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Nanocrystalline Diamond Electrolyte-Gates in Field Effect Transistor for a Prolific Aptasensing HIV-1 Tat on Hydrogen-terminated Surface

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ABSTRACT

Nanocrystalline diamonds have recently gained great attention to circumvent the current hurdles, with their appealing properties such as high-surface-area to volume ratio, low-background current, wide potential window, biocompatibility, and chemical stability. The nanocrystalline diamonds electrolyte-gated field-effect transistor (NCD-EGFET) can operate directly in solution without involving gate oxides in bringing the hydrogen-tethered moieties and facilitates the p-type surface conductivity. This research investigated on Trans-activator of transcription (Tat) protein; a powerful viral gene activator that plays a pivotal role in the primary stage of the human immunodeficiency virus type 1 (HIV-1) replication. Dose-dependent interactions of HIV-1 Tat on NCD-EGFET-based RNA aptamer sensing surface were monitored and attained the detection down to 10 fM. The linear regression curve with 3σ estimation professed the sensitivity range to be 31.213 mV/log$_{10}$ [Tat Concentration]M and the limit of detection of 6.18 fM. The selectivity analysis of NCD-EGFET was conducted with different proteins from HIV (Nef and p24) and Bovine Serum Albumin. Furthermore, to practice in the clinical application, HIV-1 Tat was spiked into the human blood serum and it displayed the genuine non-fouling interaction with the aptamer. The attained high-performance signal enhancement with nanocrystalline diamond-biosensing aids to circumvent the issues in the current diagnosis.

Keywords: Aptamer, Electrolyte-gated Field Effect Transistor, HIV-1 Tat, Nanocrystalline Diamonds.

1. INTRODUCTION

Human immunodeficiency virus (HIV) has become a widespread disease throughout the world and ~70 million people were found infected. HIV predominantly comprises of 15 types of protein and ssRNA genome with two strands, which make the entire structure of the virus. HIV encodes three enzymes, namely reverse transcriptase, protease, and integrase [1]. The supplementary proteins such as trans-activator of transcription (Tat), viral protein unique (Vpu), regulator of virion (Rev), viral infectivity factor (Vif), negative regulatory factor (Nef), P6, and viral protein r (Vpr) are also present in HIV [2]. HIV disseminates throughout certain body fluids that strikes the defence system, specifically the CD4 cells, also known as T cells. These unique cells help the defence system of the body to eliminate the infections. Throughout time, HIV can demolish most of these cells causing the body unable to fight against the infections and diseases. Different screening test have been developed to identify the existence of HIV in human especially for the

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early stage of the disease [2]. The developments of various screening test for HIV have attracted many researchers to improve the technique and quality of the device for the early detection of HIV [3].

HIV-1 Tat protein is an important component for viral replication and activation transcription of HIV-1 gene products [4]–[6]. HIV-1 Tat, a small protein consists of 86–101 amino acids which include the N-terminal, C-terminal glutamine-rich regions, basic, cysteine-rich core, and proline-rich. HIV-1 Tat experiences plentiful post-translational modifications like methylation, phosphorylation, ubiquitination, and acetylation [7], which further increase the abundance of HIV-1 Tat synergy with the host proteins by providing HIV-1 Tat with more flexible conformation changes and/or contacting interfaces. It is vital in the transcription of viral RNA and amplifies the quantity of protein produced by linking itself to the viral RNA. Although HIV-1 Tat dominates numerous functions such as controlling the cellular processes by interrelating with different cellular structures [8]–[11]; it is fundamentally included in the transcription of integrated HIV-1 proviruses [12]. In the current research, HIV-1 Tat is chosen as a target protein due to its existence during the early stage of HIV-1 infection. By detecting HIV-1 Tat, the highly risked individuals can detect virus transmission in the early disease stage regularly to reduce the risk of HIV pandemic.

Throughout this research, RNA aptamer was used as a sensing probe for the recognition of HIV-1 Tat (target). RNA aptamer is a single-stranded oligonucleotide which competent of binding to a specific target with high sensitivity, specificity and affinity. Aptamer-target interactions are based on the affinity binding and the non-covalent bonding exists between the aptamer and the target molecule (aptamer-target complex). This interaction can be caused by hydrogen bonding, electrostatic interactions, aromatic stacking, hydrophobic interaction, or van der Waals interactions [13]. The introduction of aptamer as aptasensor has built up several applications in the science, with various advantages such as more invulnerable to regeneration and degradation, hence, binding affinities and specificities can simply be controlled and promoted by rotational design or by molecular immobilization techniques on biochips. It can differentiate among chiral molecules and capable to define a target molecule site with chemical stability at stringent conditions [14]–[16]. On the other hand, merging of molecular biology and nanoelectronics has led to designate a new field known as bioelectronics. The introduction of polycrystalline diamond-based field-effect transistor (FET) as the sensing device for the detection of HIV brings a novelty due to its promising chemical stability, broad potential window, small background current, and biocompatibility [17]. Although few works have been reported on HIV-1 Tat detection, the study of HIV-1 Tat in spiked human on the nanocrystalline diamond was not yet presented.

Nanocrystalline diamond (NCD) is a carbon-based unique material for bioelectronic systems due to its above features and it is a favourable combination for optical, electrical, and mechanical properties. Regardless of the stability NCD, the surface chemistry of the diamond can be easily modified to control its physical properties, for example, surface wettability, electron affinity, and electrical conductivity. Regarding this, the hydrogen-terminated surface (H-terminated) shows a hydrophobic characteristic and displays the p-type induce surface conductivity through an intrinsic diamond. Meanwhile, the oxygen terminated (O-terminated) surface exhibits a hydrophilic and extremely resistive [18]. The prominent reasons to implement the above features into the FET are due to its promising properties such as mass production, miniaturization, standardization, low price, and its flexible configuration for simple measurement [19].

Herein, this study has demonstrated the binding activity of HIV-1 Tat protein with probe RNA aptamer in human blood serum in terms of dose-dependent analysis on hydrogen-terminated nanocrystalline diamond electrolyte-gate FET by electrical performance. It combines both the chemical stability and biocompatibility of nanocrystalline diamond surface under electrolytic condition. Furthermore, this study has shown that nanocrystalline diamond can acts as transducing channel, as it provides a large surface to volume ratio thus improving the performance of FET in terms of sensitivity, selectivity and specificity on HIV-1 Tat detection.

2. MATERIALS AND METHODS

2.1 Materials and Reagents

RNA aptamer used in this study has a split configuration, in which 5'-UCGGUCGAUCGCUUCAUAA-3'-NH_2 as probe aptamer and 5'-GAAGCUUGAUCCCGAA-3' is the aptamer derived as the second strand. Both aptamers were purchased from Trilink Biotechnologies, USA. The recombinant HIV-1 Tat stored at -75°C was purchased from Immuno Diagnostics, Inc. Bovine serum albumin (BSA) was purchased from Sigma Aldrich. AB male human serum was purchased from Nano Life Quest Sdn Bhd. Phosphate buffered saline solution (PBS), sodium saline citrate (SSC) and all other chemicals, unless mentioned, were purchased from Sigma Aldrich. The equipment used in this research was Keithley 6487 picoammeter and Keithley 2400 source meter.

2.2 Fabrication of the Nanocrystalline Diamond Electrolyte Gated Field-Effect Transistor (NCD-EGFET)

Nanocrystalline diamond (NCD) thin films were grown on silicon (Si) substrate for 4.5 hours by a chemical vapour deposition (CVD) process in a microwave ellipsoidal cavity reactor. The gas pressure was set at 30 mbar, microwave power of 1000 W and gas mixture as 1% methane (CH_4) in hydrogen gas (H_2) were used. The deposition temperature was in the range of 550-600°C, which led to the growth of approximately 450 nm thick nanocrystalline diamond film with grain sizes of ~250 nm [20]. NCD thin films were additionally hydrogenated in hydrogen plasma for 10 minutes at 600°C to prompt the surface conductivity giving the H-terminated NCD films. The beginning of the FET fabrication process starts with photolithographic masks were utilized on H-terminated NCD films using a positive MA-15 photoresist to specify three FET channels and three pair of gold patches that serve as source and drain with ohmic contacts of the holes accumulation layer of the FET. The gold patches were conceived by thermal evaporation [10 nm of titanium (Ti) and ~50 nm of gold (Au)] accompanied by the lift-off procedure using acetone. The samples were treated in oxygen radio frequency plasma at 300 W for one minute to achieve the insulating O-terminated areas, which surround the 20 μm wide and 60 μm long stripes H-terminated channels that connecting the source and drain contacts. The samples were cleaned by acetone and the area between contacts and channels was covered with a positive photoresist AR-P-3220 with a thickness of 4 μm. The final photolithographic step generated the aperture of 60 μm × 20 μm to clarify the active gate area.

2.3 Receptor-Target Bio-recognition for Different Concentration Detection and Selectivity of Receptor

The immobilization of chemically-modified RNA aptamer was conducted on the active gated surface of the NCD-EGFET. To trigger the carboxylic functional groups on the sample, every sample was initially treated with a mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.1 M N-hydroxysuccinimide (NHS) with ratio 1:1 for 1 hour [17], [21]. Then, the first strand of RNA aptamer was diluted with 3× SSC, 0.4 M EDC, and 0.1 M NHS to a final concentration of 10 μM. A 3 μl of the solution was manually drop-casted on the surface of the gate channel and incubated at 38°C for 2 hours in a humidified chamber, followed by a thorough cleansing with PBS. The binding of target HIV-1 Tat with the probe RNA aptamer was done at room temperature for 1 hour with various concentrations of HIV-1 Tat at different time. The concentration of the second strand aptamer used was proportional to the concentration of Tat protein used. After that, Tris–HCl buffer solution was used as a rinsing agent to avoid the nonspecific binding. An 8.3 M urea solution was used to remove the tested HIV-1 Tat from the active gate channel to achieve the regeneration process in order to test dose-dependent HIV-1 Tat. For the selectivity study, three different proteins with 100 pM concentration were used. These proteins were bovine serum albumin (BSA), negative regulatory factor (Nef), and p24. Both Nef and p24 are protein existed in HIV. For the real application of the developed aptasensor, human

serum with 100 times dilution factor was selected to study the effect of HIV-1 Tat binding to RNA aptamer. For this study, 100 nM of HIV-1 Tat was spiked in human serum and the detection procedures were carried out identical to the detection of 100 pM HIV-1 Tat in the standard solution.

3. RESULTS AND DISCUSSION

NCD-EGFET was fabricated to identify the interaction of RNA aptamer and binding activity of HIV-1 Tat protein in standard solution and spiked human serum on the active gate channel of the device. The binding of RNA aptamer on different concentration of HIV-1 Tat in standard solution was studied in details giving the sensitivity and the limit of detection of the device. Following the sensitivity study of the NCD-EGFET, the selectivity and specificity have been conducted to determine the performance of the device towards other biomolecules. In the final part of this paper, the spiking analysis was conducted. HIV-1 Tat was spiked into human blood serum to observe the interference of human blood serum on HIV-1 Tat detection.

3.1 Fabrication of the Nanocrystalline Diamond Electrolyte Gated Field-Effect Transistor (NCD-EGFET)

A fabricated NCD-EGFET device for HIV-1 Tat detection using RNA aptamer is illustrated in Figure 1(a). The NCD-EGFET biosensor detection operates based on the change with the surface charges [22]. The dominant carriers inside a nanocrystalline diamond are holes and the density of surface holes will increase or decrease in respect to the number of biomolecules charges, either positively or negatively charged ions that bind on the surface of active gate channel of NCD-EGFET. So, this characteristic defined a p-type field-effect transistor. As the surface of the gate channel is dipped in the electrolyte solution (PBS), the density carrier of the gate channel was regulated by the field effect of the biomolecules electric charge nearby the solid surface. To prove the functionality of NCD-EGFET, the measurement of I_{ds}-V_{ds} was conducted using an Ag/AgCl as a reference electrode in 1 mM PBS at pH 7.4. Figure 1(b) shows the image of 20 μm width active gate channel, observed under the scanning electron microscope (SEM) at 453× magnification, while Figure 1(c) shows the binding structure of HIV-1 Tat on the H-terminated NCD-EGFET channel with RNA aptamer as sensing probe by the activation of EDC-NHS. The RNA aptamer was immobilized on the active gate channel surface of NCD-EGFET and incubated for 2 hours in a humified chamber at 38°C. Later, HIV-1 Tat was introduced to the probe RNA aptamer accompanied by aptamer-derived second strand. These interactions form a duplex structure of aptamer in the existence of HIV-1 Tat, causing a significant change in the gate potential [21].

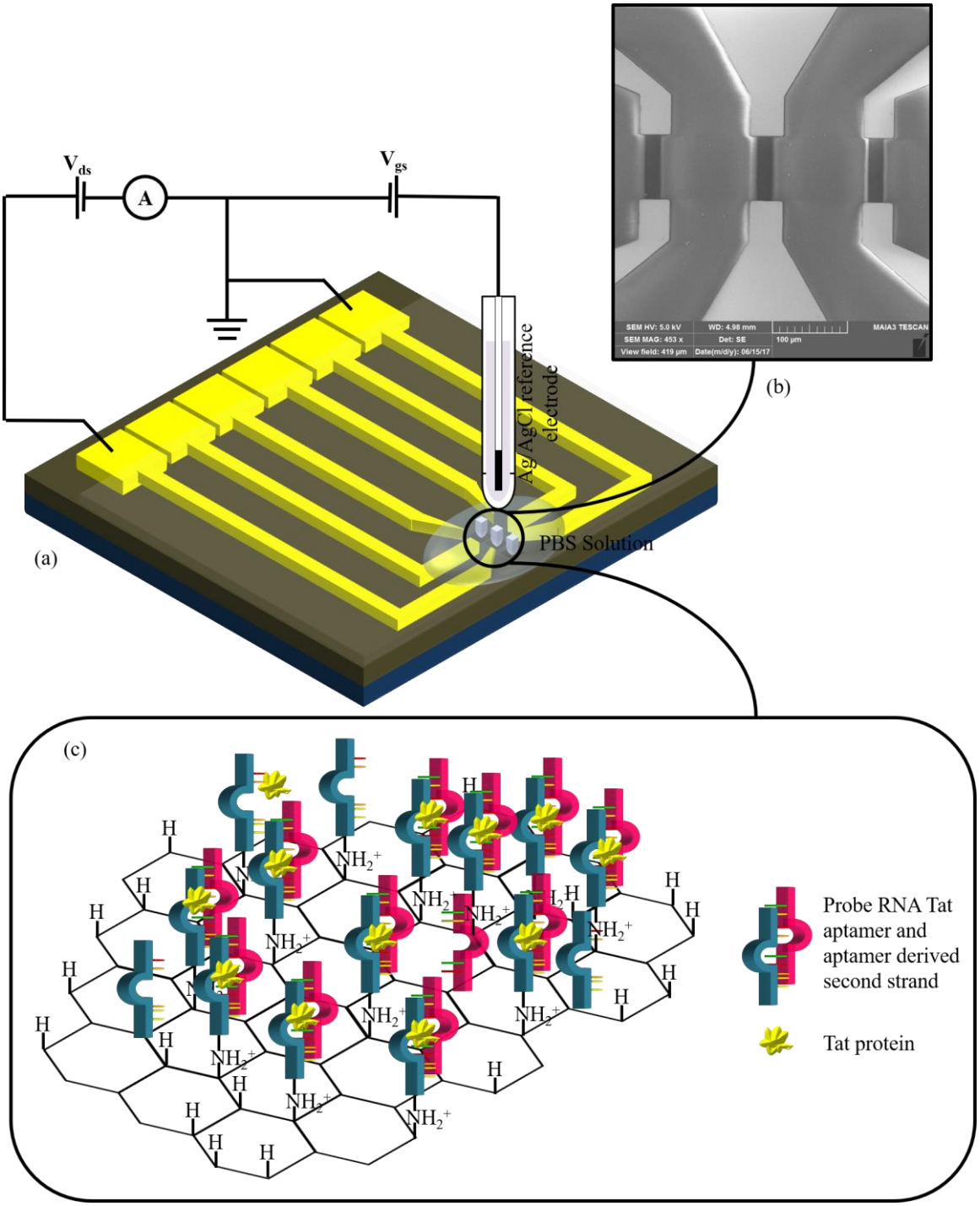

Figure 1. The fabricated NCD-EGFET: (a) in 1 cm × 1 cm dimension with three pair of source and drain contact pads for different gate channel, (b) with H-terminated gate channels of 20 µm width observed under the scanning electron microscope (SEM), and (c) the binding mechanism of HIV-1 Tat protein on NCD-EGFET via RNA aptamer as sensing probe.

3.2 Interaction of HIV-1 Tat on Split Aptamer: Dose-Dependent Binding of HIV-1 Tat and Linear Regression Analysis

The interaction of the HIV-1 Tat on split aptamer was studied in detail by observing the value of gate potential difference (ΔV_{gs}). Figure 2(b) shows a gate potential shifted by 174.80 mV when 100 pM HIV-1 Tat was introduced to RNA probe aptamer on the active gate channel surface of NCD-EGFET. This interaction caused the shift of gate potential towards the negative direction with

respect to the potential charge changes created by the biomolecular interaction. This result confirmed that the NCD-EGFET responses were only observed when specific interaction between recognition molecules (aptamer and HIV-1 Tat) takes place on the active gate surface. Based on previous studies, it has been revealed that HIV-1 Tat rich in positively charged ions [23], [24]. With the isoelectric point (pI) of 9.88, it is verified that HIV-1 Tat is positively charged at pH 7.4. The positively charge HIV-1 Tat affects the current reduction. Due to the reduction of electron transfer from source to drain, the gate potential shifted to a negative direction (M.F. Fatin *et al.,* 2019). Thus, it can be postulated that HIV-1 Tat binding cause the gate potential to deflect in the negative direction for the I_{ds}–V_{gs} transfer characteristic. Since the diagnosis of HIV-1 Tat on the NCD-EGFET biosensor is based on the modification in the charge allocation on the channel surface, the difference in molecular charges was observed, which verified the result obtained in this research. Further, when the RNA aptamers formed the duplex in the presence of HIV-1 Tat (Figure 2(a)), the fixed charge is doubled and the number of reallocation charges increases, thus the system decreases the amplitude of the current. This is primarily due to the repulsion between the positive charges of HIV-1 Tat with the positive hole carriers of the substrate within the sensing area. This work proves that the detection of HIV-1 Tat in standard solution was successfully done and the desired RNA aptamer specifically adapted for HIV-1 Tat interaction.

The binding activity between aptamer-immobilized H-terminated NCD and HIV-1 Tat was also performed in control measurement without using any probe. A 100 pM concentration of HIV-1 Tat was dropped on the bare NCD-EGFET and incubated for 1 hour at room temperature. I_{ds}-V_{gs} measurement was performed to understand the binding activity of HIV-1 Tat on the surface of an active gate channel without RNA aptamer. Only a small change in gate potential (18.52 mV) was obtained and the result was shown in Figure 2(c). The minor changes in the gate potential were insignificant thus indicated that HIV-1 Tat was not fully bound to the surface of NCD-EGFET. Weak binding of HIV-1 Tat was noticed towards the NCD-EGFET surface shows that detection of HIV-1 Tat on NCD-EGFET sample cannot be done without the existence of RNA aptamer, further indicates the specificity.

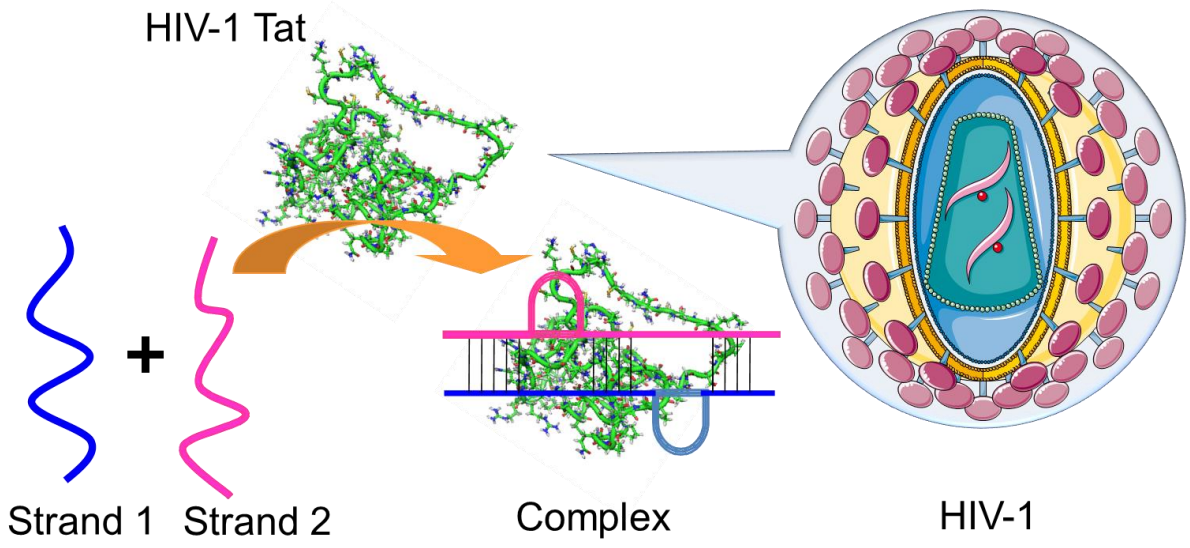

Figure 2(a). Binding mechanism of strand 1 RNA aptamer and aptamer-derived second strand with HIV-1 Tat from HIV-1 virus, forming a complex structure for detection strategy.

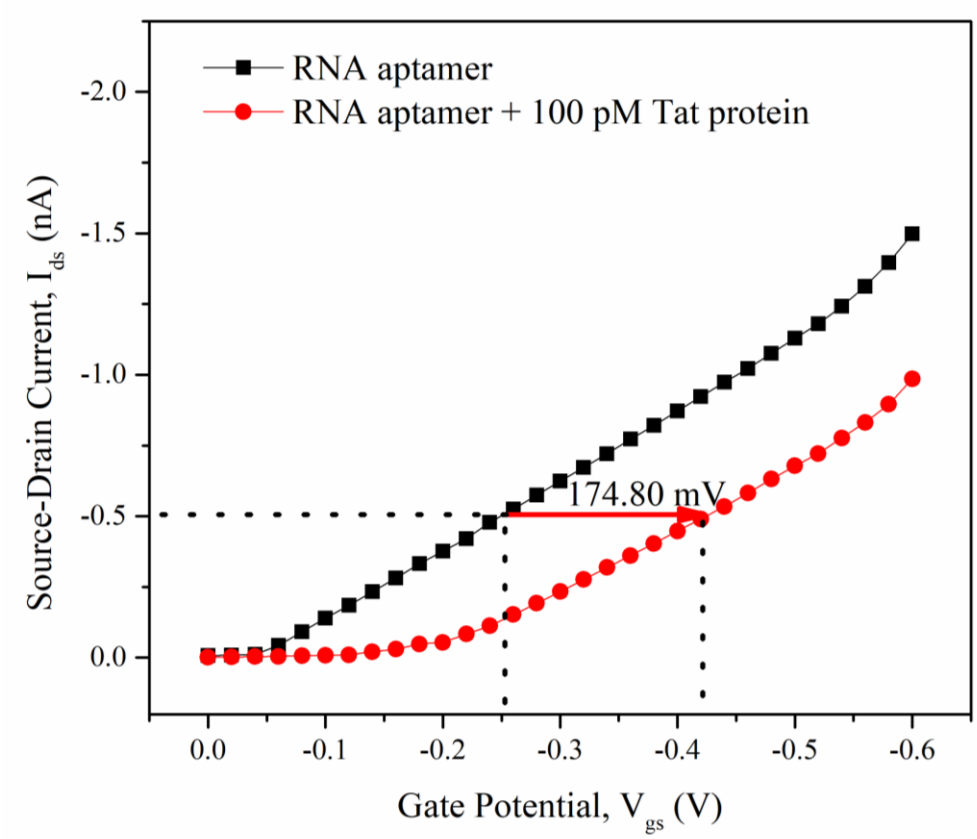

Figure 2(b). The I_{ds}-V_{gs} of NCD-EGFET with and without 100 pM HIV-1 Tat protein, measured in 1 mM PBS at pH 7.4.

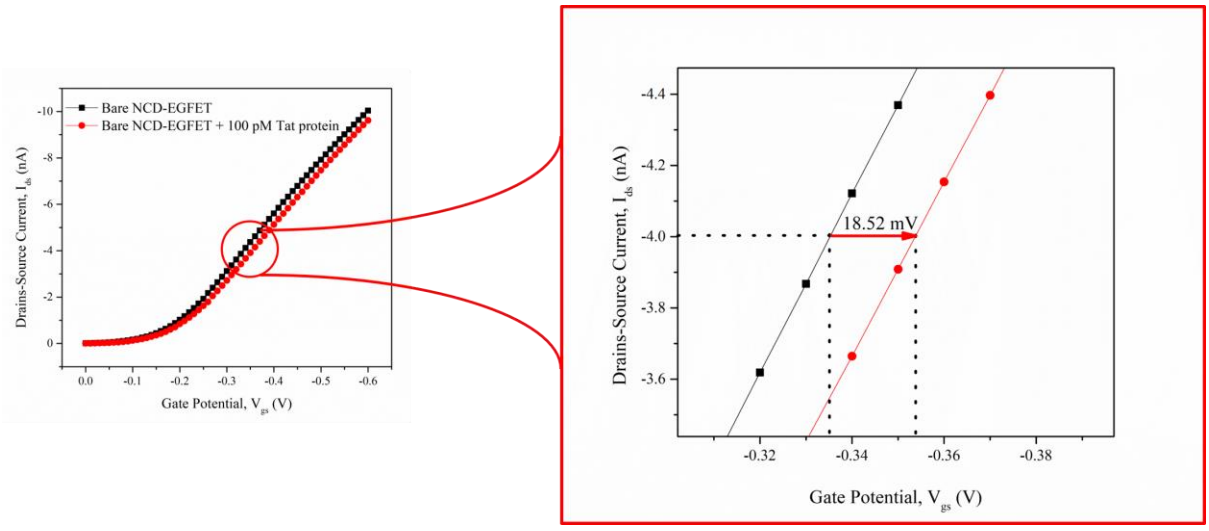

Figure 2(c). The I_{ds}-V_{gs} of NCD-EGFET with and without RNA aptamer immobilization. Small changes in gate potential show the non-specific binding of Tat protein to the active gate channel of the device.

The effect of different concentrations of the HIV-1 Tat was studied in details. Figure 3(a) and (b) displays the gate potential behaviour depending on the concentration of HIV-1 Tat protein. The concentrations of HIV-1 Tat were varied from 100 pM down to 10 fM to identify the interactions on binding activity in the standard solution. The measurement was conducted on the same device to ensure the accuracy of the shifting gate potential. When 10 pM HIV-1 Tat was introduced to the RNA probe aptamer, a 128.90 mV change in gate potential was recorded. On the other hand, a shifting of 117.10 mV was determined after the binding of 1 pM HIV-1 Tat. The shifting of gate

potential keep reducing after 100 fM and 10 fM of HIV-1 Tat were bound on RNA probe aptamer with 62.90 mV and 55.40 mV, respectively. The shifting in gate potential decline with the concentration gradient of HIV-1 Tat owing to the aspect number of charged molecules that reduce dramatically with HIV-1 Tat concentration gradient. The interaction between molecules inside the electrolytes diminished when fewer molecules interacted with the RNA probe aptamer. Furthermore, the lowest concentration for detection, which is the limit of detection (LOD) of HIV-1 Tat was analyzed as in Figure 3(c). The biosensor produced a linear response of relative change in the measured V_{gs} over the logarithmic HIV-1 Tat concentration from 10 fM to 100 pM with the LOD of 6.18 fM, based on calculation from the standard deviation of the response and the calibration curve's slope [26]. According to other researchers, the limit of detection of HIV-1 Tat was recorded at 0.6 nM using multiwall carbon nanotubes-FET based biosensor [27], 1 pM concentration when using SPR enhanced ellipsometry [28] and 10 nM using the calorimetric detection [25]. From the experiment conducted throughout this research, it can prove that the used of NCD based EGFET is much more sensitive towards HIV-1 Tat protein detection compared to other methods and materials. The calibration curve slope in Figure 3(d) was obtained from the linear response of V_{gs} with the increase of HIV-1 Tat concentration was from 10 fM to 100 pM, representing the sensitivity of the device, which is 31.213 mV/$\log_{10}$ [Tat concentration] M.

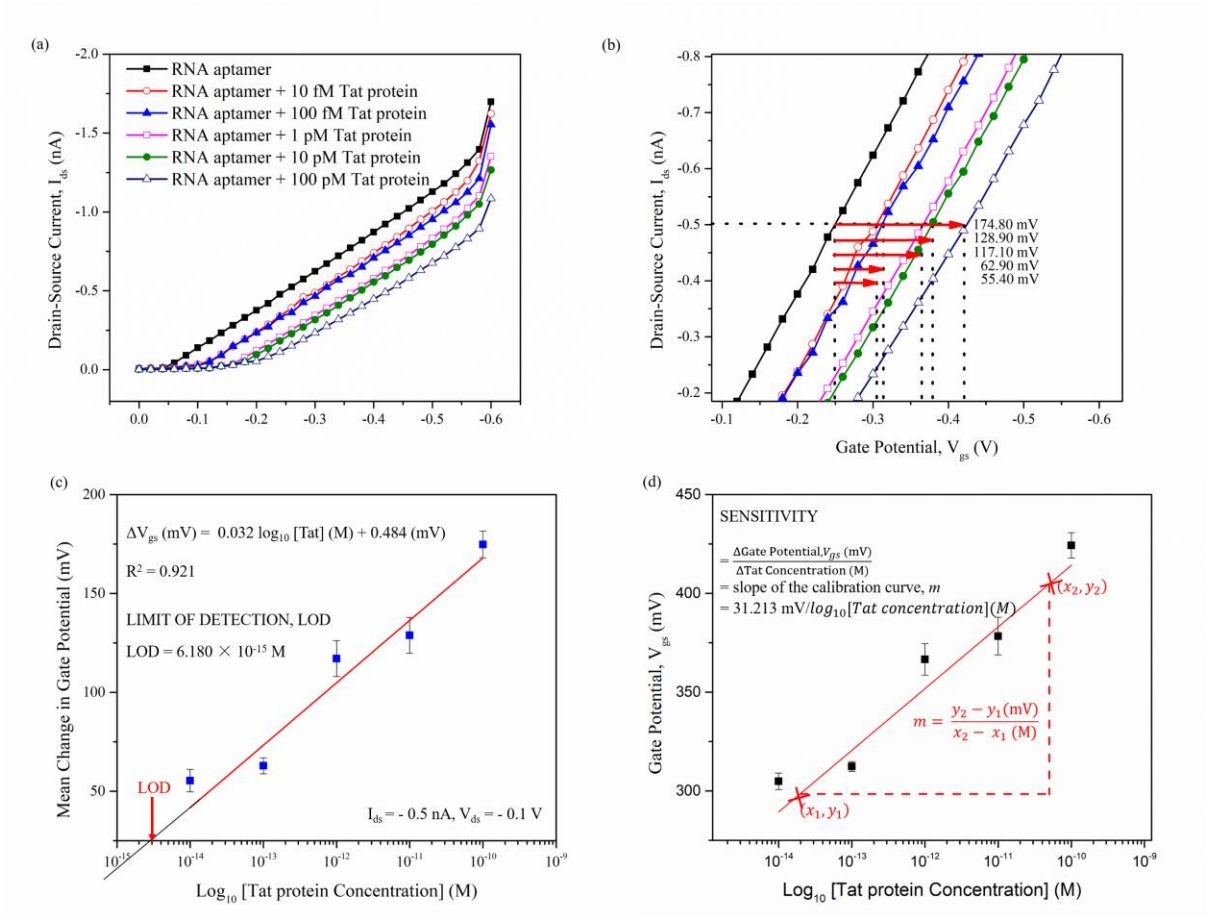

Figure 3. Detection of Tat protein target at different concentration (100 pM to 10 fM) using the NCD-EGFET biosensor at sweep V_{gs} of 0 V - -0.6 V with V_{ds} = -0.1 V. (a) I_{ds}-V_{gs} transfer characteristic at different concentration of Tat protein, (b) Enlarge of I_{ds}-V_{gs} response at different concentration of Tat protein, (c) Calibration curve of the relative change in V_{gs}, shows the LOD, (d) V_{gs} response curve of the biosensor at different Tat protein concentrations, shows the sensitivity of the device.

3.4 Analytical Performance of Nanocrystalline Diamond-Electrolyte Gated Field Effect Transistor

The analytical characteristics of NCD-EGFET biosensor in terms of selectivity and specificity were investigated. The selectivity of protein binding on RNA aptamer was studied in details by using different types of protein, which includes BSA, Nef, and p24. The interactions of these proteins with the RNA aptamer on the gate channel surface during the binding process were identified. The proteins were integrated on the probe RNA aptamer to replace the HIV-1 Tat and performed the experiments independently. First, BSA was integrated on the device followed by Nef and p24. The concentration of proteins tested was standardized at 100 pM.

Based on Figure 4, the shifting in gate potential was observed when biomolecules were integrated to the probe RNA aptamer. During the integration of BSA, the gate potential deflected towards the positive direction by ~38.50 mV. The shifting in gate potential was caused by the lack of BSA binding to the probe aptamer. The interaction was obstructed with the BSA molecules hence there is a hindrance in the binding with the probe [29]. A shifting of ~10.00 mV towards the positive direction was measured during the integration of Nef on the NCD-EGFET. Even though Nef was present in HIV as one of the accessory proteins, the deviation in gate potential was negligible since it was in a positive direction. In contrast, the shifted in gate potential is approximately 11.90 mV towards negative direction when p24 was introduced in the binding analysis. The fluctuation in gate potential was observed since p24 slightly binds to the probe RNA aptamer, and p24 is abundantly found in the early phase of HIV-1. Yet, the result cannot be used as the change in gate potential was insignificant compared to the shifting of gate potential obtained for HIV-1 Tat binding. This result reveals that the probe RNA aptamer is specifically and selectively binding only with HIV-1 Tat.

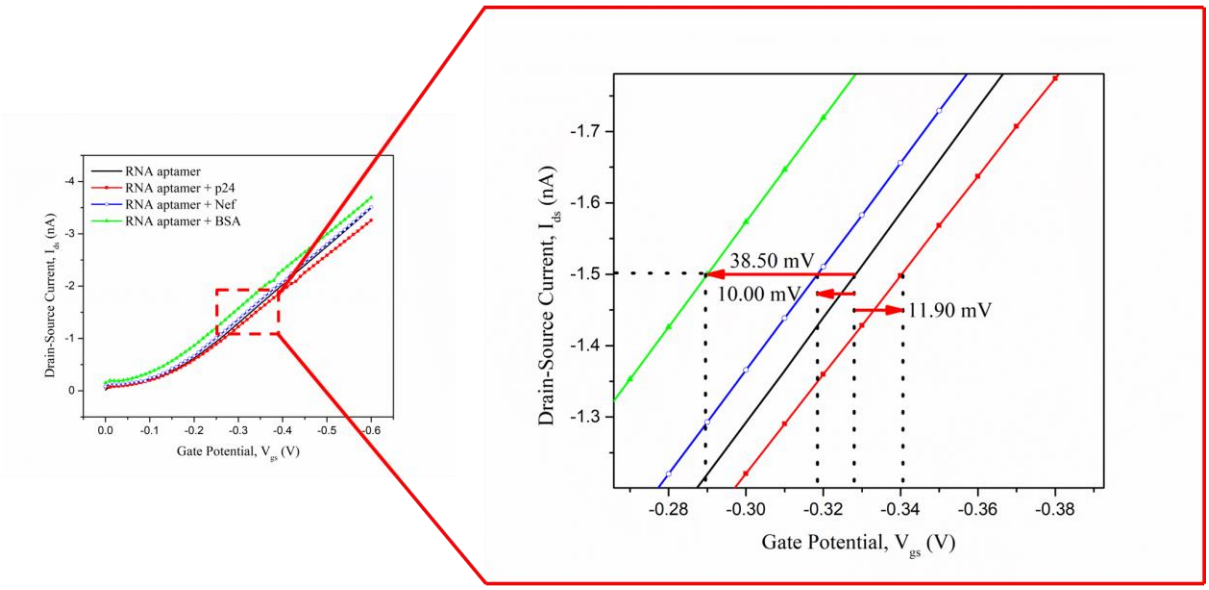

Figure 4. The I_{ds}-V_{gs} of NCD-EGFET in various proteins such as p24, Nef and BSA for the selectivity study. For p24, Nef, and BSA, the gate potential tends to shift no more than 40 mV meanwhile for Tat protein, a 174.80 mV was shifted in gate potential.

3.5 High-Performance Measurement: Spiking Analysis

Additionally, the binding of probe RNA aptamer with HIV-1 Tat in spiked human serum was studied to evaluate the interference effect from human serum in the interaction events and to practice towards the clinical sample analysis. Human serum with 100 times dilution factor was

used as a medium for the HIV-1 Tat. The binding activity was observed by measuring the gate potential shifted when HIV-1 Tat spiked in human serum passed to the RNA aptamer on the active gate channel surface. Human serum alone was used as a control experiment to investigate the binding of human serum to the probe RNA aptamer in the absence of HIV-1 Tat. Figure 5 shows the changes in gate potential on the surface channel of NCD-EGFET with and without HIV-1 Tat in human serum. There is a shift of 34.50 mV in gate potential when clear human serum bound to the RNA aptamer indicating a low interference effect of human serum in the binding event. This result was certified that the human serum contains different non-specific molecules so that a slight non-specific binding occurs during the immobilization process as shown before [30]. As soon as HIV-1 Tat was spiked in the human serum, the change in gate potential shows a significant difference (142.80 mV). The results expressed the specificity of RNA aptamer to HIV-1 Tat. From this work, we could prove that the detection of HIV-1 Tat in human serum was successfully done and the desired RNA aptamer specifically adapted for HIV-1 Tat interaction.

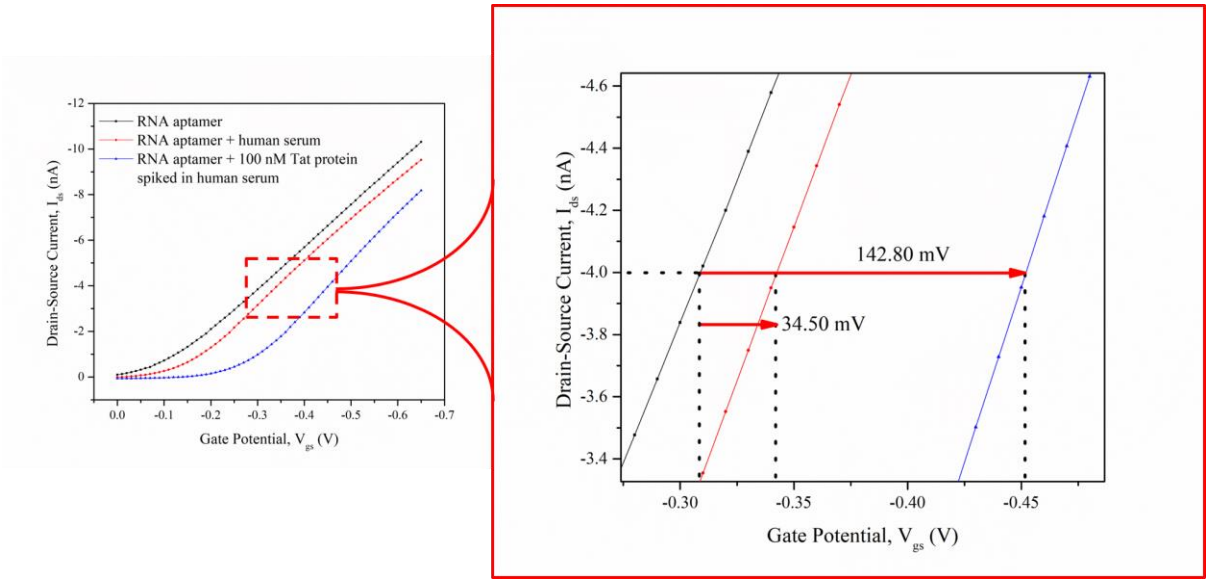

Figure 5. The I_{ds}-V_{gs} of H-terminated NCD-EGFET in human serum with and without the presence of HIV-1 Tat protein. In human serum binding activity, a small change of 34.50 mV was presented while 142.80 mV was observed when HIV-1 Tat protein spiked in human serum.

4. CONCLUSIONS

In summary, this research has successfully determined the interactions of dose-dependent HIV-1 Tat on NCD-EGFET with the limit of detection down to 10 fM. The gate voltage deviated was in a negative direction as the HIV-1 Tat approaches the active gate surface of H-terminated NCD-EGFET channel. The selectivity of RNA aptamer towards various biomolecules was examined and the RNA aptamer was specifically and selectively binds the HIV-1 Tat. The binding of 100 nM HIV-1 Tat to RNA aptamer in human serum was successfully performed by spiking experiment. The sensitivity and selectivity of the device show a successful development of a highly specific biosensor that can be adapted to clinical monitoring of biological diagnosis via nanocrystalline diamond approach for detecting HIV-1 Tat and provides the potential of nanocrystalline diamond bio-interfaces in clinical biosensor applications.

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