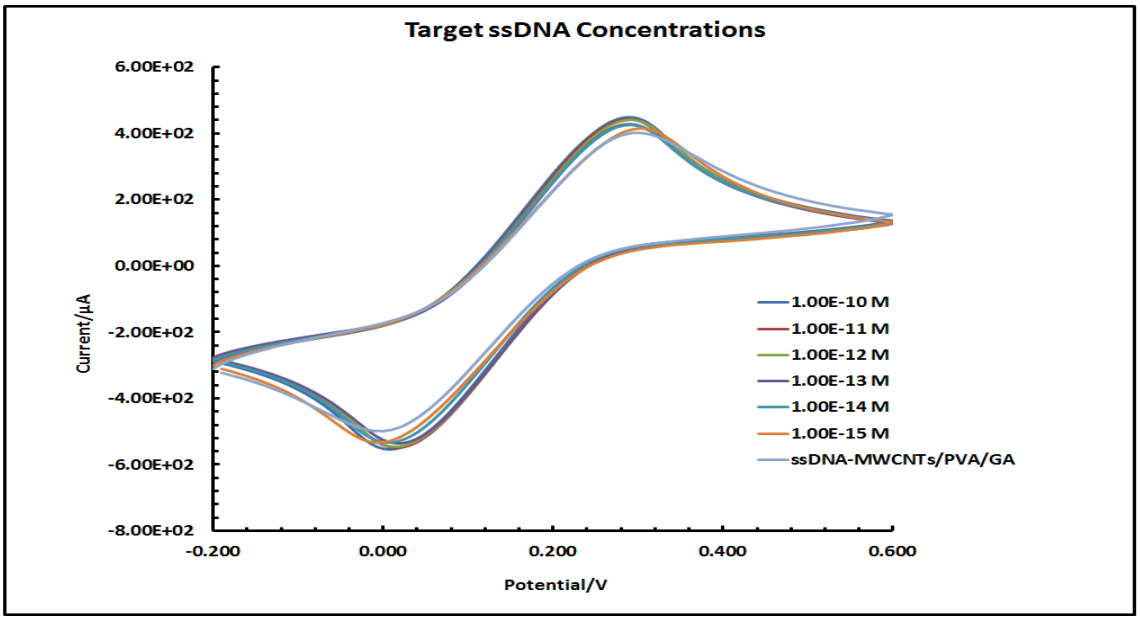


Figure 5.5. Cyclic voltammetry of ssDNA-MWCNTs/PVA/GA in 0.5 M NaCl, 50 mM $K_3[Fe(CN)_6]$ prepared in 50 mM PB (pH 8) after hybridization with different concentrations of target ssDNA sequence (1×10^{-10} M to 1×10^{-15} M) followed by MB incubation.

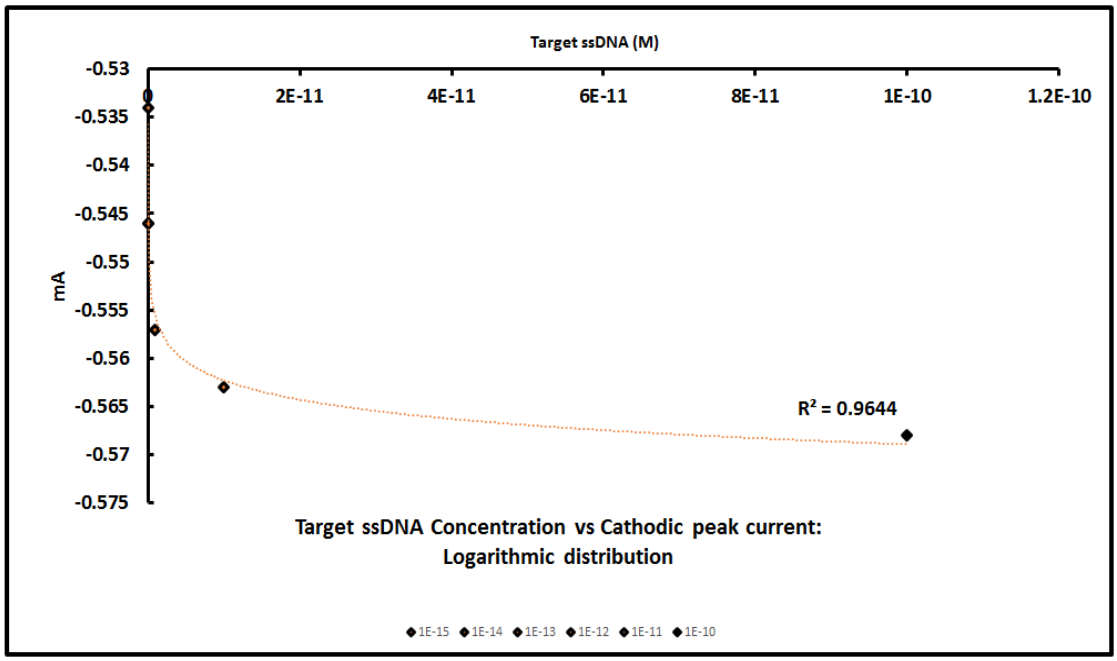


Figure 5.6(a). Plot of various concentrations of target ssDNA (1×10^{-10} M to 1×10^{-15} M) vs. I (mA) cathodic peak current showing logarithmic distribution with $R^2 = 0.9644$.

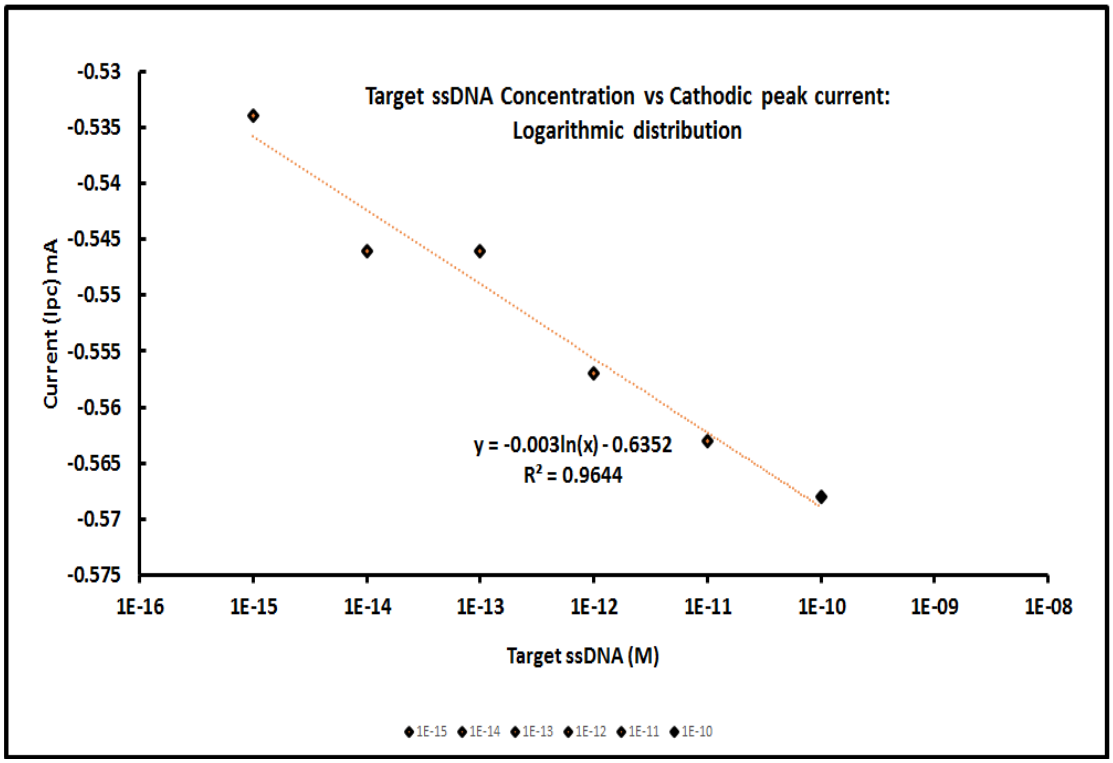


Figure 5.6(b). Plot of various concentrations of target ssDNA (1×10^{-10} M to 1×10^{-15} M) vs. I (mA) cathodic peak current showing linearity in logarithmic distribution with $R^2 = 0.9644$

5.3.4. Hybridization specificity of ssDNA probe or interference study:

MB can specifically bind and readily intercalate with guanine bases of dsDNA. Thus, MB incubation shows totally different electrochemical response with double and single stranded DNA. Before measuring current, each time the electrode was incubated with 20 µM MB. It is evident from the **Figure 5.7** that current is increased after its hybridization with complementary strand (1×10^{-15}) compared to before hybridization. Thus, the increase in the current after hybridization was due to accessibility of guanine bases in hybridized dsDNA. Magnitude of current also corresponds to the extent of hybridization **(Figure 5.6(a) & 5.6(b))**. **Figure 5.7** also explains that ssDNA probe designed with 20 bases shows very high specificity towards its complementary strand and easily discriminate between complementary and non-complementary target sequences. This ability of identifying complementary strand is crucial for the successful detection by the sensor and this step was performed by measuring reduction peak current. Electrochemical response at cathodic peak was higher for 1×10^{-15} M complementary ssDNA target compared to before its hybridization at ssDNA-MWCNTs/PVA/GA. This is due to MB intercalation and its stability at hybridized dsDNA complex. CV response after 1×10^{-15} M PupE ssDNA incubation was also measured indicating no inference in the sensor signal. Even after PupE ssDNA incubation at the electrode, the current was found to be decreased at both the anodic and cathodic peak. This suggests non-specific adsorption at the electrode surface where MB could not intercalate and it hindered the current being an insulator rather than increasing current. This study clearly demonstrates that the sensor is highly specific to its complementary target and does not bind with the random sequences of ssDNA **(Figure 5.8)**. Thus, high specificity is obtained by carefully designing the ssDNA probe and optimizing the hybridization conditions.

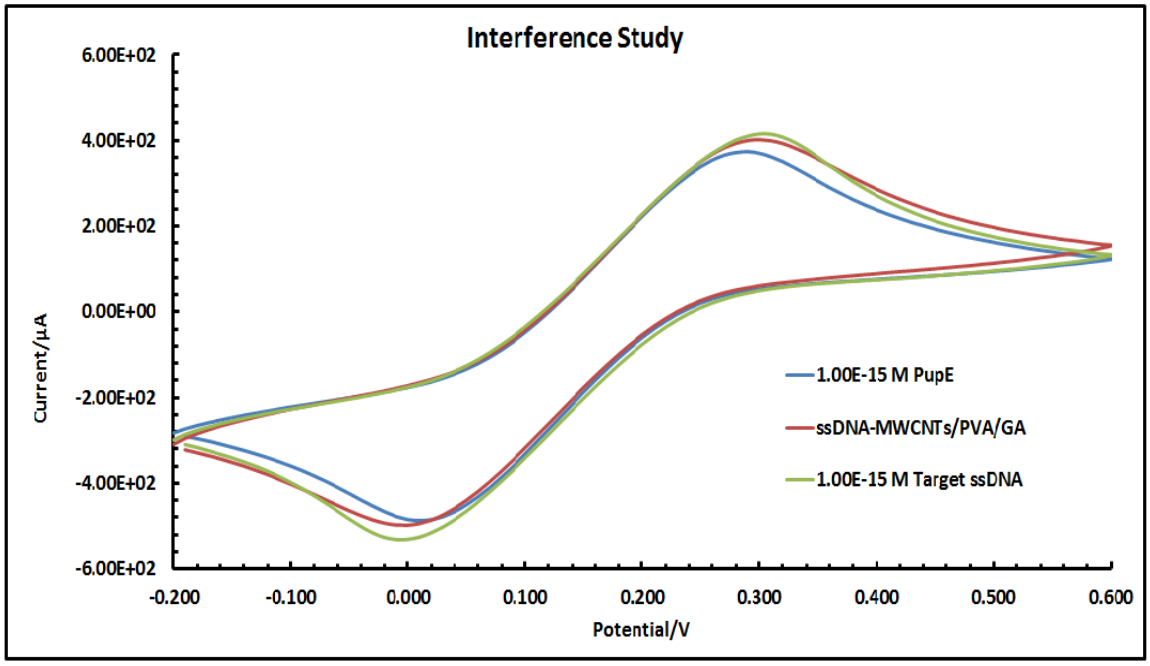


Figure 5.7. Cyclic voltammetry of ssDNA-MWCNTs/PVA/GA in 0.5 M NaCl, 50 mM $K_3[Fe(CN)_6]$ prepared in 50 mM PB (pH 8) after hybridization with target complementary ssDNA (1×10^{-15} M) and non-complementary PupE (1×10^{-15} M) after MB incubation.

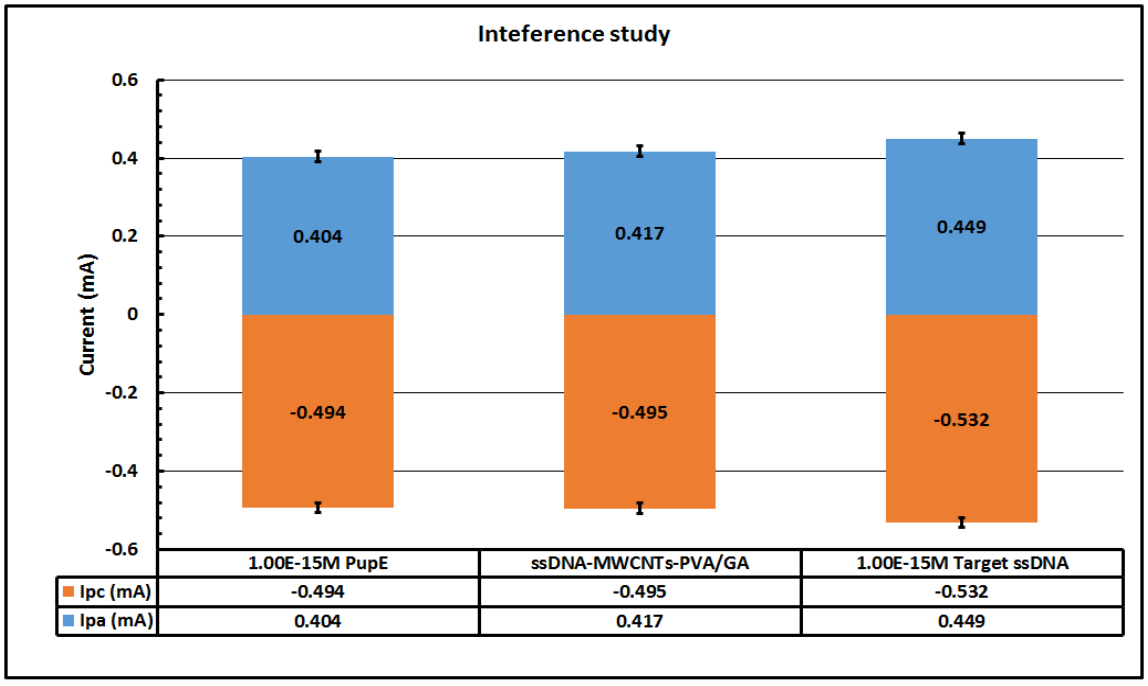


Figure 5.8. Plot of complementary (1×10^{-15} M) and non-complementary target sequences (1×10^{-15} M) vs. current (mA) of cathodic peaks after MB incubation.

5.3.5. Electrode regeneration:

The electrode regeneration of the used sensor is an essential part of developing a reusable sensor. In this study, two regeneration methods were employed to reuse the sensor as described in the experimental design. In one method the strong acid and strong alkali solutions were used for regeneration. High and low pH extents used here could lead double stranded nucleic acid on the electrode surface to dissociate into single strands and get desorbed into the washing solutions when ultrasonicated. Higher pH changes could lead to deformation of hydrogen bonds between the two strands of DNA. However, the method can pose a risk to the integrity of bases in the probe due to acidic treatment. While in the second method developed in our laboratory, the electrode was only treated in water as washing solution at 62 ˚C. Here, 62 ˚C is higher than the Tm=59.35 (the temperature at which two strands get dissociated from each other). Since, water alone is used to regenerate electrode, it avoids the possible damage to electrode due to the use of harsh chemicals. Acidic solution used to regenerate the electrode might cause harm to either ssDNA probe or PVA membrane or both. The electrode treated by both the methods was compared by running cyclic voltammetry as seen from the **Figure 5.9** it is evident from the experiment that alkali-alkaline solutions used are not that much effective to regenerate the electrode instead it reduces its performance capacity. Strong acidic solution might have damaged the ssDNA probe, weaken the ester bond between ssDNA-MWCNTs complex or even might have damaged the PVA membrane. Water treated electrode at 62˚C also showed slightly lower current response compared to the unused electrode surface. This might be due to weakening of ester bonds between ssDNA probe sequence and MWCNTs and changes in the spatial arrangements of ssDNA probes at high temperature. This temperature effect might have caused the sensor to lose its original activity during regeneration. But, while comparing both the regeneration methods, water treatments seems to be more successful.

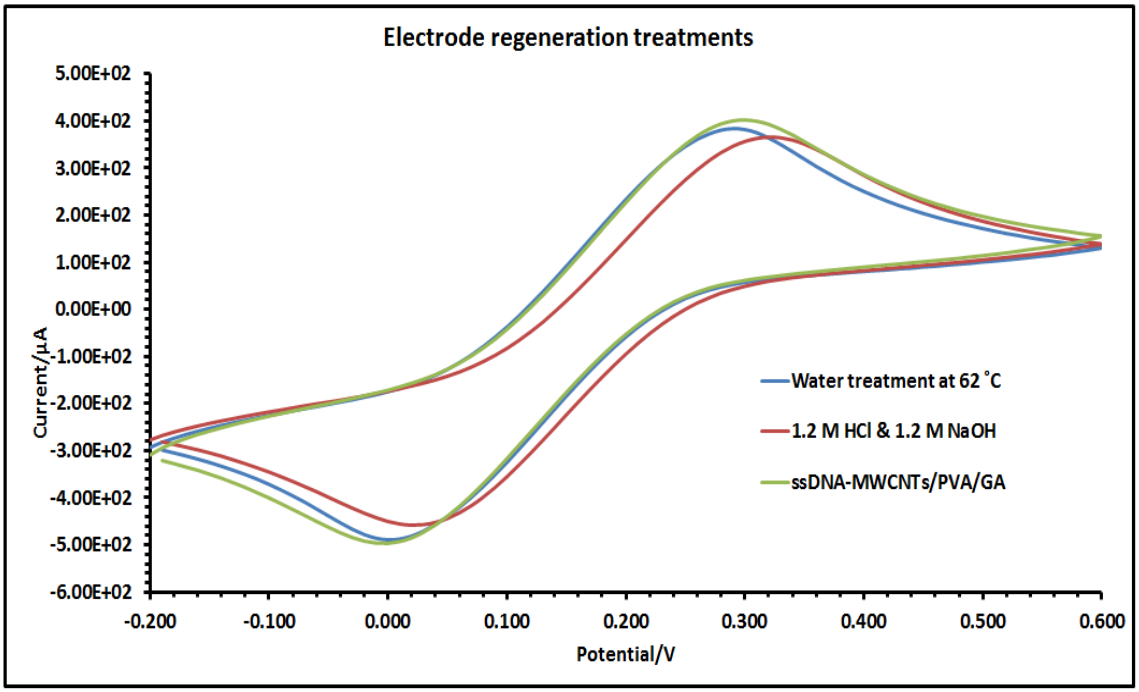


Figure 5.9. Cyclic voltammetry of water and alkali-alkaline solutions treated electrode in 0.5 M NaCl, 50 mM $K_3[Fe(CN)_6]$ prepared in 50 mM PB (pH 8).

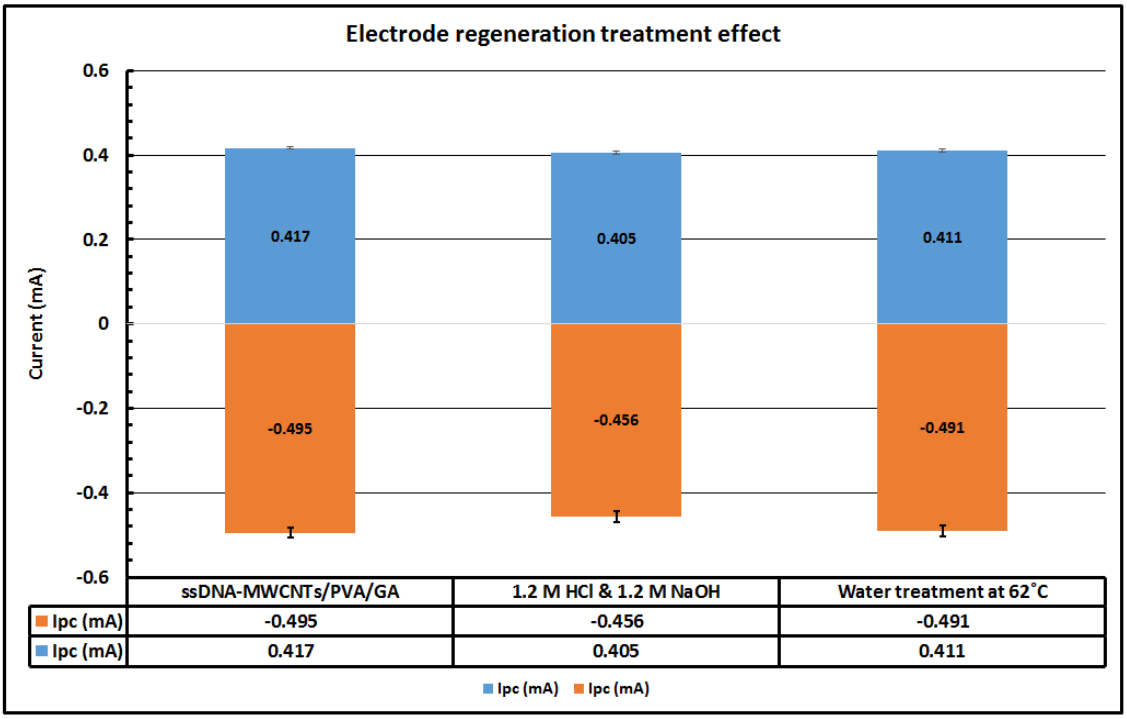


Figure 5.10. Plot of treated electrodes for regeneration vs. current (mA) of cathodic peaks.

5.4. Conclusion:

An electrochemical DNA chip was developed to study the hybridization specificity of Nsp3 gene probe using MB as a hybridization indicator. The method is simple, rapid and cost effective. Results suggest that ssDNA probe was successfully bound to carboxylic acid group modified MWCNTs via esterification. MWCNTs were properly dispersed into PVA matrix and stable film was formed on the electrode surface. Despite holding ssDNA probe, MWCNTs also provide high surface area and electrical conduction. The reduction current signal of MB increased after increasing target ssDNA concentration. Increase in magnitude of reduction current reflects the extent of hybridization onto the electrode surface. Complementary target ssDNA of Nsp3 protein gene was successfully detected by this genosensor in the concentration range from 1×10^{-8} to 1×10^{-15} M with the linear range from 1×10^{-10} to 1×10^{-15} M. The genosensor showed excellent specificity towards its complementary target sequence. Both the sensitivity and specificity are better than PCR. The sensor can be replicated with the error of ±4.92% to ±2.60% at anodic and cathodic peak respectively.

In summary, the authors propose an electrochemical method to make a genosensor which can detect SARS-CoV-2 virus. The DNA sensor proposes a strategy which can skip the second step of RT-PCR (i.e. amplification of cDNA). Directly the presence and concentration of cDNA can be detected by this sensor chip. To check the sensor's performance against real SARS-CoV-2 positive samples, government's approval along with biosafety measures are required. The sensor can be used to scan number of samples which can further offer its statistical analysis for its performance. The sensor can be compared with commercially available assays for its performance.