

Ultrasensitive Electrochemical Sensor Based on Vertical Graphene for SARS-CoV-2 Protein N Detection

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Abstract. Monitoring and controlling infection is required in order to prevent the progression of the coronavirus severe acute respiratory syndrome 2 (SARS-CoV-2). To accomplish this goal, the development and implementation of sensitive, quick and accurate diagnostic methods are essential. Electrochemical sensors have exposed large application possibilities in biological detection due to the advantages of high sensitivity, short time-consuming and specificity. Here, we report the improvement of a sensitive electrochemical sensor capable of detecting the presence of the SARS-CoV-2 virus using graphene-modified interdigitated working electrodes functionalized with antibodies targeting the SARS-CoV-2 *nucleocapsid protein* (N protein).

Key-words: EIS detection; electrochemical biosensor; graphene; SARS-CoV-2.

1. Introduction

The global epidemic of coronavirus disease in 2019 (COVID-19) was caused by the rapid spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1]. SARS-CoV-2

is a coronavirus with a spherical shape and diameter of around 130 nm [2, 3] with 'spike-like structures' all over its surface. Rapid diagnostic methods for viral detection are of paramount importance for fast initial diagnosis, especially during the pandemic period.

Biosensors are analytical devices that convert a biochemical/biological reaction into a measurable physico-chemical signal that is directly proportional to the analyte concentration. The typical structure of a biosensor consists of two elements: i) a contact surface to which the biological component selectively interacting with the analyte of interest is bound; and ii) a transducer for detecting the analyte binding event at the contact surface. The advantages of using biosensors compared to other conventional biochemical assays such as immunoassays and *polymerase chain reaction* (PCR) based strategies are fast response time (normally a few minutes) and high specificity.

One of the challenges in biosensor development is the efficient capture of the biological recognition bioreceptor. Nanomaterials such as magnetic nanoparticles, metal nanoparticles and carbonaceous nanomaterials have been intensively investigated as attractive candidates to enhance the amount of bioreceptor immobilization and hence signal enhancement at very low concentrations of biological analytes, *low limit of detection* (LOD). Among the studied nanomaterials, graphene and its derivatives such as *graphene oxide* (GO), *reduced graphene oxide* (rGO), CVD graphene, vertical graphene, and so on are being studied worldwide for the development of biosensors (electrochemical and field-effect transistors) [5].

Because COVID-19 is a major public health concern, its control through early discovery is essential. Epidemic prevention can be done using simple, easy to use, fast, sensitive, specific, mass produced and budget technologies. SARS-CoV-2 is a single-stranded positive-stranded RNA virus, 80–220 nm in diameter and has 12–24 nm vesicular rod like spikes. The outer coating of the virus is composed of four structural proteins, the S protein, the E protein, the M protein and the N protein, having different roles [6].

Electrochemical, point-of-care biosensors can be used for rapid detection of SARS-CoV-2 viral genetic material (RNA), viral antigens and antibodies. Electrochemical DNA biosensors have single-strand DNA or complementary target DNA fixed on the electrode. Alafeef *et al.* developed a paper-based electrochemical biosensor enhanced with graphene and gold nanoparticles (AuNPs) capped with highly specific antisense oligonucleotides that can recognize the N gene. It has a limit of detection of 6.9 copies/ μL with almost 100% accuracy, sensitivity, and specificity [7]. Heo *et al.* developed an electrochemical biosensor for nucleic acid amplification-free RNA detection using the CRISPR/Cas13a trans-cleavage reaction. Their biosensor has a limit of detection of 4.4×10^{-2} fg/mL and 8.1×10^{-2} fg/mL of ORF and S genes, respectively [8]. Ke *et al.* developed a graphene FET-based electrochemical sensor with a low LOD of ~ 0.1 and ~ 1 fg/mL for RNA and antibodies, respectively [9]. Beduk *et al.*, developed a *laser-scribed graphene* (LSG)-based disposable electrochemical biosensor with 3D Au nanostructures. The sensor has a *limit of detection* (LOD) of 2.9 ng/mL [10]. Eissa *et al.* developed an electrochemical biosensor integrated into a cotton tip that served as a detector and a sample collector at the same time for N protein detection. They used screen-printed electrodes modified with carbon nanofibers. They achieved a LOD of 0.8 pg/mL [11]. Shan *et al.* developed a nanomaterial-based sensor array for COVID-19 detection from exhaled breath. The sensors are composed of different gold nanoparticles that can swell or shrink when exposed to *volatile organic compounds* (VOCs), which causes changes in the electric resistance [13]. Lasserre *et al.* reported an antigen-based electrochemical aptamer sensor based on EIS and thin-film gold electrodes for S1 protein detection. They achieved a LOD of 80 ng mL $^{-1}$ [14]. Mahari *et al.* developed a *printed circuit*

board (PCB)-based electrochemical device that can detect S protein in spiked saliva samples in 30 s with an LOD of 90 fM [15].

Based on the data provided by these studies, in this research, we chose to test the functionality of the developed biosensors developed with a lower antigen concentration, 1 aM.

In the following section, the microfabrication processes of the electrochemical sensor are detailed, alongside the functionalization and characterization of the sensor. In the results section, the sensor is tested for three different concentrations, and the results are analyzed in terms of cyclic voltammetry and Nyquist diagram. The papers end with concluding remarks.

2. Microfabrication of the Electrochemical Sensor

2.1. Electrochemical sensor fabrication

The basic process of interdigitated electrodes microfabrication based on vertical graphene is photolithography: the optical transfer of 3 photolithographic masks on a substrate covered with photoresist. The following steps are used to fabricate the electrochemical sensor and configure the electrodes, and an overview of the process is presented in Fig.1:

- Substrate preparation (cleansing of the Si type p wafer in piranha solution);
- SiO₂ growth – 300 nm by thermal oxidation at 1000°C;
- Photolithography of the first mask – for the configuration of the electrodes (2 working electrodes, counter electrode and reference electrode, electrical circuit, pin-out followed by lift off);
- TiN deposition – 50 nm through RF-Sputtering;
- Vertical graphene growth through PECVD (Nanofab 1000, Oxford Instruments, UK);
- Photolithography of the second mask – configuration of working electrodes, vertical graphene etching in RIE (Reactive Ion Etching, Etchlab 220, Sentech Instruments, Germany);
- Photolithography of the third mask – Al₂O₃ deposition through ALD, reference electrode metallization with Ag and lift off;
- Routing of the individual sensors according to the geometry;
- Chlorination of the reference Ag electrode.

The technological flow for the fabrication process of the electrochemical sensor with modified interdigitated electrodes with *vertical graphene* (VG) is shown in Fig. 1. The design of the COVID-19 electrochemical sensor was planned to use vertical graphene material-based working electrodes functionalized with the anti-COVID-19 N protein antibody.

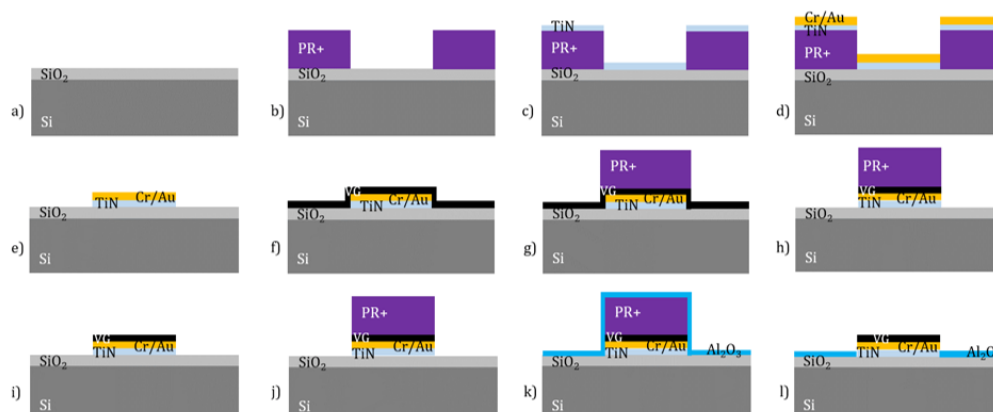


Fig. 1. a) 300 nm SiO₂; b) configuration of metallic layers; c) 50 nm TiN; d) 150 nm Au; e) lift-off process f) VG growth with PECVD method; g) photoresist; h) VG etching in RIE; i) photoresist removal; j) photoresist; k) 15 nm Al₂O₃; l) lift off process (Reprinted with permission from © IEEE [12]).

2.2. Specific functionalization for SARS-CoV-2 detection

The functionalization of the electrochemical sensor with vertical graphene modified interdigitated gold electrodes was carried out with the purpose of increasing the sensitivity of the sensor. These carbon-modified sensors have been used in the development of devices for the detection of SARS-CoV-2 viral nucleocapsid protein (N-protein or SARS-CoV-2 nucleoprotein).

We chose to decorate the carbonaceous materials in the sensor structure with gold nanoparticles to exploit several advantages, such as increasing the surface-to-volume ratio to improve detection limits and for the immobilization of antibodies specific to the SARS-CoV-2 nucleocapsid anti-protein (SARS-CoV-2 anti-N/anti-nucleoprotein/anti-SARS-CoV-2 nucleocapsid antibodies).

Gold nanoparticles (AuNPs) were obtained by reduction of chloroauric acid (HAuCl₄ · 4H₂O, Sigma Aldrich, USA) with sodium citrate (C₆H₅O₇Na₃ · 2H₂O, Sigma-Aldrich, USA). The principle of nanoparticle chemical synthesized is to reduce Au (III) to Au (0). Chloroauric acid solution (6×10^{-4} M) was used as a metal precursor, and sodium citrate solution (1%) was used as the reducing agent.

The surface characteristics and size of the synthesized AuNPs were evaluated using *scanning electron microscopy* (SEM) with Nova NanoSEM 630 field emission scanning electron microscope (FE-SEM) equipment (FEI Company, USA) equipped with EDX detector (EDAX TEAM™, USA).

Immobilization of AuNPs on the surface of the VG electrodes was performed by silanizing the materials with aqueous solution of (3-Aminopropyl) triethoxysilane (APTES) (10%), for 30 min. Subsequently, the sensors were thoroughly washed with deionized water. Then, after drying at room temperature, the sensors were kept in the oven for 10 minutes at 120°C to prevent elution of APTES.

After decorating the VG interdigitated electrodes with AuNPs, the anti-SARS-CoV-2 nucleocapsid antibodies were immobilized on the AuNPs using cysteamine (10mM) and glutaraldehyde solution (2.5%) as crosslinking agents, accordingly to the method adapted from [4] (Fig. 2 I)).

2.3. Characterization of the electrochemical sensor

Morpho-structural characterization of carbon nanomaterials is an essential step for quality and stability control, important characteristics for integration into applications.

Morphological characterization involves the use of *scanning electron microscopy* (SEM) and *atomic force microscopy* (AFM) to analyze the material in terms of the shape of the layers, the size of the graphene domains and their functionalization.

Field Emission Gun Scanning Electron Microscope (FEG-SEM) -FEI Nova NanoSEM 630 (FEI) was used for SEM investigations. *Atomic force microscopy* (AFM) analysis was performed using the Witec alpha 300S GmbH Germany system in contact mode with a Si₃N₄ AFM cantilever (125 μ m length, 40 N/m force constant and 300 kHz frequency). Surface parameters are calculated using Witec Project FIVE 5.0 software.

Structural characterization is performed using Raman spectroscopy which allows to obtain information on the shape and type of chemical bonds in the developed materials.

Raman spectra were acquired using a Witec Raman Spectrometer (Alpha-SNOM 300 S, WiTec. GmbH, Germany) using a 532 nm diode pumped solid state laser with a maximum power of 145 mW. The incident laser beam with a spot size of approximately 1.0 with two objectives attached (50x and 100x) to a Thorlabs MY100X-806 microscope.

In Fig. 2 II, The VG-modified interdigitated electrodes are characterized using scanning electron microscopy. The first image shows VG-coated interdigitates, the second image represents a VG-coated interdigitate, and in the third image the VG modified interdigitated electrodes are decorated with AuNPs. The characteristic VG morphology is observed, with vertically increased walls.

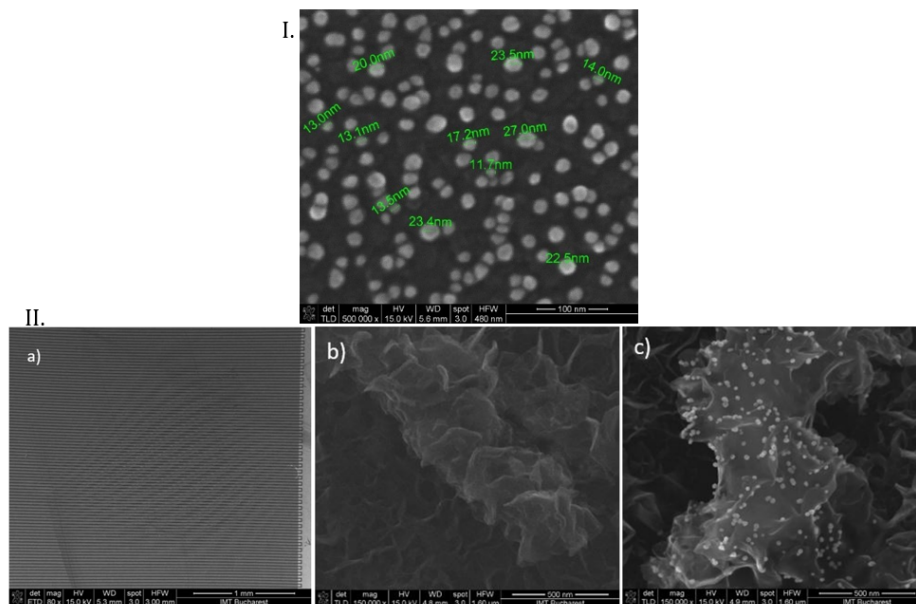


Fig. 2. I. SEM Image of the AuNPs.; II. SEM images for a) interdigitated electrodes modified with VG, b) VG and c) AuNPs/VG (Reprinted with permission from © IEEE [12]).

After increasing the VG on the gold electrodes of the electrochemical sensor, the functionalization followed (according to the functionalization section).

For VG topography and structural properties, we used AFM characterization (Fig. 3). AFM identifies the attachment of AuNPs on the tops/edges of the VG sheets by the roughness parameters highlighted in Table 1.

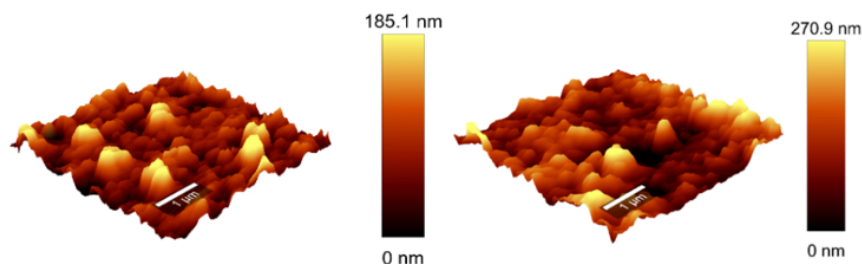


Fig. 3. 3D AFM image- A) control VG and B) VG decorated with AuNPs. Contact AFM on $5 \times 5 \mu\text{m}$ area.

Table 1. Roughness parameters: VG- before and after decoration with AuNPs

Probe	SDR (%) Surface area ratio	SDQ (nm) Root mean square gradient	Sa (nm) Arithmetic average	Sq (nm) Root mean square average
VG – control	11.811	0.533488	27.8198	37.3939
VG – AuNPs	16.652	0.636141	46.7088	58.3215

The spectrum of the AuNPs-decorated VG sample (Fig. 4) shows the characteristic graphene bands: G band at 1570 cm^{-1} , D band at 1336 cm^{-1} , 2D band at 2670 cm^{-1} , D+G band at 2928 cm^{-1} and 2D' at 3206 cm^{-1} . The $I_{\text{D}}/I_{\text{G}}$ ratio is 1.3 and $I_{2\text{D}}/I_{\text{G}}$ is 1.02.

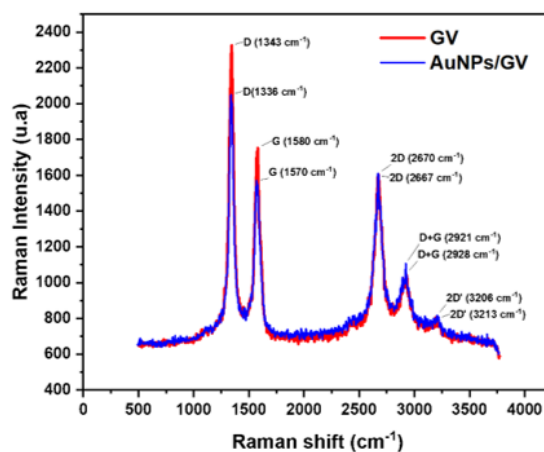


Fig. 4. Raman spectra acquired on electrochemical sensor with vertical graphene (VG) modified gold electrodes before and after AuNPs decoration.

3. Results and Discussions

To demonstrate the functionality of the developed electrochemical sensor, VG electrodes decorated with AuNPs conjugated with specific anti-protein N antibodies (anti-protein nucleocapsid of SARS-CoV-2 virus) were incubated at room temperature for one hour with 10 μ L protein N solution (SARS-CoV-2 - recombinant nucleoprotein) (HyTest, Finland), at different concentrations: 1nM, 1fM and 1 aM. After incubation, the electrochemical sensors were washed with PBS (pH 7.4) and then electrochemical measurements were performed: *Cyclic Voltammetry* (CV) and Electrochemical Impedance Spectroscopy (Nyquist diagram) using a PGSTAT204 potentiostat/galvanostat with FRA32M: *Electrochemical Impedance Spectroscopy* (EIS) module.

Fig. 5 shows the cyclic voltammogram for the vertical-graphene-enhanced electrochemical sensor compared to the SARS-CoV-2 anti-protein N antibody functionalized VG electrochemical sensor (according to the reported functionalization process) and the SARS-CoV-2 anti-protein N-1nM/Anti-protein N antibody/VG electrochemical sensor. Cyclic voltammetry was recorded in the presence of an electrolyte solution containing redox mediator $[\text{Fe}(\text{CN})_6]^{3-/4-}$, sensitive to functional groups present on the surface of vertical graphene-modified electrodes. The appearance of reversible oxidation-reduction peaks is observed indicating a rapid charge exchange between the electrolyte and the electrode after functionalization and Protein N attachment.

The cyclic voltammogram for the VG-witness sensor shows a potential difference of $\Delta E_p \approx 810$ mV, compared to the potential difference for the electrochemical sensor with VG functionalized with SARS-CoV-2 anti-protein N antibody where the potential difference is $\Delta E_p \approx 340$ mV, and in the case of Protein N detection, with a concentration of 1nm, we identified a potential difference of $\Delta E_p \approx 160$ mV. In addition to the potential difference, there is also a visible increase in current. This decrease in potential difference may be due to anchoring of S-H bonds. Increased sensitivity of the electrochemical sensor is observed after functionalization and detection of Protein N at the nm level.

In the Nyquist diagram (Fig. 5 b)) the imaginary impedance ($-Z''$) is plotted against the real impedance (Z'). The linear variation of the imaginary impedance versus the real impedance corresponding to low frequencies is due to Warburg diffusion. The Nyquist plots differ for the 3 stages of the sensor, the impedance spectra overlap over a very narrow range at high frequencies ($\approx 1\text{MHz}$), where electron transfer occurs, then it varies significantly in the low frequency range ($< 1\text{MHz}$).

From the semicircular curve corresponding to high frequencies, we can determine the charge transfer resistance (Rct). Compared to the control sensor: $R_{ct} \sim 1200 \Omega$, after protein N attachment, Rct drops to $\sim 175 \Omega$.

Figure 6 a) shows the cyclic voltammogram for the vertical-graphene-enhanced electrochemical sensor compared to the SARS-CoV-2 anti-protein N antibody-functionalized VG electrochemical sensor (according to the reported functionalization process) and the SARS-CoV-2 anti-protein N-1fM/Anti-protein N antibody-VG electrochemical sensor. Cyclic voltammetry was recorded in the presence of an electrolyte solution containing redox mediator $[\text{Fe}(\text{CN})_6]^{3-/4-}$, sensitive to functional groups present on the surface of vertical graphene-modified electrodes.

The cyclic voltammogram for the VG-witness sensor shows a potential difference of $\Delta E_p \approx 730$ mV, compared to the potential difference for the VG electrochemical sensor functionalized with SARS-CoV-2 anti-protein N antibody where the potential difference is $\Delta E_p \approx 710$ mV, and in the case of Protein N detection, with a concentration of 1fm, we identified a potential difference of $\Delta E_p \approx 630$ mV.

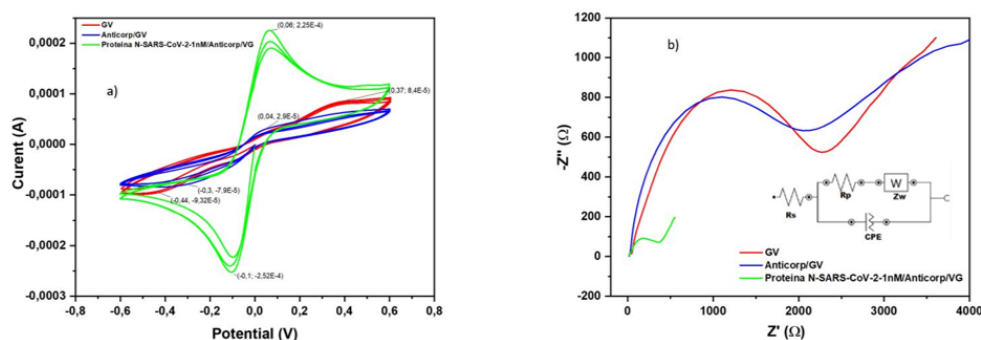


Fig. 5. a) Cyclic voltammogram and b) Nyquist plot ($-Z''$ vs Z') of 1mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1M KCl in phosphate buffer saline (PBS, pH=7.4) for VG, Antibody/VG, Protein N-SARS-CoV-2- 1 nM concentration.

From the semicircular curve corresponding to high frequencies, we can determine the charge transfer resistance (R_{ct}). Compared to the control sensor: $R_{ct} \sim 950 \Omega$, after Protein N attachment, R_{ct} decreases to about $\sim 720 \Omega$.

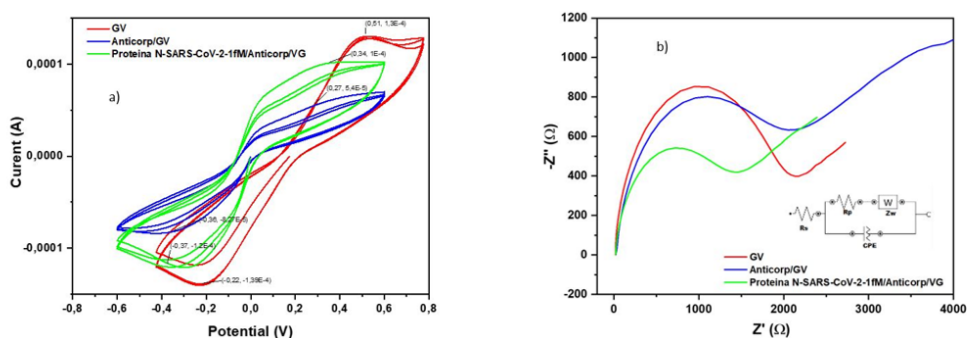


Fig. 6. a) Cyclic voltammogram and b) Nyquist plot ($-Z''$ vs Z') of 1mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1M KCl in phosphate buffer saline (PBS, pH=7.4) for VG, Antibody/VG, Protein N-SARS-CoV-2- 1 fM concentration.

Figure 7 a) shows the cyclic voltammogram for the vertical-graphene-enhanced electrochemical sensor compared to the SARS-CoV-2 anti-protein N antibody functionalized VG electrochemical sensor (according to the reported functionalization process) and the SARS-CoV-2 anti-protein N-1aM/Anti-protein N antibody/VG electrochemical sensor. Cyclic voltammetry was recorded in the presence of an electrolyte solution containing redox mediator $[\text{Fe}(\text{CN})_6]^{3-/4-}$, sensitive to functional groups present on the surface of vertical graphene-modified electrodes.

The cyclic voltammogram for the VG-witness sensor shows a potential difference of $\Delta E_p \approx 770 \text{ mV}$, compared to the potential difference for the VG electrochemical sensor functionalized with SARS-CoV-2 anti-protein N antibody where the potential difference is $\Delta E_p \approx 280 \text{ mV}$, and in the case of Protein N detection, with a concentration of 1 aM, we identified a potential difference of $\Delta E_p \approx 140 \text{ mV}$.

From the semicircular curve corresponding to high frequencies, we can determine the charge transfer resistance (R_{ct}). Compared to the control sensor: $R_{ct} \sim 1080 \Omega$, after Protein N attachment, R_{ct} increases to about $\sim 1400 \Omega$.

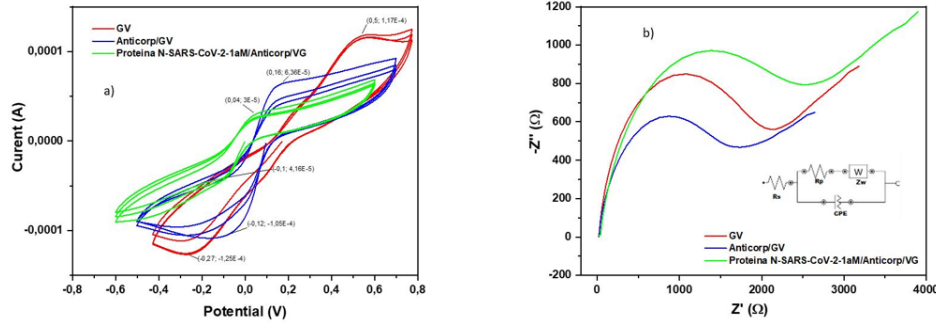


Fig. 7. a) Cyclic voltammogram and b) Nyquist plot ($-Z''$ vs Z') of $1\text{mM } [\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1M KCl in phosphate buffer saline (PBS, $\text{pH}=7.4$) for VG, Antibody/VG, Protein N-SARS-CoV-2- 1 aM concentration (Reprinted with permission from © IEEE [12]).

In all the cases, the Randels Circuit was used to fit the experimental data. The initial circuit was modified by placing in parallel the *Constant Phase Element* (CPE).

To evaluate the results obtained at different concentrations, in Fig. 8 the cyclic voltammogram and the Nyquist plots contain only the values obtained for the three different concentrations 1 nM , 1 fM and 1 aM , for the N-Protein-SARS-CoV-2-x/Antibody/VG measurements. At the 1nM concentration, in the cyclic voltammogram, Fig. 8 a), high values of currents are obtained at a low potential difference and the surface becomes more conductive after the attachment of the protein N. At low concentration the conductivity is decreasing in the same manner as the concentration of the N protein decreases, hence the results obtained in the Nyquist plot in Fig. 8 b). The highest values of the charge-transfer resistance are obtained for the 1aM concentration of N protein where the conductivity is the lowest, while the opposite is happening the case of the 1nM concentration, with a low charge-transfer resistance and high conductivity.

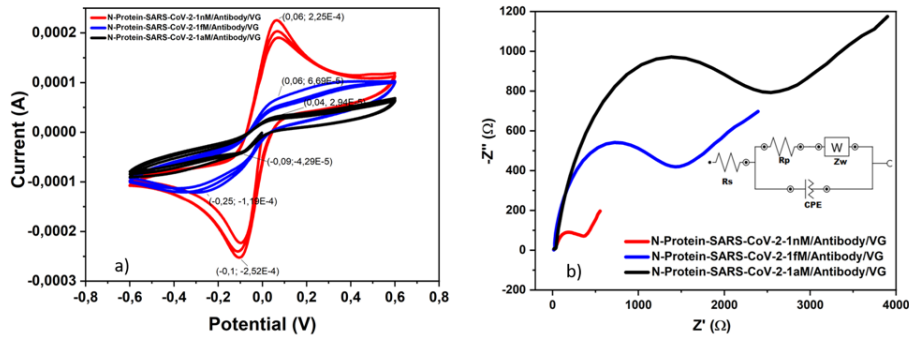


Fig. 8. a) Cyclic voltammogram and b) Nyquist plot ($-Z''$ vs Z') of $1\text{mM } [\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1M KCl in phosphate buffer saline (PBS, $\text{pH}=7.4$) for GV, Antibody/GV, Protein N-SARS-CoV-2- for 3 different concentrations: 1nM , 1fM , 1 aM (Reprinted with permission from © IEEE [12]).

4. Conclusions

In this work we have presented an electrochemical sensor modified with vertical graphene gold interdigitated electrodes capable of detecting the N protein in the SARS-CoV-2 virus at very low concentrations. The main fabrication method used was photolithography combined with multiple thin layer deposition methods and etching processes. The sensor is decorated with AuNPs to increase its sensitivity and specifically functionalized with the anti-SARS-CoV-2 nucleocapsid antibodies; in this way the only target of the sensor is the SARS-CoV-2 viral nucleocapsid protein. The advantage of targeting the N protein comes from the fact that it is highly conserved and is present in all the variants of the virus.

The Electrochemical Impedance Spectroscopy method was used for detection. Through both tests, cyclic voltammetry and EIS, the N protein was detected in very low concentrations, by comparing the VG witness sensor, with the VG sensor with antibodies and finally with the VG sensor with the N protein. The cyclic voltammogram shows that the conductivity decreases with concentration and this result was highlighted in the Nyquist plot, by having the lowest impedance at the highest concentration, because in the case of the 1nM concentration, the charge-transfer resistance was the smallest.

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