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An Impedimetric Genosensor for Ultrasensitive Detection of SARS-Cov-2 Genome Based on 3D Reduced Graphene Oxide and Gold Nanoparticle Composite

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Abstract

Nowadays the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become an endemic disease throughout the world on the other hand intensive worldwide vaccination programs decreased the severity of the affection but early virus detection and disease diagnosis are still important healthcare management of infectious disease control. Therefore, in this research, we introduce an electrochemical genosensor based on a DNA probe that can hybridize directly to the viral genome or its transcripts and therefore does not need cDNA synthesis following RNA extraction from patient samples, a necessary and challenging step in routine RNA virus detection methods like Real-time PCR. Altogether, in this research, an electrochemical biosensor based on a virus-specific probe with thiol modification was designed and immobilization of the probe was carried out through self-assembly by thiol binding on reduced graphene oxide (RGO) and gold nanoparticles composite-modified pencil graphite electrode (PGE). The hybridizations of probe and target sequences were analyzed by electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) methods. The linear range was found to be within 10^{-12} - 10^{-6} M and the limit of detection (LOD) was at 3×10^{-13} M. The time of 20 minutes was chosen as the optimal hybridization time. The results showed that the fabricated biosensor can be recovered and reused up to 6 times. This means significant time, and expense savings when compared with other conventional detection methods for this virus. Therefore, this biosensor is suggested for clinical applications especially when time and sensitivity are the most limited elements.

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Introduction

SARS-CoV-2 is an airborne RNA virus that has led to an infectious pandemic coronavirus disease, COVID-19. Different methods are applied to identify viruses, including cell culture, antigen test, polymerase chain reaction (PCR), real-time PCR, and RT-PCR (1). These methods have some disadvantages such as low specificity and time-consuming. As the risk of viral infections has increased, novel virus detection methods are being developed. Electrochemical biosensors do not have many limitations and disadvantages compared with conventional methods. Their merits are high sensitivity, fast, easy application, and portability. Based on related literature, they had been designed and fabricated for detection of different viruses such as VHSV (2), HIV (3), hepatitis B (4), hepatitis E (5), influenza Poultry (6), and Zika (7). Sabzi et al., (8) designed an electrochemical DNA biosensor capable of detecting human papilloma virus with a detection limit of 1.2 ng/µl. To detect the E6 gene from human papillomavirus (HPV16) Campos-Ferreira et al. (9) developed an electrochemical genosensor with a detection limit of 16 pg/µl. As documented in the bibliography, Balvedi et al. (10) designed a DNA-based electrochemical biosensor with a graphite electrode modified with 4-aminothiophenol (4-ATP) polymer for the detection of Epstein-Barr virus (EBV), capable of detecting 17.32 $nmol.L^{-1}$ of the target oligonucleotide. In a research, Dong et al. (11) designed a thiolar tetrahedral DNA probe to detect the hemagglutinin gene sequence of influenza A virus (H7N9) and used it to fix on the surface of a gold electrode, and in conclusion, the detection limit of this biosensor was found to be 100 femtomol. Shakoori et al., (12) presented an electrochemical biosensor to detect the hepatitis B virus in which gold nanorods were used as gold electrode surface modifiers to immobilize the single-stranded DNA (ssDNA) probe, which resulted in a detection limit of $10^{-12} \times 2$ M. Also, the fabrication of an electrochemical DNA biosensor with a detection limit of 0.65 picomolar for the detection of hepatitis A virus cDNA using a screen-printed gold electrode was reported by Manzano et al., (13). In a study, Shawky et al., (14) reported an achievement for developing an electrochemical genosensor with a DNA probe to detect hepatitis C virus (HCV) with a detection limit of 4.57 IU/µl. In another study, Mohammadi et al. (15) reported the fabrication of DNA-based electrochemical biosensor for the detection of influenza type A (H1N1) using a platinum electrode modified by cobalt oxide with a detection limit of 0.28 ng/µl. A DNA-based electrochemical biosensor for the detection of Ebola virus (EBV) was introduced (16). They reported a detection limit of 4.7 nM by immobilizing the thiolar probe on the screen-printed gold electrode. Moattari et al. (2) reported the DNA-based biosensor for the detection of Viral hemorrhagic septicemia virus (VHSV) with a detection limit of 125 pM.

Biosensor platforms are promising candidates, as they can be developed and integrated with inexpensive materials and substrates to be applied for point-of-care testing (POCT) aims (17).

Due to the importance of the pathogenesis of the recent coronavirus, reliable detection of this virus as a quick, accurate, and timely method is crucial to prevent its spread; therefore, it is very important to design a diagnostic biosensor with high sensitivity and accuracy to identify this coronavirus. In this study, a specific probe was designed in such a way that the selected probe is connected to the virus genome and to multiple copies of mRNA produced from virus replication, this will increase the sensitivity of the biosensor. Pencil graphite electrode (PGE) as a transducer was selected because of its low price, availability, renewable, and suitable mechanical surface. PGE was used for supporting the oligonucleotide probes and transducer for the hybridization.

This research aimed to design and fabricate an electrochemical biosensor based on 3D reduced graphene oxide (RGO) and gold nanoparticles (GNPs) composite immobilized on disposable PGE for SARS-Cov-2 detection with high sensitivity.

Materials and Methods

Graphite powder was obtained from CDH India and other materials such as $HAuCl_4$. $3H_2O$ (Sigma), $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, DMF, and DTT (Merck) and the solutions used in the study were prepared with double

distilled water (DDW).

All electrochemical tests were performed using a potentiostat/galvanostat electrochemical device with impedance analysis (Ivium Co. Vertex 1 model, Netherlands) which is connected to a three-electrode system on one side and a computer on the other side. A three-electrode system including a working electrode (Pentel graphite pencil lead with a diameter of 0.7 mm), a silver/silver-chloride electrode (in a 0.3 M potassium-chloride solution) was used as a reference electrode, and platinum as an auxiliary electrode. They were placed in a cell containing 5 mM Fe $(CN)_6^{-4/3-}$ containing 0.10 M potassium chloride as electrolyte. Cyclic voltammetry was performed in a potential range of 0.60 to -0.20 V with a scan rate of 50 mV/s and impedance measurements in a frequency range within 100 kHz-10 mHz and at a potential of 150 m Volt. All potentials are mentioned relative to the silver/silver chloride reference electrode.

The voltammograms were recorded using Ivium 2.231 software and the equivalent circuit was then obtained using the software and charge transfer resistance (Rct) was calculated accordingly. Microsoft Excel 2013 and Origin 2019 software were also used to draw the graphs.

1. Specific ssDNA (probe) design

To design a suitable probe for the detection of the SARS-CoV-2, the unique part of the genome of this virus and even in the SARS-CoV was used, which is the closest virus to it in terms of genomic similarity is also not there. This is the QTNSPRA peptide sequence, which is a part of the highly expressed S1/S2 cleavage region of Spike virus. For this purpose, the inverse and complementary nucleotide sequence (5'-CAGACTCAGACTAATTCTCCTCGGCGGGCA-3') as a probe was used. Therefore, the probe is selected and designed in such a way that it can be directly connected to the virus genome as well as to multiple copies of mRNA produced from virus replication, which will greatly increase the sensitivity of the biosensor.

2. ssDNA sequences

Thiolar single-stranded DNA probe: 5'-SHTGCCCGCCGAGGAGAATTAGTCTGAGTCTG-3'

Complementary strand: 5'-CAGACTCAGACTAATTCTCCTCGGCGGGCA-3', which is similar to the desired sequence of the virus. To evaluate the sensitivity of the designed biosensor, the mismatch sequences with one, two, and three wrong nucleotides were evaluated.

mismatch (1 nucleotide): 5'-CAGACTCATACTAATTCTCCTCGGCGGGCA -3'

mismatch (2 nucleotides): 5'-CAGACTCAGACTAATTCTCCGCGGCGGGCA -3'

mismatch (3 nucleotides): 5'-CAGACTCAGACTAATTCTCCTCGGCGAGCA -3'

Sequence stocks were then prepared with sterile distilled water at a concentration of 100 µM and stored at -20ºC. To break the disulfide bonds created between the thiol groups of Probe, 1M solution of dithiothritol (DTT) was used in a ratio of 5:1 (dithiothritol: ssDNA). After keeping for 15 minutes at room temperature to remove DTT, ethyl acetate was added to the vial at a ratio of 1:1 volume/volume, vortexes for 30 seconds, then the upper phase was discarded. The final step was repeated three times.

3. RGO synthesis

The synthesis of GO was performed according to the modified Hummer method using graphite (18). The graphene reduction step was also done using a sodium borohydride solution. Before use, RGO was dissolved in dimethylformamide (DMF) and placed in a water bath sonicator for homogenization.

4. Fabrication of Geno sensor

One of the important steps in biosensor design is proper modification of the working electrode surface, which is done by stabilizing different layers. Also, to immobilize ssDNA through self-assembly, thiol modification at the 5' end of the oligonucleotide sequence was used to immobilize virus-specific ssDNA. Activating and cleaning the graphite electrode surface was done by voltammetry methods. The graphite electrode was then placed in an electrochemical cell containing 0.10M acetate buffer solution and 0.020M sodium chloride with pH=4.8 and the solution was stirred at a speed of 200 rpm. A potential of +1.4 V was applied to the silver/silver-chloride reference electrode for 300 seconds, and the surface of the electrode was then washed with deionized water. The high surface-to-volume ratio in nanoparticles leads to more immobilization of ssDNA on the surface of the PGE, so that the measurement of the target occurs in a larger concentration range and leads to late saturation of the surface with the target. RGO and GNPs were used for modification of the working electrode. Modification of PGE with RGO and GNPs composite was carried out as follows, the activated PGE was placed in the RGO solution for 90 minutes to immobilize the RGO on the surface of the PGE. Then to remove excess particles from the surface, the electrode was placed in 0.1 M phosphate buffer solution and the potential range -0.3 up to 0.7 was applied to the solution with a scan rate of 50 mV.

In the case where GNPs are used, the thiolar ssDNA is self-assembled (vertical mode) on the surface of the electrode, which increases the sensitivity of the biosensor. GNP immobilization was carried out with the electrodepositing method (19). To electrochemically deposit GNPs on the surface of the electrode, the modified electrode with RGO was placed in a 3 mM HAuCl4. 3H2O and 0.10 M potassium nitrate and the potential was applied in the range of -0.5 to 1.5 with a scan rate of 5 mV/s to form a layer. In the next step, to regenerate the excess gold ions trapped on the surface of the electrode, the electrode was placed in a 0.1 M potassium nitrate solution and the potential range of 0.5 to 1.5 V with a scan rate of 50 mV was applied by cyclic voltammetry.

Results and Discussion

1. RGO characterization

The structure of synthesized G, GO, and RGO was investigated using FTIR (Figure 1). The peaks in 3420, 1724, 1570, and 1035 cm^{-1} are respectively related to O-H groups, C=O bands (indicating oxidation, the intensity of this peak decreased in the RGO), C=C and hydroxyl groups.

2. Characterization of fabricated genosensor

CV and Electrochemical Impedance Spectroscopy (EIS) techniques were used to investigate the various stages of geneosensor preparation. In Figure 2A, the peak related to the RGO/GNPs composite has increased in comparison to bare PGE due to the increase in electron transfer. After immobilization of thiolar Single-Stranded DNA (ssDNA) on the RGO/GNPs composite, the electron transfer decreased due to the presence of oligonucleotides with a negative charge. After the hybridization of target DNA by ssDNA, the electron transfer was further reduced compared to the ssDNA due to the electrostatic repulsion between the negative charges of DNA and $Fe(CN)6^{-4/3-}$ (20). The Nyquist plot resulting from EIS is shown in Figure 2B. After immobilization of the RGO/GNPs composite on the PGE surface, the Rct decreased. After ssDNA stabilization, the Rct increased due to the presence of the ssDNA with a negative charge on the electrode surface and inhibition of electron transfer. Also, after the hybridization of target DNA and ssDNA, an increase is observed in the Rct.

3. pH optimization

Since the first important parameter for the immobilization and fabrication of the biosensor is the pH of the solution, no immobilization of the designed oligonucleotide on the electrode surface was observed in acidic and alkaline solutions. Therefore, the steps related to the immobilization of oligonucleotides were investigated by the EIS technique, and the pH range where the highest Rct was observed was selected as the optimal pH (Figure 3). Therefore, according to the results obtained in all stages of the work, ssDNA modified with thiol group was dissolved in the pH range of 7 and immobilized on the electrode surface.

Mansor et al. (20) showed that the response of the sensor was maximum at pH=7 and therefore they

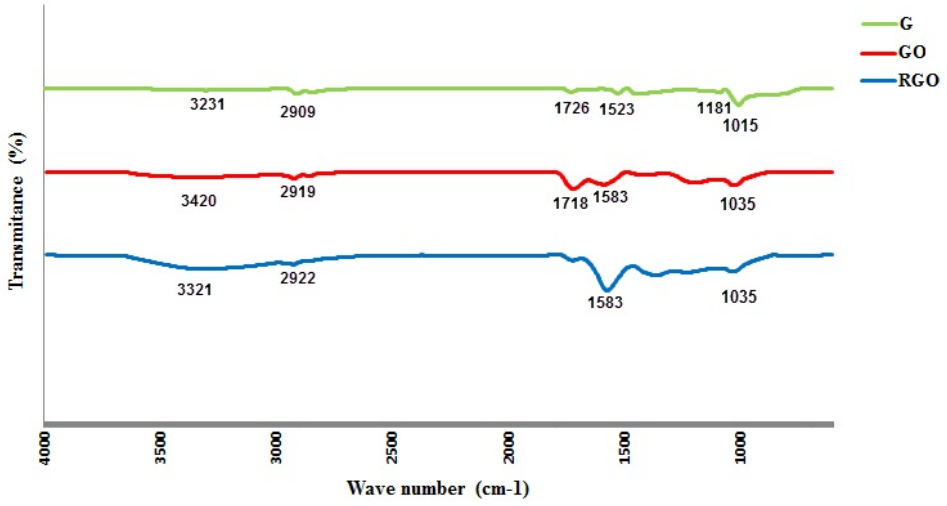

Figure 1. FTIR spectra of G, GO, and RGO

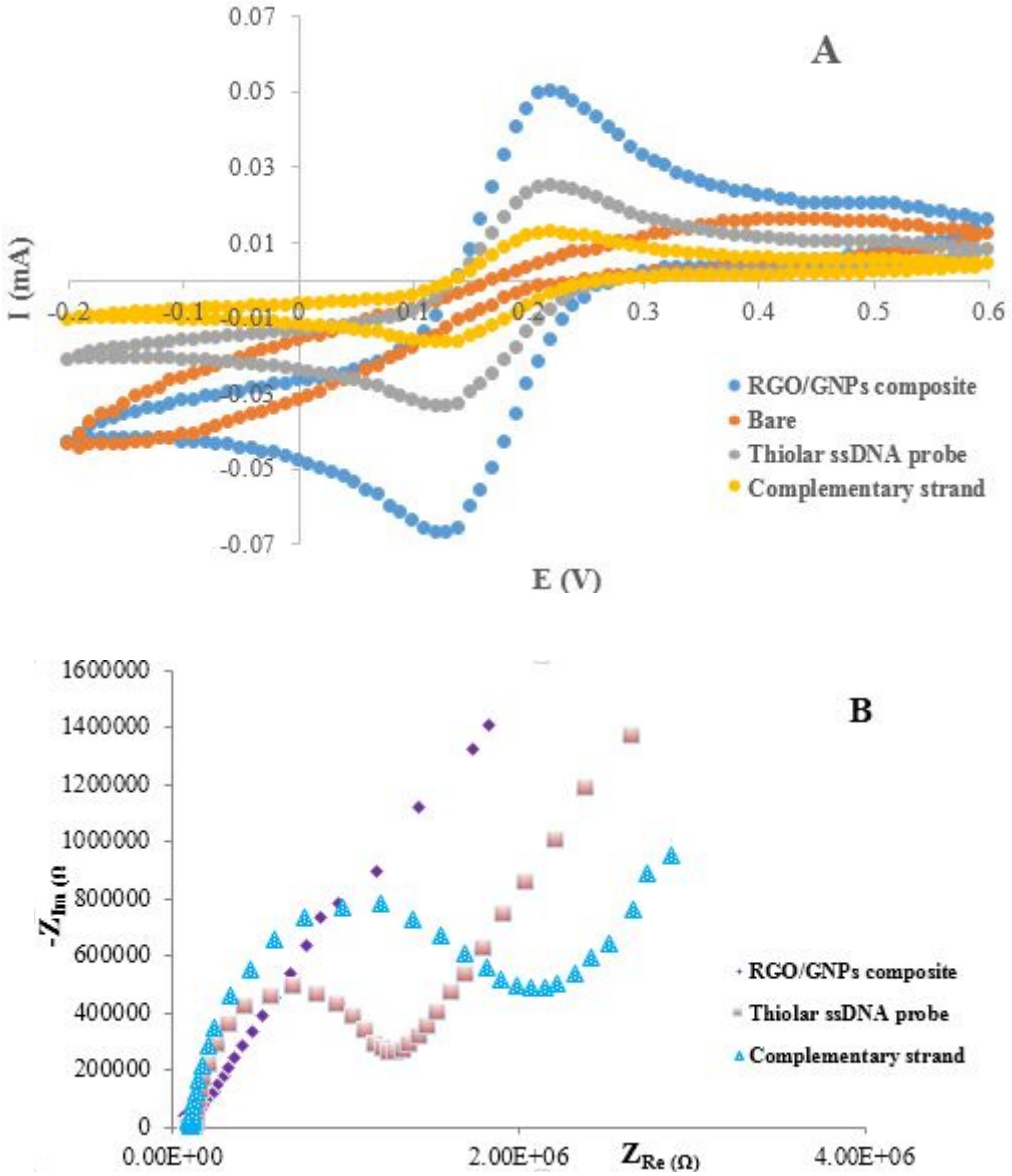

Figure 2. The voltamogram (A) and Nyquist plots (B) for different modifications of electrode surface in 5 mM $Fe(CN)_6^{4-/3-}$ solution with a ratio of 1:1 and 0.1 M sodium chloride electrolyte with a scan rate of 50 mV/S and potential range of – 0.2 to 0.6 V. EIS Conditions: potential of 150 mV and frequency range of 100 KHz-10 mHz.

selected the optimal pH of 7 for DNA detection. The increase in DNA-based biosensor response at pH=7 is related to the effective hybridization at this pH. In acidic conditions, due to the protonation reaction of phosphodiester in DNA, the solubility of DNA molecules is reduced, which leads to the reduction of DNA hybridization.

4. Optimization of specific ssDNA immobilization time

To optimize the specific ssDNA immobilization time on the electrode surface, the PGE modified with RGO and RGO/GNPs was placed in the ssDNA solution for different times of 10, 20, 40, 60, 80 and 100 minutes, and the EIS technique was then performed in a 5 mM $Fe(CN)6^{-4/3-}$ containing 0.10 M potassium chloride as electrolyte.

The Nyquist curve obtained from the EIS technique and fitted with the equivalent circuit (Randless) and Rct was achieved. An example of the equivalent circuit used is shown in Figure 4.

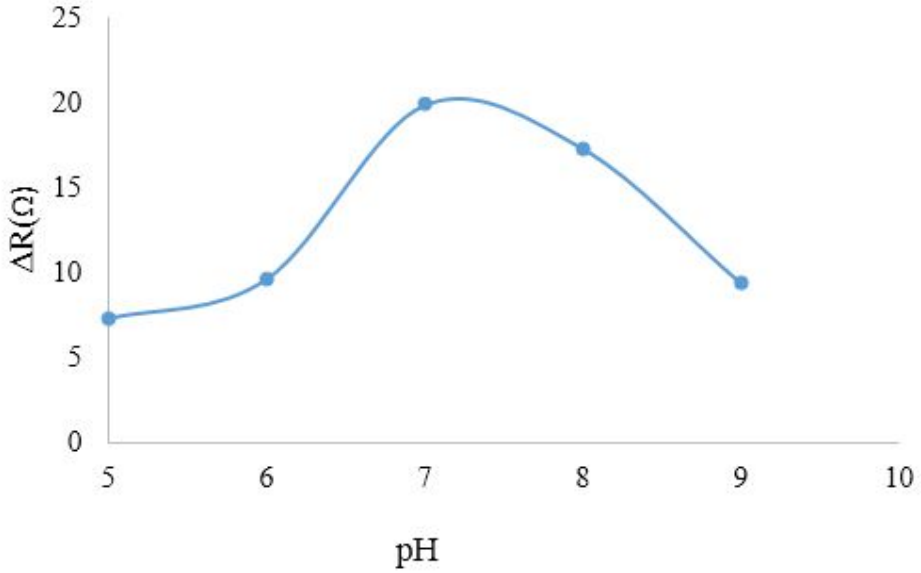

Figure 3. Effect of different pH to immobilize ssDNA using Electrochemical impedance technique (EIS) in $Fe(CN)_6^{4-/3-}$ solution

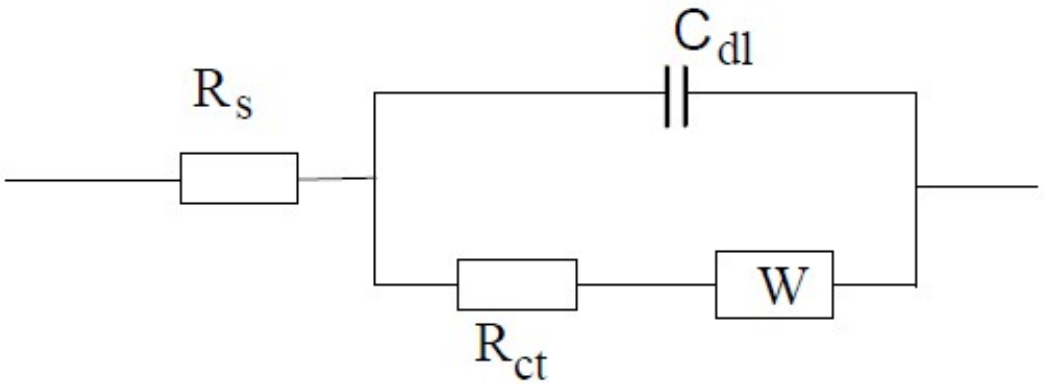

Figure 4. Randless equivalent circuit (system under the control of kinetics of charge transfer and mass transfer).

The results of the Rct data revealed that the Rct value increases from 10 to 40 minutes, and after 40 minutes, the Rct value did not show a significant increase. The reason for this observation can be interpreted that probably after 40 minutes, the active sites on the electrode surface were completely connected with ssDNA. Therefore, 40 minutes was chosen as the optimal time for immobilizing specific ssDNA on the surface of RGO/GNP-modified PGE.

5. Optimization of hybridization time with complementary strand

As shown in Figure 5, the Rct increased with the immobilization of specific ssDNA, which is due to the accumulation of negative charge on the electrode surface and the creation of electrostatic repulsion between the electrode surface and $Fe(CN)_6^{-4/-3-}$ electrolyte. When the hybridization was done with the complementary strand, the Rct showed a greater increase, which is due to the accumulation of more negative charge due to the placement of the complementary strand, and as a result, the Rct showed a greater increase compared with when the specific ssDNA is immobilized on the electrode surface. Similar results were reported in the study of Izuan et al. (21). To choose the optimal time for hybridization, the designed diagnostic biosensor was placed near the complementary strand at different times (10, 15, 20, 25, and 30 minutes). As shown in Figure 3, with the increase of time from 10 to 20 minutes, the amount of Rct increases, but from 20 minutes to 30 minutes, the amount of Rct is constant, so the time of 20 minutes was chosen as the optimal hybridization time, therefore the response time of the biosensor (the time taken to determine the test result) is 20 minutes.

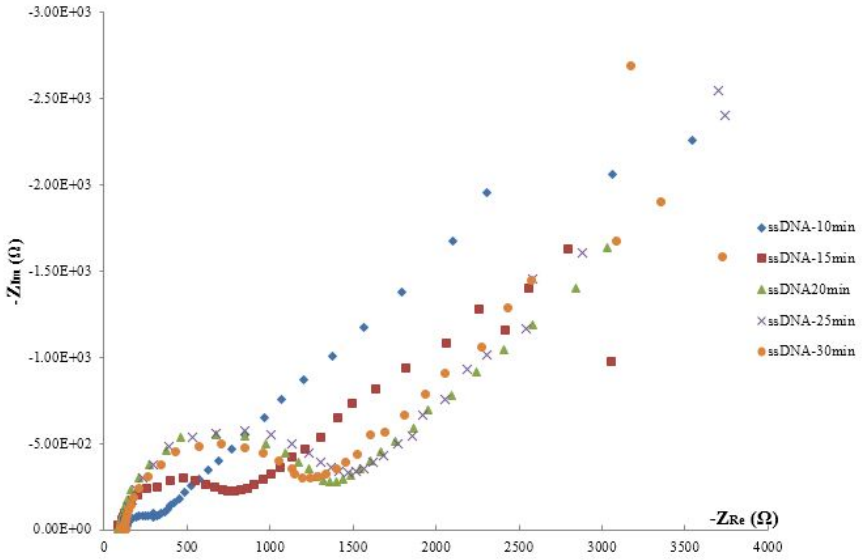

Figure 5. Nyquist curve related to different times (10, 15, 20, 25, and 30 minutes) for hybridization of ssDNA with complementary strand. Hybridization conditions: 2×10^{-6} M concentration of complementary strand at 45 °C and pH = 7

6. Calibration curve, linear range, and limit of detection (LOD)

After optimizing the parameters affecting the sensitivity of the biosensor, using the EIS technique, the Rct data (in 5 mM $Fe(CN)_6^{-4/3-}$) in the presence of different concentrations of target DNA (in optimal conditions) was illustrated. For each concentration, Rct was measured three times. The linear range was found to be within 10^{-12} - 10^{-6} M and the LOD was at 3×10^{-13} M (Figure 6).

7. Biosensor recovery

To recover the fabricated biosensor after exposure to the target DNA, it was placed in 0.1 M sodium solution for 4 minutes to break the hydrogen bonds between the

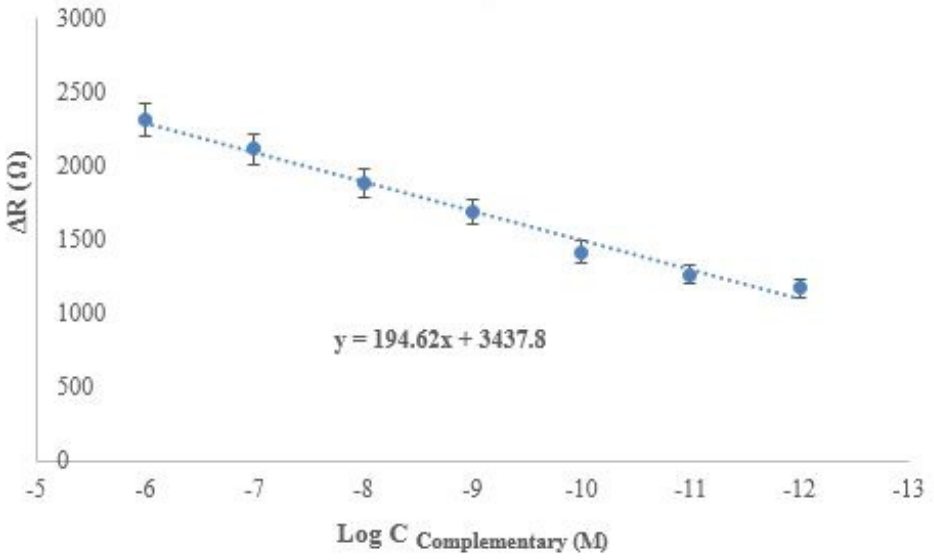

Figure 6. Calibration curve based on the resistance difference measured in 5 M $Fe(CN)_6^{4-/3-}$ solution containing 0.1 M

Table 1. Recovery of biosensor surface in 0.1 M sodium solution

Recovery process	Rct (Ω)
1	1215
2	1229
3	1198
4	1203
5	1196
6	1184
7	1064
8	913

probe and the target, and its charge transfer resistance was then measured. The results showed that the fabricated biosensor can be recovered and reused up to 6 times (Table 1).

Relatively similar times for electrode recovery have been reported in other studies. For instance, Zhang et al. (2008) recovered a DNA-based sensor by placing the electrode in a 0.5 M sodium solution for 2 min. Chun et al. (22) reported that alkaline solutions affect the reaction between the aptamer and the target molecule and cause instability of the immobilized aptamer. Unlike enzymes and antibodies, diagnostic layers of nucleic acids can be easily synthesized and can be recovered for reuse (23-25).

8. Specificity of the biosensor

The degree of specificity of the biosensor against other viruses including viral hemorrhagic septicemia (VHS), influenza A virus subtype H1N1 and *infectious bronchitis virus* (IBV) viruses was accomplished. In Figure 7, a significant difference between the COVID-19 virus and other viruses is illustrated.

Our biosensor is more sensitive in comparison with the biosensor of Balvedi et al. (10) for detection of EBV by using a graphite electrode modified with 4-aminothiophenol polymer,the biosensor of Ilkhani and Farhad (16) for the detection of Ebola virus through a thiolar probe on the screen printed gold electrode, and the biosensor of Moattari et al. (2) for recognition of VHSV a graphite electrode and RGO/GNPs. However, the biosensor of Dong et al. (11) for detection of Influenza A virus and the biosensor of Manzano et al., (13) for the detection of Hepatitis A virus by application of gold electrode, and that of Shakoori et al., (12) for recognition of Hepatitis B virus using gold nanorods (Table 2).

Conclusion

To identify the coronavirus in a fast and accurate approach, the inexpensive surface of the PGE was used, which showed good compatibility with ssDNA. The modification of the working electrode surface was done using RGO and RGO/GNPs and the electrodeposition method, which reduces the cost of biosensor fabrication. Due to the use of RGO/GNPs to modify the surface, it was possible to immobilize ssDNA by self-assembly method, which also showed a great effect on increasing the sensitivity of the biosensor. The results proved that ssDNA does not bind to RGO/GNPs without

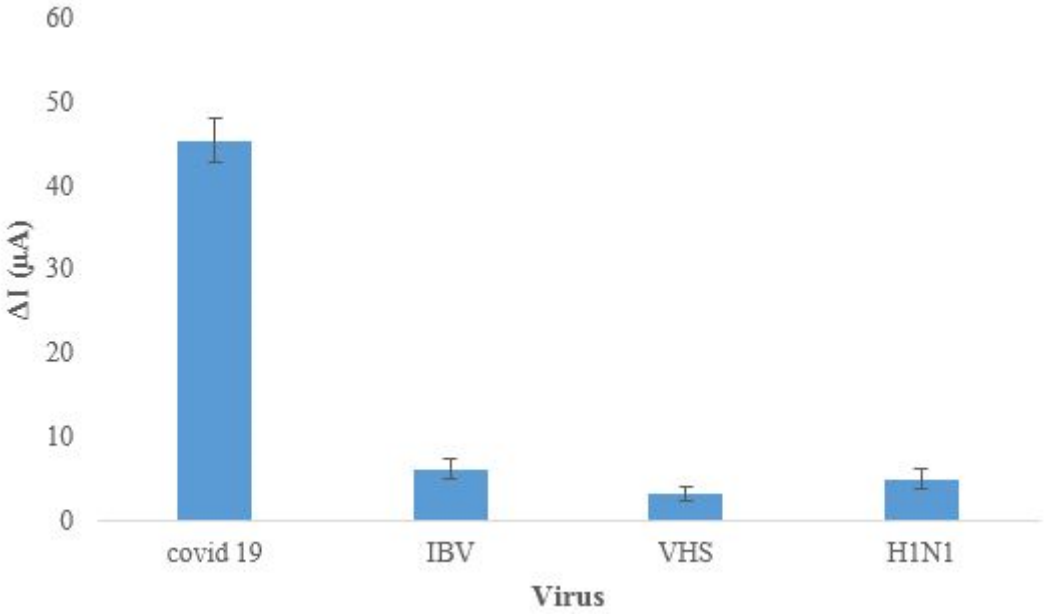

Figure 7. Specificity of the designed biosensor with IBV, VHS, and H1N1 viruses

Table 2. Comparison of the proposed biosensor with other DNA-based biosensors for virus detection

Type of Virus	limit of detection (LOD)	Electrode	Reference
Human papilloma virus	1.2 ng/µl	pencil graphite (lead) electrode (PGE)	Sabzi et al., (8)
Human papillomavirus	16 pg/µl.	PGE	Campos-Ferreira et al. (9)
Epstein-Barr virus (EBV)	17.32 $nmol.L^{-1}$	graphite electrode modified with 4-aminothiophenol polymer	Balvedi et al. (10)
Hepatitis B virus	2×10^{-12} M	gold nanorods	Shakoori et al., (12)
Hepatitis A virus	0.65 picomolar	gold electrode	Manzano et al., (13)
Influenza type A	0.28 ng/µl	platinum electrode modified by cobalt oxide	Mohammadi et al. (15)
Ebola virus	4.7 nM	thiolar probe on the screen printed gold electrode	Ilkhani and Farhad, (16)
Viral hemorrhagic septicemia virus	125 pM	graphite and gold nanoparticle	Moattari et al. (2)
SARS-CoV-2	13×10^{-9} M	PGE	Our study

modification of the thiol functional group, and by using this type of modification of the electrode surface, the effect of interaction and non-specific connections with other sequences is eliminated in the test conditions due to the binding of DNA to gold through thiol is very strong. In non-specific binding, DNA is attached to the gold surface through its bases, while in specific binding there is only one binding point through the thiol group. The designed probe increases the sensitivity of the biosensor. The short response time of the biosensor and low detection limit, high sensitivity, user friendly and suitable recovery are the advantages of the designed diagnostic biosensor as well as no necessary need for cDNA synthesis by using the reverse transcription reaction. Therefore, fabricated biosensors can be used for COVID-19 detection.

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