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Improving immunoassay detection accuracy of anti-SARS-CoV-2 antibodies through dual modality validation

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A novel test strategy is proposed with dual-modality detection techniques for COVID-19 antibody detection. The full-length S protein of SARS-CoV-2 was chemically immobilized on a glass surface to capture anti-SARS-CoV-2 IgG in patient serum and was detected through either Electrochemical Impedance Spectroscopy (EIS) or fluorescence imaging with labeled secondary antibodies. Gold nanoparticles conjugated with protein G were used as the probe and the bound GNP-G was detected through EIS measurements. Anti-human-IgG conjugated with the fluorescent tag Alexa Fluor 488 was used as the probe for fluorescence imaging. Clinical SARS-CoV-2 IgG positive serum and negative controls were used to validate both modalities. For fluorescence-based detection, a high sensitivity was noticed with a quantification range of 0.01–0.1 A.U.C. and a LOD of 0.004 A.U.C. This study demonstrates the possibility of utilizing different measurement techniques in conjunction for improved COVID-19 serology testing.

1. Introduction

Coronavirus disease 2019, known as COVID-19, has caused a worldwide epidemic since 2020, and was declared by the World Health Organization (WHO) as a pandemic in March of 2020 (Organization 2020). Aside from the fact that Reverse Transcription-Polymerase Chain Reaction (RT-PCR) tests are taken as the gold standard for diagnosis of COVID-19 by direct detection of viral material in the body, serology testing has been considered an important complement to RT-PCR (Carter et al., 2020). SARS-CoV-2 is the virus causing COVID-19 and is comprised of four essential proteins: the spike protein (S protein), envelope protein (E protein), membrane protein (M protein), and nucleocapsid protein (N protein) (Krajewski et al., 2020). The S protein is mainly responsible for receptor binding and transportation of the virus into cells to be infected (Lu et al., 2020). Most serology testing commercially available or under development utilize the interaction of anti-SARS-CoV-2 antibody (IgG, IgM, or both) with parts or whole of either the S or N proteins of SARS-CoV-2 (Food and Administration), a strategy that has been proved efficient and usually comes with high specificity. For instance, combined IgG/IgM serology testing approved with Emergency Use Authorization (EUA) by the U.S. Food and Drug Administration (FDA) should have 90% in positive percent agreement (PPA) and 95% in negative percent agreement (NPA) with RT-PCR (Food and Administration). However, this also means false results are possible and are quite ubiquitous in diagnosing early infections during the initial 7 days after symptom onset (Serre-Miranda et al., 2021). Huge variations in performance for various test kits have also been observed. In a comparison study, a total of 11 commercial serological tests were...
assessed (Serre-Miranda et al., 2021). For the peak sensitivity of IgG detection after 21 days after symptom onset, the lowest sensitivity was only 68.8% for the SD test kit and the highest was 90.6% for the Snibe test kit. The other 9 kits exhibited intermediate values. For all types of sensitivity tests, either IgG or IgA/IgG or combined, none reached 100% sensitivity. Therefore, improving or assuring the sensitivity of a test should still be the focus for test developers. One possible strategy is to use two or more independent modalities simultaneously to improve the test credibility (Ogawa et al., 2009; Wang 2020). Another way is to achieve a lower limit of detection (LOD), making it less likely to obtain false negative results in samples with low levels of antibodies, especially for early-stage infections (Giri et al., 2021).

In this study, we developed an anti-SARS-CoV-2 antibody assay, with dual modality validations: Electrochemical Impedance Spectroscopy (EIS) and fluorescence labeling. EIS is an electrochemical non-faradic measurement that produces an impedance spectrum with an AC voltage applied. To achieve this, interdigitated electrode arrays (IDEs) are deposited onto a glass surface, followed by S protein immobilization on gaps between electrodes. Gold nanoparticles labeled with protein G were used as the probing conjugated secondary antibody, whose binding was verified through impedance changes measured by a potentiostat. Fluorescence labeling based on fluorophore-tagged anti-human-IgG is also used to detect the anti-SARS-CoV-2 IgG. This study indicates the possibility of improved accuracy for the assay with both validation methods combined.

2. Material and methods

2.1. Materials and reagents

Borofloat glass wafers were purchased from Swift Glass Co., Inc. Elmira Heights NY, and sliced as substrates for the deposition of IDEs and surface modification. IDEs were deposited through a standard photolithographic method as reported before (Abdelrasoul et al., 2018). SARS-CoV-2 spike protein was provided by Canadian Food Inspection Agency, with the method of immobilization briefly described in the supporting information. The anti-SARS-CoV-2 antibody-positive and negative serum samples were obtained from Alberta Precision Laboratories. Polyvinyl alcohol (98–99%, Mw 41241) and Tween 20 was purchased from Alfa Aesar (Haverhill, USA). The SYLGARD 184 silicone elastomer kit was purchased from DOW. Phosphate-buffered saline (PBS, 10X, pH 7.4), 3-Aminopropyltriethoxysilane 99% (APTES), N-(3-(dimethylamino)propyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide 98% (NHS), succinic anhydride 99% (SA), Poly(ethylene glycol) 2-mercaptoethyl ether acetic acid (SH-PEG5000-COOH, Mw 5000) were purchased from Sigma-Aldrich (Oakville, Canada). Bovine serum albumin (BSA, 98%, IgG free, Protease free), Alexa Fluor® 488 AffiniPure Goat anti-Human IgG (H + L) were purchased from Jackson Immunoresearch (USA). Ethyl acetate, ethanol, acetic acid and isopropanol were purchased from Sigma-Aldrich Canada Co. (Oakville, Ontario). Gold (III) chloride trihydrate (HAuCl4) and trisodium citrate dihydrate were purchased from Sigma-Aldrich (Oakville, Canada), The Pierce™ recombinant protein G and Nunc MaxiSorp™ high protein-binding capacity 96 well ELISA plates were purchased from Thermo Fisher Scientific (Whiby, Canada). Ultrapure water (18.2 MΩ/cm) was obtained from Millipore equipment (Milli-Q water) for sample preparation and washing.

2.2. Characterizations

The thickness of the films formed by surface functionalization were measured using ellipsometry (M-2000V J.A. Woollam Ellipsometer). Silicon wafers with 500 nm layer of thermally-deposited SiO2 were used as substrates for functionalization. For the APTES and SA layer, the Cauchy model (An = 1.30, Bn = 0.01, Cn = 0.001) was used for thickness fitting. For S protein layer, the Cauchy model (An = 1.45, Bn = 0.01, Cn = 0.001) was also used for thickness fitting. The scanning wavelength ranged from 300–700 nm and the incidental angle was set to 70°. The size and zeta-potential of the gold nanoparticles (GNPs) were measured with a Malvern Zetasizer Ultra at 25 °C. Zeta-potentials were measured under Hepes buffer (0.1 mM, pH 7.4). SEM characterization was performed on a ZEISS Sigma FE-SEM.

2.3. Surface functionalization with S protein

The substrates were first cleaned by immersing them sequentially in acetone, isopropanol, and ultra-pure water with sonication, and then put into a Reactive Ion Etching (RIE) instrument for plasma treatment (100 sccm O2, 150 mT pressure, 150 W RF, 120 s). 5% APTES in ethanol was prepared and was used to immerse the substrates for 3 h at room temperature (MacKay et al., 2017). Afterwards, 100 mM succinic anhydride in ethyl acetate was prepared for incubation overnight at room temperature. A PDMS mask was bonded on top of the substrate to separate IDE units and areas under liquid. EDC/NHS solution containing 14 mg of EDC (73 mM) and 28 mg of NHS (243 mM) in 1 mL of MES buffer (10 mM, pH = 5.5) was added to the substrate surface and incubated at room temperature for 15 min. Afterwards, the EDC/NHS solution was replaced with an S protein solution (0.1 mg/mL, PBS, pH 7.4), and incubated for 1 h at 37 °C. 1% PVA solution (PBS, pH 7.4) was then added and incubated at room temperature to block unfunctionalized regions.

2.4. Immunoassays

EIS-based immunoassays were analyzed with a SP-200 Potentiostat (BioLogic Sciences Instruments). The serum sample was diluted 10 times with 1% PVA in PBS, followed by incubation on the substrate for 1 h at 37 °C. After serum incubation, the substrate was washed three times with PBS containing 0.05% Tween 20, followed by three washes with PBS. After that, GNP-G solution in PBS was added onto the substrate and incubated for 3 h at room temperature. After GNP-G incubation, the substrate was washed twice with PBS before EIS measurement. Prior to all EIS measurements, the IDEs were immersed in PBS (10 μM, pH 7.4), and the |Z| at 10 kHz on the EIS spectra was taken for calculation.

For fluorescence-labeling immunoassays, all steps were the same as with the EIS-based assay, with the exception of using Alexa-anti-IgG (5 μg/mL) as the fluorophore-labeled secondary antibody. Fluorescence images were taken with a ChemiDoc MP Imaging System and analyzed with Image-Pro plus (v 6.0). The Relative Fluorescent Units (RFU) were obtained by averaging the fluorescence intensity of all pixels within the area of interest. In addition, a reference test was done with similar protocols, but was close to a typical ELISA process. In brief, S protein solution (0.1 mg/mL, PBS, pH 7.4) was added into wells of a 96-well plate and incubated overnight at 4 °C, followed by blocking for 1 h with 1% PVA in PBS. Serum samples diluted with 1% PVA in PBS were added and incubated for 1 h at 37 °C. After serum incubation, the wells were washed three times with PBS containing 0.05% Tween 20, and then three times with PBS. After that, Alexa-anti-IgG (5 μg/mL) was added and incubated for 1 h at room temperature, followed by washing with PBS two times.

Enzyme-Linked Immunosorbent Assay (ELISA) was used to identify anti-SARS-CoV-2 antibody positive or negative serum. S protein solution (PBS, pH 7.4) was added into the wells of a 96-well plate and incubated overnight at 4 °C, followed by blocking for 2 h with PBS containing 0.05% Tween 20 and 3% skim milk. Serum samples diluted 10 times with PBS containing 0.05% Tween 20 and 1% milk were added and incubated for 1 h at 37 °C. After serum incubation, the wells were washed five times with Tris-buffered saline containing 0.2% Tween 20 (TBST, pH 7.4). HRP-conjugated Protein G solution (500 ng/mL) in PBS (pH 6.0) was then added and incubated for 1 h at room temperature, followed by washing five times with TBST. 100 μL of TMB substrate solution was added and the reaction was quenched by the addition of 2N hydrogen peroxide.
3. Results and discussion

3.1. Characterization of S protein immobilization

Surface chemistries were utilized for immobilization of S proteins by the following method: 1) immobilization of 3-Aminopropyltriethoxysilane (APTES) to introduce amino groups; 2) conjugation of APTES with succinic anhydride (SA) linker molecules; 3) immobilization of S protein. For characterization, ellipsometry was performed to identify increases in thin-layer films after each step. Films of APTES and SA were measured with a thickness of 1.1 nm and 0.9 nm, respectively (Fig. S1). These measured thicknesses are close to the reported values as mono-layers (Liang et al., 2014; van der Maaden et al., 2012). After S protein immobilization, an additional layer of 5.0 nm was formed. Measuring the protein layer thickness, we estimated the surface density of the immobilized S protein to be 653 ng/cm², which may suggest partial exposure of RBD regions (see Supporting Information for more details).

3.2. Characterization of GNPs

GNPs used in our study were prepared according to a previously-reported method (Kimling et al., 2006), and characterized through Dynamic Light Scattering (DLS). The Z-average size for GNPs upon synthesis was determined to be 14.09 nm. This increased to 24.61 nm after conjugation with Polyethylene glycol (PEG), and further increased to 27.77 nm after protein G conjugation (Table S1). The zeta-potential value also revealed changes to surface charge after functionalization. The zeta-potential for bare GNPs upon synthesis was –47.61 mV and increased to –8.02 mV after conjugation with PEG. The zeta-potential value of –29.25 mV for the GNPs with conjugated protein G indicates that the GNP-G were negatively charged. Considering the 7.4 pH of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) as the measurement buffer, this result is in agreement with the isoelectric point of protein G at 4.55 (Specialists). Therefore, the conjugation of protein G on GNPs was confirmed. SEM images for bare GNPs were also obtained, which showed spherical GNPs with a uniform size distribution. The average spherical diameter was obtained as 17.86 nm (Fig. S2).

3.3. Immunoassay based on EIS measurement

For the equivalent circuit of the IDEs, three factors are involved to determine the absolute impedance (|Z|) (Randvir and Banks, 2013): Dielectric capacitance (C₀), electrical double layer capacitance (C_DL) and solution resistance (Rₜ) (Fig. 1 left). Among them, C_DL is the parameter that varies upon adsorption of GNPs on IDEs, which causes |Z| to decrease.

3.3.1. Immunoassay based on EIS measurement

To quantify the difference of impedance before and after GNP-G incubation, ΔZ is introduced and is calculated through the following equation:

\[
\Delta Z = \frac{Z_{\text{GNP-G}} - Z_{\text{serum}}}{Z_{\text{serum}}} \times 100\%,
\]

where \(Z_{\text{GNP-G}}\) is the |Z| after incubation with GNP-G, and \(Z_{\text{serum}}\) is the |Z| after serum incubation, prior to GNP-G incubation. The value of |Z| varies at different frequencies and was found to have optimal signal sensitivity and stability at 10 kHz. Therefore, |Z| at 10 kHz was used for \(\Delta Z\) calculation.

In our study, the anti-SARS-CoV-2 antibody-positive and negative serum samples were validated using ELISA prior to incubation on a functionalized surface, with the result shown in Fig. S3. To test the performance of the IDEs, we first applied a “one chip, one test” strategy, which was to include both positive samples and negative controls on working units of one chip and analyze the results separately for each chip. The results are shown in Fig. 2 for all five chips tested, and the ΔZ values of positive serum incubated working units are consistently higher than the negative ones. This is in consistency with the expectation that |Z| changes with GNP-G binding. To determine the statistical difference on each chip, the one-tailed p-values for the T-test were calculated (Ludbrook, 2013). The p-value is less than 0.1 for chip #3 and #4, and is
more than 0.1 for chip #1, 2 and 5. A chip-to-chip difference in AZ was also noticed. Such a difference may be caused by quality control difficulties when fabricating the chips, such as rare defects in the electrode that can adversely affect the chip performance. Nevertheless, it may still be applicable for serology testing. For each separate test, negative serum can be used as an internal control. By subtracting the negative control value from the sample value, a DiffAZ is then obtained. From Table S2 we found that the DiffAZ for the positive sample studied is consistently positive, which can be seen as the validation of this modality. Detection of SARS-CoV-2 antibodies through the EIS technique has also been validated before, based on a commercial platform with a label-free feature (Rashed et al., 2021). Similar to what was obtained in our study, large error bars seem to be inevitable when it comes to clinical positive samples in comparison to ELISA, making it difficult to distinguish from negative samples.

3.4. Immunoassay based on fluorescence measurement

For the study based on fluorescence measurements, the same surface modification strategy and serum sample as for the IDE chips was used. Fluorescence was quantified by the average fluorescence intensity (RFU) of areas with serum incubation, and the test result on three substrates is shown in Fig. 3a. As shown in Fig. 3a, all positive serum samples resulted in a fluorescence intensity of ~30000 RFU, and all negative serum samples exhibited intensities below 10000 RFU. Despite this, the negative samples (Fig. 3b, area 4, 5 and 6) displayed higher fluorescence over blank controls without serum incubation (Fig. 3b, area 7 and 8), which is likely due to nonspecific interactions. The difference between positive and negative serum samples is much more pronounced than that obtained in the IDE tests, with p < 0.01 for all three tested substrates. Additionally, the results were more consistent among different substrates.

3.4.1. Effect of anti-SARS-CoV-2 IgG concentration

Further investigation was done with varied anti-SARS-CoV-2 IgG concentrations (Fig. S4). The result showed an increasing fluorescence intensity from 16060 RFU to 29783 RFU, with the concentration ranging from 0.01 to 0.1 A.U.C. (defined as arbitrary unit for anti-SARS-CoV-2 IgG concentration). To evaluate the binding efficiency for immobilized S protein to anti-SARS-CoV-2 IgG antibodies, a comparison study with S protein immobilized through non-specific adsorption on a 96-well plate was performed (Fig. S4b). A unilateral trend was also observed within the same concentration range. However, we found that at the low concentration of 0.01 A.U.C., the difference between positive and negative sera are much less distinguishable, with 31 RFU for positive and 29 RFU for negative. Such a result reveals the advantage of surface chemistry for S protein immobilization, which show a higher binding affinity towards anti-SARS-CoV-2 IgG and is more sensitive in detection than physical adsorption. For the fluorescence assay, the high interference from nonspecific adsorption may affect the result accuracy. By filtering serum with a 100 kDa filter membrane, decreased RFU for all samples were observed. For the negative serum sample, the RFU significantly decreased, approaching the blank control’s RFU without serum incubation (Fig. S5). To estimate the limit of detection (LOD) for the fluorescence-based assay, a T-test analysis was done with further decreasing concentrations of anti-SARS-CoV-2 IgG. For optimal resolution and reliability, the “one chip, one test” strategy was used. The result shown in Fig. S6 gives the p-values of four T-tests. It was found that for three concentrations (0.01, 0.007 and 0.004 A.U.C.) the difference is significant (p < 0.05) and is insignificant (p > 0.05) at the lowest tested concentration (0.001 A.U.C.). Thus, the LOD of this test can be determined to be 0.004 A.U.C. This LOD is low in comparison to that of the ELISA method, where the optimal quantification range generally falls between 0.01 and 10 μg/mL (Roy et al., 2020). To the best of our knowledge, there has not been any commercial serology testing using the same surface functionalization and antigen/fluorescence labeled anti-IgG for probing anti-SARS-CoV-2 IgG. The closest one is a chemiluminescence immunoassay (CLIA) modality by the Snibe Maglumi 2000 series which uses both S and N proteins for detection of IgG or IgM (Lippi et al., 2020b; Selingerova et al., 2021). One study revealed a high degree of concordance of test results between this CLIA and ELISA (Lippi et al., 2020a).

3.4.2. Effect of serum and Alexa-anti-IgG incubation time

Whether a serology testing is suitable for rapid testing depends on the time it takes. To this end, the dependence of time of serum incubation and Alexa-anti-IgG incubation were investigated. The result shown in Fig. S7 reveals the effect of serum incubation time on fluorescence intensity. The RFU, for both positive and negative samples, grows rapidly between 15 and 60 min, and then remains almost unchanged for an elongated period (Fig. S7a.). For Alexa-anti-IgG incubation time, the fluorescence intensity established rapidly during the initial 15 min, and then gradually increased within 60 min (Fig. S7b.). In another study, the required incubation between S protein and COVID-19 antibodies was found to be no less than 20 min to yield a sufficient fluorescence signal (Xu et al., 2021).

We may conclude that for optimal intensity or resolution, 60 min of incubation time for serum and greater than 60 min of incubation time for Alexa-anti-IgG are necessary. Considering test efficiency, 15 min of serum incubation and 15 min of Alexa-anti-IgG incubation, or ~30 min of total test time, seem to be feasible.

3.4.3. Effect of concentration of S protein and Alexa-anti-IgG

It has been shown before that a protein layer was formed with 0.1 mg/mL of S protein via EDC/NHS immobilization, and the successful detection of COVID-19 antibody was proved through an immunoassay. We then further examined a fluorescence immunoassay with a lower S protein immobilization concentration of 0.01 mg/mL. From the result shown in Fig. S8, the RFU for positive serum sample surprisingly decreased to the same value as for the negative serum. This suggests the concentration of 0.01 mg/mL is too low to form sufficient surface binding sites to the COVID antibody. We speculate that the S proteins immobilized at a lower surface density may mis-orientate and will therefore be unable to bind the target antibody. In addition, we also performed an immunoassay with different Alexa-anti-IgG concentrations, ranging from 2 to 8 μg/mL. As shown in Fig. S9, similar RFU values were achieved for both positive and negative serum, with no obvious increase or decrease as a function of concentration. Such a result may indicate a minor influence of this probing reagent to the detection, and the insensitivity of Alexa-anti-IgG concentration in the formation of the antigen-primary antibody-secondary antibody complex.
3.5. Result analysis for dual modality feasibility

Our proposed dual modality COVID-19 serology test includes both impedance and fluorescence-based detection methods. The yielded data for two detection methods, as well as ELISA, is statistically summarized and compared in Table 1. The p-value indicates the difference between the positive and negative data groups, and is 8.09 × 10⁻¹¹ for ELISA, 9.59 × 10⁻⁴ for impedance-based detection, and 4.48 × 10⁻¹⁸ for fluorescence-based detection. This suggests fluorescence-based detection has the highest resolution and impedance-based detection has the lowest resolution.

Impedance-based detection has the advantage of instantaneous measurements for a large number of samples and is suitable for POC applications. The IDE chips can be fabricated at a low cost through microfabrication in mass production for disposable use. In future testing with a large number of positive samples, it is possible to define a cut-off value for DiffAZ to be used as a criteria for identification of anti-SARS-CoV-2 antibody-positive cases. Where the increased possibility of false positive results due to a low cut-off value is concerned, the fluorescence-based method may work as a good complement by bringing the advantages of improved accuracy. The high resolution of the fluorescence method makes it possible to quantify antibody titers with elongated incubation time. Samples to be tested with impedance-based detection can be easily shared for testing with fluorescence-based detection, and thus additional sampling is not necessary. One possible scenario for the application of this dual modality technique is to first screen samples of a large population through impedance-based detection in POC situations. Then, the identified suspicious positive samples can be further tested with fluorescence-based detection to validate and quantify these results. This test strategy has the advantage of quick screening at a low cost and does not compromise accuracy.

4. Conclusions

In this study, two independent anti-SARS-CoV-2 antibody immunoassays were developed. Both the surface immobilization of S protein and surface modification with GNPs were characterized. Both EIS-based and fluorescence-based detection measurements were separately validated using clinical samples. For fluorescence-based detection it was determined to have a quantification range of 0.01–0.1 A.U.C. with a LOD of 0.004 A.U.C. and with a minimal total test time of 30 min. The unique feature and high similarity of these two methods provides the possibility of both working in conjunction to improve COVID serology testing.

CRediT authorship contribution statement

Yuhao Ma: Methodology, Investigation, Writing – original draft, Investigation. Daniel To: Writing – review & editing. Jie Zeng: Methodology. Lian C.T. Shoute: Methodology. Meng Wu: Resources. Shawn Babiuik: Resources. Ran Zhuo: Resources. Carmen Charlton: Resources. Jamil N. Kanji: Resources. Lorne Babiuik: Funding acquisition. Jie Chen: Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biox.2022.100176.

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