Effect of Recombinant Antibodies and MIP Nanoparticles on the Electrical Behavior of Impedimetric Biorecognition Surfaces for SARS-CoV-2 Spike Glycoprotein: A Short Report

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Abstract: Electrochemical immunosensors are often described as innovative strategies to tackle urgent epidemiological needs, such as the detection of SARS-CoV-2 main biomarker, the spike glycoprotein. Nevertheless, there is a great variety of receptors, especially recombinant antibodies, that can be used to develop these biosensing platforms, and very few reports compare their suitability in analytical device design and their sensing performances. Therefore, this short report targeted a brief and straightforward investigation of the performance of different impedimetric biorecognition surfaces (BioS) for SARS-CoV-2, which were crafted from three commonly reported recombinant antibodies and molecularly-imprinted polymer (MIP) nanoparticles (nanoMIP). The selected NanoMIP were chosen due to their reported selectivity to the receptor binding domain (RBD) of SARS-CoV-2 spike glycoprotein. Results showed that the surface modification protocol based on MUDA and crosslinking with EDC/NHS was successful for the anchoring of each tested receptor, as the semicircle diameter of the Nyquist plots of EIS increased upon each modification, which suggests the increase of $R_{ct}$ due to the binding of dielectric materials on the conductive surface. Furthermore, the type of monoclonal antibody used to craft the BioS and the artificial receptors led to very distinct responses, being the RBD5305 and the NanoMIP-based BioS the ones that showcased the highest increment of signal in the conditions herein reported, which suggests their adequacy in the development of impedimetric immunosensors for SARS-CoV-2 spike glycoprotein.

Keywords: covid; coronavirus; biosensor; electrochemistry; immunosensors; molecularly-imprinted polymer

1. Introduction

The advent of the SARS-CoV-2 pandemic led many researchers to develop new biosensing technologies, amongst which electrochemistry thrived as a reliable and affordable way to promote point-of-need testing [1]. This technology is based on the intrinsic electric properties of matter and allows for the determination of the concentration of compounds either through the electric current that is generated from redox processes, or changes in the electric behavior of the sensing surface [2–4]. The performance of electroanalytical detection has been compared to standard analytical techniques based on spectrophotometry, as well as other optical physics approaches such as surface plasmon polaritons, Kretschmann configurations, and extraordinary optical transmission, allowing it to be used either as a...
complimentary method or standalone biosensing technology [5,6]. In this regard, numerous authors detailed the use of voltammetry and electrochemical impedance spectroscopy (EIS) as analytical techniques in the detection and quantification of SARS-CoV-2 antigens, such as its spike and nucleoproteins [7]. To all accounts, these technologies allowed the crafting of portable sensing platforms whose performance often rivalled highly refined diagnostic tools.

Moreover, novel point-of-care (POC) devices are designed to be flexible in terms of the biofluids being analyzed. While blood still retains its role as the gold standard for diagnostic purposes, several alternative body fluids have already been explored, giving encouraging results for noninvasive rapid testing [8]. Among these, saliva has been identified as a promising candidate for disease diagnostics due to its rich availability in biomarkers, continuous renovation, and ease of collection [9]. Salivary biomarkers have been already identified for several pathologies, such as cancer [10], neurodegenerative diseases [11], and systemic diseases [12]. Viral infections constitute another challenging target that salivary analysis could tackle, and some examples already surfaced relative to SARS-CoV-2 [13] detection.

Although the development of electrochemical biosensing devices for SARS-CoV-2 has been extensively demonstrated and validated by researchers worldwide [14], the approaches taken in these studies were vastly distinct. While the majority of authors resorted to the detection of the spike glycoprotein, some works concerned the detection of the nucleoprotein through a range of recombinant antibodies [15,16]. Even though these differences may not pose an issue at first glance, it must be noted that there is a long list of recombinant antibodies for SARS-CoV-2 antigens, and each combination yields different binding responses, which can have deep implications on the reliability of the sensor [17,18].

Intrinsic to the use of antibodies in biosensing applications are their limitations in terms of stability. It is well reported that the sensing platform must be carefully crafted and optimized in order to reach adequate stability [19]. On the same hand, researchers have to carefully consider which reagents, solvents, and conditions shall be taken during the surface modification of the sensing substrate; hence most biological macromolecules have a narrow stability range in regards to the physicochemical features of the environment where they are placed [20]. In fact, these limitations led to the selection of a small number of anchoring and crosslinking protocols that can be performed within a physiological medium, without extreme heating or shifts in the acidity/alkalinity of the environment.

Owing to the vast number of recombinant antibodies for SARS-CoV-2, and their varied binding behavior to the antigens, as well as the limited stability of these molecules, some groups have proposed the use of artificial receptors, namely molecularly imprinted polymers nanoparticles (NanoMIP) in the development of reliable and robust biosensing platforms [21–23]. Molecular imprinting technologies are based on the topological imprinting of targets on the surface of a polymer, thereby leading to a cavity of shape and size equal to that of the target [24,25]. This very feature grants MIP-based sensors their acknowledged selectivity, while the polymer nature confers stability due to the inherent robustness of polymeric networks [26]. Indeed, several authors detailed the benefits of applying MIP and NanoMIP in sensing technologies, and some reported their application in the development of MIP-based SARS-CoV-2 immunosensors [27,28], thus paving the way for the development of innovative analytical platforms to address urgent needs in medicine.

Considering the need to better understand how the responses of recombinant antibodies vary in bioanalytical applications, and also how MIP technologies stand in comparison to these approaches, this short report targeted a straightforward investigation and comparison of the performance of different BioS based on impedimetric detection for SARS-CoV-2, which were crafted from three commonly reported recombinant antibodies, and commercial NanoMIP. The selected NanoMIP were chosen due to their reported selectivity to the receptor binding domain (RBD) of SARS-CoV-2 spike glycoprotein. We hope our investigation sheds light on the importance of the adequate selection of recombinant antibodies in
the development of biosensing technologies, as well as showcasing the benefits of using NanoMIP in sensing applications.

2. Materials and Methods

2.1. Reagents and Solutions

The salts NaCl, KCl, Na$_2$HPO$_4$, and KH$_2$PO$_4$ were used to prepare a phosphate-buffered saline (PBS), pH 7.4. 11-mercaptoundecanoic acid (MUDA) was prepared into an ethanolic solution at 10 mmol·L$^{-1}$. The solutions of N-ethyl-N’-(3-(dimethylamino)propyl) carbodiimide and N-hydroxysuccinimide (EDC/NHS, 1:2) were prepared in PBS at 0.1 mg·µL$^{-1}$. Bovine serum albumin (BSA) was prepared at 0.1% (w/v) in PBS and 1% (v/v) Tween 20%. H$_2$SO$_4$ was prepared into a 0.5 mol·L$^{-1}$ solution, which was used to pretreat the sensing substrate before the modifications. Moreover, potassium hexacyanoferrate (III) K$_3$[Fe(CN)$_6$] and potassium hexacyanoferrate (II) K$_4$[Fe(CN)$_6$] were used to prepare a 1 mmol·L$^{-1}$[Fe(CN)$_6$]$^{3−/4−}$ solution in 5 mmol·L$^{-1}$ PBS containing 0.1 mol·L$^{-1}$ KCl. The NanoMIP as well as non-imprinted polymer nanoparticles (NIPnps, built of the same material as the NanoMIP) were acquired from MIP Discovery (Bedfordshire, UK), and diluted at 1:10 (v/v) in PBS. All solutions, except the NanoMIP, [Fe(CN)$_6$]$^{3−/4−}$ and H$_2$SO$_4$, were freshly prepared before analysis. All reagents were purchased from Sigma and were of analytical grade when applicable. Furthermore, all aqueous solutions were prepared with ultrapure water.

2.2. Antigens and Recombinant Antibodies

In this work, SARS-CoV-2 spike glycoprotein RBD was used as antigen, and was purchased from Native Antigen Company (Oxford, UK). The recombinant monoclonal antibodies herein used were of immunoglobulin G-class, namely: R107, RBD1106, and RBD5305, and were kindly provided by MIP Discovery (Bedfordshire, UK).

2.3. Gold Electrodes Pretreatment in Acid Solution

The BioS used in this work was based on gold screen-printed electrodes (Au-SPE). The Au-SPE comprised three electrodes, namely: working (Au), counter (Au), and reference (AgCl ink). Au-SPE were purchased from Metrohm, DropSens (model DRP-250AT). All Au-SPE were subjected to pretreatment on H$_2$SO$_4$ solution at 0.5 mol·L$^{-1}$. For this purpose, 50 µL of the solution was carefully dropped atop the Au-SPE, covering all electrodes. Thereafter, two voltammetric assays were conducted in a row. The first pretreatment assay consisted of single-scan fast-cyclic voltammetry (CV), whose conditions were: preconditioning at an electric potential ($E$) of +2.0 V for 5 s; $E$ deposition set to −0.35 V for 10 s; 3 s of equilibration between the $E$ window of −0.30 V and +1.55 V; and $E$ step of 0.001 V. The resulting rate was set to 4.0 V·s$^{-1}$. Following the first pretreatment step, the second protocol employed a standard CV, which was performed for 4 cycles. The settings for the standard CV were: 2 s of equilibration between the $E$ window of −0.30 V and +1.55 V; and $E$ step of 0.001 V. The resulting rate was set to 4.0 V·s$^{-1}$. The E step was set to 0.001 V and the scan rate was set to 0.1 V·s$^{-1}$.

2.4. Gold Substrate Modification

The surface modification herein selected was based on the spontaneous formation of self-assembled monolayers (SAM) on gold [29], and was followed by a crosslinking protocol based on EDC/NHS chemistry [30,31]. The SAM-forming domain herein selected was MUDA, whose aliphatic long chains have been reported to allow adequate anchoring of macromolecules such as antibodies onto surfaces [32]. Owing to the terminal carboxylic motif in MUDA, this compound is therefore compatible with EDC/NHS, which drove us to select this crosslinking method in our study. Furthermore, EDC/NHS is also compatible with the primary amines of amino acid residues in the antibodies, as well as the primary amines of the NanoMIP and NIPnps (information disclosed by the manufacturer).

The pretreated Au-SPE was modified by sequential drop-casting protocols. Between each step, the electrodes were thoroughly rinsed with ultrapure water and dried with a
nitrogen jet. The volume for the drop-casting was 50 µL, which was enough to cover all electrodes. The SPE was stored inside glass Petri dishes, which were laid horizontally on a flat surface. The incubations were performed either at 4 °C in the fridge (MUDA and BSA steps), or at room temperature (EDC/NHS and Antibody/NanoMIP step) The first step consisted of adding the MUDA solution, followed by 1 h incubation. The second step consisted of adding the EDC/NHS followed by 1 h incubation. The third step consisted of adding either the antibody or NanoMIP, or NIPnps solution, followed by 2 h incubation. The fourth and final step consisted of adding PBS, followed by 1 h incubation. BSA was herein used as a blocking agent, in order to block unbound sites and minimize responses due to the unspecific binding of targets on the gold substrate. 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The EIS Nyquist plot was used to gather electric information on the sensing surfaces, moreover, equivalent circuits were proposed according to the Randles equivalent topology [33,34]. This classic circuit comprises the resistance of the solution (R₀) in series with the capacitance of the double layer (C_{dl}), the latter of which is in parallel with the resultant of the series association of charge transfer resistance (R_{ct}) and Warburg diffusion element (W). A slight variation of this circuit was also used to model the electric behavior of the BioS, wherein the C_{dl} was swapped for a constant-phase element (CPE). This was performed due to the fact that CPE is reported to better describe the conductive and dielectric properties of solid electrodes [35]. The circuits herein used in the fitting are shown in Figure 2.

![Randles equivalent circuit](image)

**Figure 2.** Randles equivalent circuit considering: (A) C_{dl} and (B) CPE.

3. Results

3.1. Electrochemical Features of the Modified Surfaces

In order to monitor the surface modification, as well as to characterize the electrochemical behavior of the BioS, EIS was used. The Nyquist plots of the experiments are shown in Figure 3, while the extracted circuit elements are disclosed in Table 1.

![Nyquist plots](image)

**Figure 3.** Nyquist plots of the electrochemical responses of the electrodes after each modification. the final modification therein represented regards the NanoMIP post-BSA-blocking, which was added to provide a sense of scaling (A). Nyquist plots of the BioS after preparation and BSA-blocking (B).
Table 1. Values of the equivalent circuit fitting of each surface modification step required for crafting the BioS with the recombinant antibodies, NanoMIP and NIPnps. The phase-shift angle (\(\Phi\)) was extracted from each fitting concerning CPE modeling of the conducting/dielectric behavior of the electrode surface. Moreover, the \(X^2\) parameter was used to evaluate the quality of each fitting. All resistances are in \(\Omega\) and capacitances in nF.

| Surface Modification Steps | \(R_{ct}\) (\(\Omega\)) | \(C_{dl}\) (nF) | \(\Phi\) | \(X^2\) (\(C_{dl}\) fit) | \(X^2\) (CPE fit) |
|---------------------------|-------------------------|-----------------|--------|-----------------|-----------------|
| AuSPE                     | 222.3                   | 5333            | 0.906  | 0.0027          | 0.0007          |
| MUDA                      | 616.4                   | 5231            | 0.936  | 0.0031          | 0.0007          |
| EDC/NHS                   | 724.6                   | 5209            | 0.931  | 0.0040          | 0.0009          |

| Immunosensor Response Post BSA-Blocking |
|-----------------------------------------|
| \(R_{ct}\) (\(\Omega\)) | \(C_{dl}\) (nF) | \(\Phi\) | \(X^2\) (\(C_{dl}\) fit) | \(X^2\) (CPE fit) |
|-----------------------------------------|
| MIP                      | 1687                | 2292         | 0.950  | 0.0028          | 0.0005          |
| NIP                      | 4458                | 1865         | 0.933  | 0.0087          | 0.0002          |
| R107                     | 1809                | 2362         | 0.970  | 0.0017          | 0.0007          |
| RBD 1106                 | 2161                | 2579         | 0.948  | 0.0022          | 0.0003          |
| RBD 5305                 | 2286                | 2507         | 0.930  | 0.0032          | 0.0004          |

Results evidenced that the semicircle of the Nyquist plots increased in diameter upon each step, which is suggestive of successful modification [4]. Moreover, the electrochemical behavior of the BioS differed from each other, being the smallest semicircle that of the NanoMIP, followed by the antibody-based BioS R107, RBD1106, and RBD5305. The longest semicircle diameter was that of the NIPnps. Parallelly, the fitted circuit components evidenced the increase in \(R_{ct}\) according to each successive modification step. Furthermore, all responses were in consonance with the behavior of solid electrodes, hence the resistive behavior denounced by the \(\Phi\) values next to 1.0 [35]; and all fittings showcased adequate quality (Figure 3 and Table 1).

The increase in semicircle diameter is extensively reported as strong evidence of successful modification, due to its relationship with \(R_{ct}\) [36]. Considering that all surface modifiers herein used are of dielectric nature, an increase in the \(R_{ct}\) is expected, as the charge transfer is hindered on the surface of the electrode. In fact, the semicircle diameter of bare Au-SPE increased about 400 \(\Omega\) upon the addition of MUDA, whose long aliphatic chains are known to promote a drop in conductivity upon its anchoring to surfaces [32]. On the other hand, the short-chained NHS promoted an increase of about 100 \(\Omega\) upon its binding to the MUDA-modified Au-SPE, thence suggesting that smaller dielectric materials promote lesser hindrance to charge-transfer; which is extensively supported by the literature [37,38].

Following the modification with the recombinant antibodies, NanoMIP and NIPnps, an increase of the semicircle diameter, and consequently, of \(R_{ct}\) was noticed. The smaller increase of the semicircle regarding NanoMIP in comparison to the other molecules could be attributed to the size of the nanoparticles (40–80 nm range) and their homogeneousanchoring to the surface [39], nevertheless, further testing would be required to confirm this. Following the same interpretation, the longer semicircle diameter of the NIPnps suggests that the absence of imprinted cavities somehow hinders the access of the redox probe to the surface of the working electrode, thereby leading to an increase in the semicircle profile, and an apparent increase in the \(R_{ct}\).

Conversely to the observed trends regarding \(R_{ct}\), the \(C_{dl}\) values seemed to be consistent in the order of 5200–5300 nF in the first modification steps, though decreased by half upon the addition of the macromolecules. This is a remarkable finding, as it suggests that the capacitance values may be somehow correlated to the ultrastructural ordering of the electrode surface. In fact, this interpretation is supported by literature, as several authors have reported that the bulkiness of macromolecules disrupts the homogeneous formation of the Helmholtz Layer, which could strengthen the imperfect capacitive behavior, and change the predicted capacitance values [40,41].
In pursuance of exploring further the effect of surface modifications on the electric parameters of the electrodes, the $R_{ct}$ values were plotted against the $C_{dl}$, so to highlight if there could be any trend between them. Results are depicted in Figure 4.

![Figure 4](image)

**Figure 4.** Plot of $C_{dl}$ versus $R_{ct}$. The inset details the linear fitting herein used, being the r2 of $-0.8262$. Data which showcased overlap: upper-left corner: MUDA and EDC/NHS; lower-central region: RBD 1106 and RBD 5305, as well as R 107 and MIP.

Results evidenced that there seems to be an inversely proportional trend between the $C_{dl}$ and $R_{ct}$ values. Moreover, the plotting of these two attributes allowed visual segregation of the dataset from the modification steps (upper-left corner), and the dataset of the BioS based on NanoMIP and recombinant antibodies (lower-central part), and NIPnps (lower-right corner).

The seemingly inversely proportional trends of the $C_{dl}$ and $R_{ct}$ values are known to occur in many systems in electrochemistry [42]. The $R_{ct}$ values are regarded as descriptors of not only hindrances to charge transfer on electrode surfaces, but also may provide information on the accessibility of the solvent, as they carry the redox probes used in most reported EIS assays [43,44]. Likewise, owing to the formation of the double layer in the surface solid electrodes upon contact with a liquid medium, the capacitance values also provide information on the interface and its integrity [45]. Although the implications of the relationship between $R_{ct}$ and $C_{dl}$ are not fully known, our results hinted that they could harness information to allow the discrimination of the origin of the dataset, thereby possibly serving as a characterization tool.

3.2. Comparison of Immunosensor Response

After evaluating the electrochemical behavior of the BioS during their manufacturing, their responses were tested upon the presence of SARS-CoV-2 spike glycoprotein. The analytical signal herein taken was the variation of $R_{ct}$, hence many other authors have disclosed this parameter as a reliable analytical descriptor in EIS. Results are depicted in Figure 5.
Figure 5. Response of the BioS after incubation with SARS-CoV-2 spike glycoprotein. The analytical signal is herein represented as a relative increase in charge-transfer resistance ($\Delta R_{ct}$).

Results showed that the recombinant monoclonal antibody RBD 5305 yields the highest change in signal upon incubation with SARS-CoV-2 spike glycoprotein. The NanoMIP exhibited the second highest change in signal, followed by the RBD1106 and R107. The response of the NIP was unreliable, as hinted by the long error bars, and was seemingly the lowest (Figure 5).

The remarkable affinity of RBD-based antibodies towards SARS-CoV-2 spike glycoprotein has been previously reported and is considered crucial in the development of BioS and therapeutic strategies [46]. RBD5305 is a full-sized chimeric recombinant monoclonal antibody, which is expressed in mammalian cells. This monoclonal antibody is based upon the combination of original wild-type variable domains of rat monoclonal antibodies and human immunoglobulin 1 constant domains [47]. On the other hand, RBD1106 and R107 are hybridoma clones from Sp2/0 myeloma and spleen cells of BALB/C mice that were immunized with recombinant SARS-CoV-2 Spike RBD [47]. These recombinant antibodies have been proven in vitro as efficient inhibitors of the interaction between recombinant RBD and the angiotensin-converting enzyme 2 [48,49], which is a critical step in the infection of cells.

The order of affinity between the monoclonal antibodies and the $\beta$ variant of SARS-CoV-2 spike glycoprotein is RBD1106 > RBD5305 > RBD107. These findings differed from what was obtained by the electrochemical BioS; hence the best response came from the modification that was conducted with RBD5305. The main feature of the $\beta$ variant is that it has four mutations in its spike protein, namely: K417N, E484K, N501Y, and D614G [47]. Nevertheless, factors other than affinity could play a role in the electrochemical response, such as successful anchoring to the surface. To all accounts, RBD5305 showed the best results in the conditions herein tested.

In a similar way to RBD5305, the NanoMIP showcased a striking increase in the signal upon being exposed to the target. This result may be hence the imprinted cavities in the NanoMIP bear the same ultrastructural topology of SARS-CoV-2 spike glycoprotein RBD. To all accounts, the reported affinity constants of the NanoMIP used in this work were $<18$ nM to SARS-CoV-2 spike glycoprotein, and heat-transfer-based immunosensors exhibited very low limits of detection to both the $\alpha$ ($\sim 9.9$ fg·mL$^{-1}$) and $\Delta$ ($\sim 6.1$ fg·mL$^{-1}$) variants of the spike glycoprotein [50]. Nonetheless, the different responses of the NanoMIP and NIPnps hint at the effect that the imprinted cavities have on the performance of the sensor, hence the varied response in the assays with the NIPnps strongly hinted at an unspecific binding. Overall, it has to be remarked that the NanoMIP response is comparable to that of a widely
used, and reliable, recombinant monoclonal antibody in the development of impedimetric
immunosensors for SARS-CoV-2 spike glycoprotein, thus demonstrating the nature of
plastic antibodies of synthetic receptors prepared by molecular imprinting technology.

4. Conclusions

This work compared the electrochemical behavior of impedimetric BioS based on
recombinant antibodies and nanoMIPs to detect SARS-CoV-2 spike glycoprotein. Results
showed that the surface modification protocol based on MUDA and crosslinking with
EDC/NHS was successful, as the semicircle diameter of the Nyquist plots of EIS increased
upon each modification, which suggests the increase of $R_{ct}$ due to the binding of dielectric
materials on the conductive surface. Moreover, the $R_{ct}$ and $C_{dl}$ values followed a seemingly
inversely-proportional trend, and their datasets allowed the differentiation of the origin of
the observations, thereby suggesting that this strategy could be used for characterization
purposes. Furthermore, the type of receptors used to craft the sensor led to very distinct
responses, the RBD5305, and the NanoMIP-based BioS showcased the highest increment of
signal in the conditions herein reported, which suggests their adequacy in the development
of impedimetric immunosensors for SARS-CoV-2 spike glycoprotein.

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