

Selective Detection and Ultrasensitive Quantification of SARS-CoV-2 IgG Antibodies in Clinical Plasma Samples Using Epitope-Modified Nanoplasmonic Biosensing Platforms

Adrianna N. Masterson and Rajesh Sardar*



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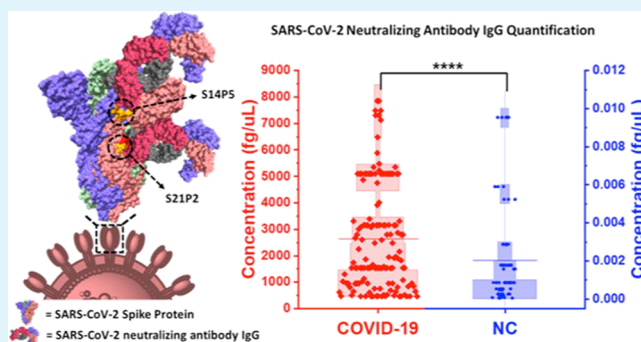
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ABSTRACT: Monitoring the human immune response by assaying (detection and quantification) the antibody level against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is important in conducting epidemiological surveillance and immunization studies at a population level. Herein, we present the design and fabrication of a solid-state nanoplasmonic biosensing platform that is capable of quantifying SARS-CoV-2 neutralizing antibody IgG with a limit of detection as low as 30.0 attomolar (aM) and a wide dynamic range spanning seven orders of magnitude. Based on IgG binding constant determination for different biological motifs, we show that the covalent attachment of highly specific SARS-CoV-2 linear epitopes with an appropriate ratio, in contrast to using SARS-CoV-2 spike protein subunits as receptor molecules, to gold triangular nanoprisms (Au TNPs) results in a construction of a highly selective and more sensitive, label-free IgG biosensor. The biosensing platform displays specificity against other human antibodies and no cross reactivity against MERS-CoV antibodies. Furthermore, the nanoplasmonic biosensing platform can be assembled in a multi-well plate format to translate to a high-throughput assay that allowed us to conduct SARS-CoV-2 IgG assays of COVID-19 positive patient ($n = 121$) and healthy individual ($n = 65$) plasma samples. Most importantly, performing a blind test in an additional cohort of 30 patient plasma samples, our nanoplasmonic biosensing platform successfully identified COVID-19 positive samples with 90% specificity and 100% sensitivity. Very recent studies show that our selected epitopes are conserved in the highly mutated SARS-CoV-2 variant “Omicron”; therefore, the demonstrated high-throughput nanoplasmonic biosensing platform holds great promise for a highly specific serological assay for conducting large-scale COVID-19 testing and epidemiological studies and monitoring the immune response and durability of immunity as part of the global immunization programs.

KEYWORDS: COVID-19, SARS-CoV-2, linear epitope, neutralizing antibody IgG, nanoplasmonic biosensing, selectivity, clinical samples



INTRODUCTION

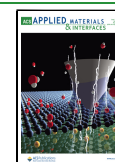
As of this writing, the World Health Organization (WHO) reported that the COVID-19 disease, which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2),^{1,2} has led to >318 million confirmed cases and more than 5.5 million deaths worldwide, with the prediction of a faster increase in cases and mortality due to a new variant, “Omicron”.³ Although vaccines are now available, with limited supplies and low inoculation rates in low-income countries/resource-limited places, effective ways to contain the spread of COVID-19 diseases still includes rapid and accurate testing methods to identify infected individuals followed by patient isolation. Current COVID-19 diagnostic techniques include molecular detection of the SARS-CoV-2 virus and antibody testing to detect humoral immune response to infection.^{4–8} Viral RNA and/or antigen levels last during a very short period of active infection. In contrast, antibodies are detectable as early as two days after the infection in some patients and their

levels persist for months in human biofluids. Therefore, crucial information such as time of exposure, disease progression, and past infection or immunity can be obtained by measuring the antibody level in biofluids (serology antibody assay).^{5,8–11} Additionally, the level of antibodies can provide insights into the human immune response by determining the level of protection the body has against the virus. Serological assays that have been developed for COVID-19 detection include techniques based on enzyme-linked immunosorbent assays (ELISA), chemiluminescent assays, lateral flow assays, and

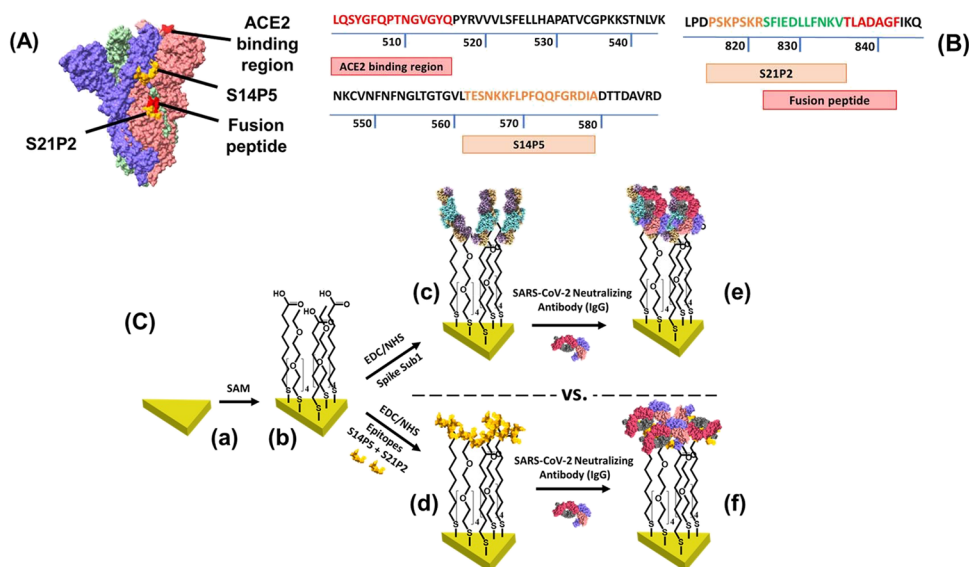
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Scheme 1. (A) Three-Dimensional Structure of SARS-CoV-2 Spike Protein; (B) Amino Acid Sequences of SARS-CoV-2 Spike Protein Regions Corresponding to the ACE2 Binding Region, S14P5, S21P2, and Fusion Peptide; and (C) Fabrication of the Nanoplasmonic Biosensing Platform for the SARS-CoV-2 Neutralizing Antibody IgG Assay: (a) Silanized Glass Surface-Bound Au TNP, (b) Au TNPs after Formation of a Mixed Self-Assembled Monolayer (SAM) of 8-Mercaptooctanoic Acid and PEG₆-SH, (c) Covalent Attachment of Spike Subunit 1 (Sub1) through Amide Coupling (EDC/NHS) to a SAM-Modified Au TNP, (d) Covalent Attachment of Epitopes (S14P5, S21P2, or Different Percent Ratios of the Two) to a SAM-Modified Au TNP, (c) and (d) Construct a Nanoplasmonic Biosensing Platform, and (e and f) SARS-CoV-2 Neutralizing Antibody IgG Binding to Nanoplasmonic Biosensing Platforms



others.^{4–6,10,12–16} However, these techniques either suffer from low sensitivity when quantifying a low abundance of biomarkers, especially at a high specificity,^{5,9,12,15,16} or are unable to differentiate immunoglobulin-G (IgG) specific to SARS-CoV-2 from other novel coronaviruses.¹⁴

To overcome these drawbacks, several plasmonic nanostructured-based (“nanoplasmonic”), solid-state serology assays have been developed for COVID-19 antibody detection.^{5,8,13,17–19} Nanoplasmonic assays utilize the unique localized surface plasmon resonance (LSPR) property of noble metal nanostructures that originates due to collective oscillation of free electrons upon light irradiation.^{20,21} Furthermore, with an appropriate choice of nanostructures, along with the suitable surface chemistry anchoring receptor molecules, highly sensitive and specific nanoplasmonic biosensors can be fabricated for protein assays in human biofluids.^{22–24} In the context of COVID-19 serology antibody assays, current plasmonic-based antibody assays lack specificity.^{8,12} Moreover, these assays use an anti-human antibody (anti-IgG, anti-IgM, and anti-IgA) as receptor molecules in the biosensor construct to detect SARS-CoV-2 antibodies (IgG, IgM, and IgA).^{5,12,17–19} However, these anti-human antibodies (anti-IgG, anti-IgM, and anti-IgA) are known to be abundant in blood and may compete with target SARS-CoV-2 antibodies for binding with anti-human antibody receptors, leading to non-SARS-CoV-2 antibody specific receptors.^{9,10,12,25} In order to develop a highly specific serology antibody assay for COVID-19, one must select a SARS-CoV-2 antibody specific receptor.

SARS-CoV-2 uses the spike glycoprotein harboring the receptor-binding domain (RBD) to co-opt the angiotensin-converting enzyme 2 (ACE2) receptor for cell entry. Therefore, blocking this binding could prevent the viruses’ entry.^{4,7,10,26} Neutralizing antibodies that are produced against

the spike protein have been shown to target RBD and prevent cellular transfection. A highly specific serology antibody assay for COVID-19 must include a receptor molecule that is a subset of the SARS-CoV-2 spike protein and a detection analyte that would be SARS-CoV-2 neutralizing antibody (IgG).^{10,15,26,27} Recent works by Ng and co-workers showed that around 18 peptides in length of the spike protein segments, called linear epitopes, have a higher binding affinity toward SARS-CoV-2 neutralizing antibody IgG, suggesting a further improvement in specificity.^{9,26} The authors reported a gold nanoparticle-based colorimetric assay to detect IgG from clinical samples. Although this colorimetric-based IgG detection is highly specific, the assay displayed a limit of detection (LOD) in the nanomolar (nM) range. Together, both highly sensitive and specific serology antibody assays are extremely important to avoid false responses and to understand antibody responses against different antigens and time-dependent maturation of antibody levels. Additionally, to aid epidemiological surveillance and post-vaccine monitoring studies at a population level, the assay should be capable of analyzing biomarkers from a small sample volume and should demonstrate high-throughput capabilities.

In this present study, we examine the hypothesis that functionalization of chemically synthesized gold triangular nanoprisms (Au TNPs), which display unique LSPR properties,^{28,29} with linear epitopes as receptor molecules, constructs a highly sensitive (attomolar LOD) nanoplasmonic biosensing platform for SARS-CoV-2 specific IgG detection (Scheme 1). Furthermore, we elucidate the binding interaction between receptors to COVID-19 specific IgG to prepare a highly specific and robust biosensing platform into a multi-well plate format for high-throughput assays. Our ultrasensitive assay allows clinical samples to be highly diluted, leading to substantial improvement in specificity by reducing non-specific

binding of unwanted biomolecules present in human biofluids. The clinical applicability of our nanoplasmonic biosensing platform is demonstrated by analyzing (detection and quantification) IgG in 121 COVID-19 positive patient and 65 healthy control plasma samples. Importantly, in the training and validation cohorts, the sensitivity/specificity for COVID-19 detection is 100/100% and 100/90%, respectively. Taken together, we believe that the high-throughput biosensing platform presented here not only provides a highly specific quantification assay for COVID-19 diagnosis but also sets up a foundation for highly specific and ultrasensitive serological assays to be developed for future virus outbreaks and pandemics.

EXPERIMENTAL SECTION

Materials. Chloro(triethylphosphine) gold(I) (Et_3PAuCl , 97%) was purchased from Gelest Inc. Poly(methylhydrosiloxane) (PMHS, $M_n = 1700\text{--}3300$), triethylamine (TEA, 98%), ACS grade acetonitrile (CH_3CN , 99.9%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 99%), *N*-hydroxysuccinimide (NHS, 98%), and 8-mecaptooctanoic acid (MOA) were purchased from Sigma-Aldrich. (3-Mercaptopropyl)-trimethoxysilane (MPTMS, 94%) was purchased from Alfa Aesar. Ethanol (200 proof) was purchased from Decon Labs. Thiolated polyethylene glycol ($\text{PEG}_6\text{-SH}$) was purchased from purePEG. Glass coverslips (18×18 mm) and RBS 35 Detergent were obtained from Fisher Scientific. No-bottom 96-well plates were purchased from Greiner Bio-One. Krazy Glue and 3M Scotch tape were purchased from Office Depot. SARS-CoV-2 spike protein and MERS-CoV Spike RBD IgG were purchased from R&D Systems, a BioTechne brand. Linear epitope polypeptide sequences (S14P5 and S21P2, sequences in Table S1) were purchased from GenScript. SARS-CoV-2 neutralizing antibody IgG was purchased from GeneTex. Human IgG1 was purchased from Acros Biosystems. All chemicals were used without further purifications. RNase free sterile water was obtained from Baxter Healthcare Corporation. All water was purified using a Thermo Scientific Barnstead Nanopure system. Antibodies, antigens, proteins, and patient samples were stored at -80°C . Phosphate-buffered silane (PBS) buffer ($\text{pH} = 7.2$) was prepared using RNase free sterile water.

Silanization of Glass Coverslips. Glass coverslips with 18×18 mm dimension were silanized based on a previously published procedure.^{19,30} Briefly, 10 glass coverslips were placed in a glass staining jar, were incubated in a 10% RBS 35 detergent solution at 90°C , and were sonicated for 15 min. The coverslips were then rinsed with a copious amount of nanopure water followed by incubation in a 1:1 (v/v) hydrochloric acid:methanol solution for 30 min at room temperature. After 30 min, the coverslips were rinsed multiple times with nanopure water and then were allowed to dry overnight in a vacuum oven at 60°C . The following day, the coverslips were allowed to cool to room temperature and then were incubated for 30 min in a 15% (v/v) solution of MPTMS in N_2 purged ethanol. After 30 min, the coverslips were sonicated for 10 min in N_2 purged ethanol three times. After ethanol sonication, the coverslips were dried in a vacuum oven for a minimum of 3 h at 120°C . The coverslips were stored at 4°C up to 1 week.

Synthesis of Gold Triangular Nanoprisms (Au TNPs). Au TNPs were chemically synthesized according to our previously published procedure.²² Briefly, 18.0 mg (0.05 mmol) of $\text{Et}_3\text{PAu(I)Cl}$ was dissolved in 40 mL of N_2 purged acetonitrile and stirred at room temperature for 10 min. Then, 38 μL (0.273 mmol) of TEA was injected to the solution and heat was applied to reach a steady 40°C temperature. At this point, 600 μL of PMHS was slowly added in an unstirred solution, and then the reaction was allowed to proceed with slow stirring. Once the color of the solution turned dark navy blue with an LSPR dipole peak position (λ_{LSPR}) of ~ 850 nm, the solution was removed from heat and was centrifuged at 7000 rpm for 10 s. The Au TNP-containing solution was then immediately transferred to previously prepared MPTMS-functionalized coverslips and incubated

for 1 h followed by rinsing with acetonitrile, dried with N_2 flow, and stored under N_2 at 4°C . For the best biosensing performance, Au TNP-attached coverslips should be used within 3 days of fabrication (prior-modification).

Fabrication of High-Throughput Nanoplasmonic Biosensing Platforms. We constructed different nanoplasmonic biosensing platforms by changing the structure of receptor molecules (SARS-CoV-2 spike subunit 1 protein, two different epitopes, and varying their ratios). High-throughput biosensing platforms were fabricated based on previously published procedures with modifications.³⁰ Au TNP-attached glass coverslips were glued to the bottom of a no-bottom 96-well plate by applying a small amount of glue around the edges of the wells on the plate and gently applying pressure on the coverslip to adhere to the well plate. The coverslip-attached plate was allowed to dry for at least an hour at room temperature. The well plate was then incubated in nanopure water to check for any leakage. For SARS-CoV-2 neutralizing antibody IgG detection, each well was first incubated in 0.3 mL of a 1.0 mM:1.0 μM ratio of 8-mecaptooctanoic acid (MOA): $\text{PEG}_6\text{-SH}$ solution overnight. Then, each well was carefully rinsed with PBS buffer to remove any loosely bound organic molecules and then incubated in 0.2 M solution of EDC/NHS in PBS buffer for 2 h at room temperature. Next, each well was rinsed with PBS buffer and then incubated in a 1.0 $\mu\text{g}/\text{mL}$ receptor molecule (SARS-CoV-2 spike subunit 1 protein or linear epitopes varying ratios) PBS solution overnight. The following day, each well was rinsed with copious amount of PBS buffer to remove any loosely bound biomolecules. The receptor-bound Au TNPs in well-format is referred as the "nanoplasmonic biosensor". Overall, a single biosensing platform contains 92 individual biosensors with four wells designated for blank references, leading to a high-throughput (e.g., multiple samples simultaneously in one instrumental run) assay. A fully functional nanoplasmonic biosensing platform is shown in Figure S1.

Determination of Binding Dissociation Constants. The binding dissociation constants (K_d) were determined by replotting the previously developed calibration curves in PBS buffer as $\Delta\lambda_{\text{LSPR}}$ versus concentration in mol/L (M). A 1:1 Langmuir model (eq 1) was used to fit the data through Origin software and to determine the binding association constant (K_a), where c is the concentration of adsorbed molecules, K_a is the Langmuir adsorption constant, and θ is the fraction coverage of adsorbed molecules (θ_{eq} is the concentration-dependent equilibrium surface coverage). The obtained K_a was then converted to K_d using eq 2.^{9,31,32}

$$\theta_{\text{eq}} = \frac{K_a c}{1 + K_a c} \quad (1)$$

$$K_d = K_a^{-1} \quad (2)$$

Development of SARS-CoV-2 Neutralizing Antibody IgG Calibration Plots. After fabrication of the biosensing platform, the LSPR extinction spectra of each biosensor were obtained in PBS buffer and the λ_{LSPR} was determined. Each biosensor was then incubated overnight in a 300 μL solution of SARS-CoV-2 neutralizing antibody IgG of different concentrations, ranging from 100.0 nM to 1.0 aM. These solutions were made in PBS buffer or 10% plasma through serial dilutions. The following day, IgG-bound biosensors were rinsed with nanopure water, and the LSPR extinction spectra of each biosensor were collected in PBS buffer and then λ_{LSPR} was determined. The difference in λ_{LSPR} ($\Delta\lambda_{\text{LSPR}}$) before and after IgG attachment as a function of concentrations was used to develop calibration plots. All calibration plots were established by taking the measurement of six individual biosensors. False positive analysis was conducted by incubating the biosensors in a PBS buffer solution without any analyte or in 10% human plasma. False negative analysis was conducted by incubating the SAM-functionalized Au TNPs (without receptor molecule) in a 100 nM IgG solution.

Quantification of SARS-CoV-2 Neutralizing Antibody IgG Levels in Patient Plasma. The study design and protocols for COVID-19 patients were evaluated by the Indiana University Institute

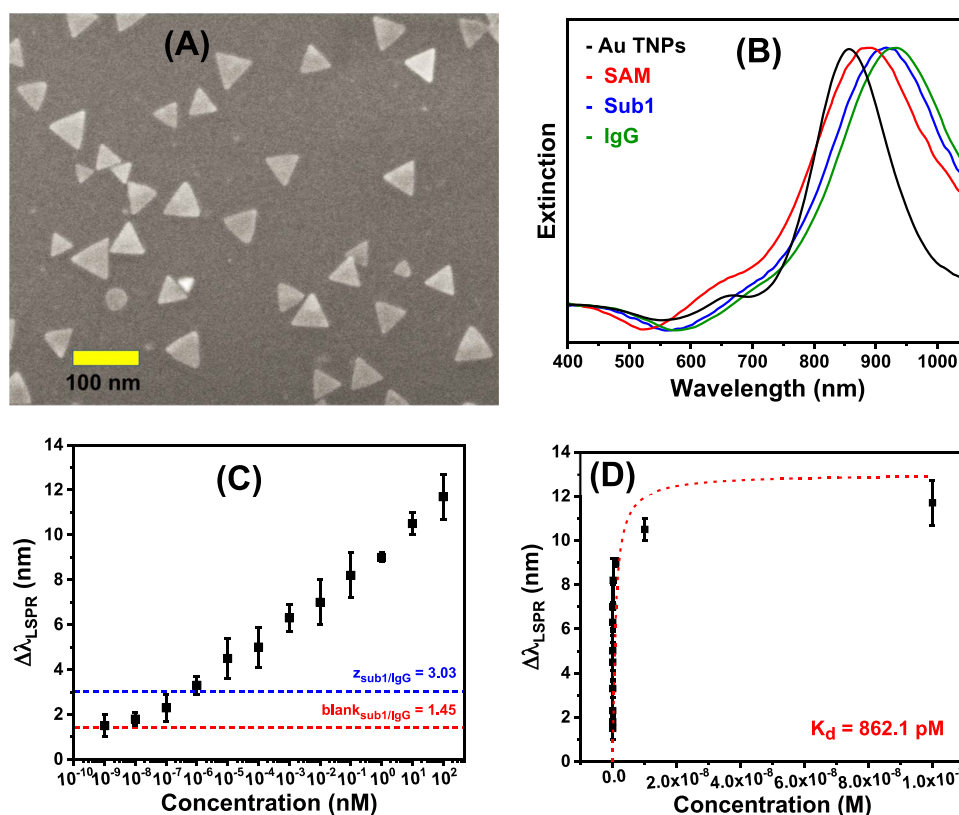


Figure 1. Structural and optical characterizations of nanoplasmonic biosensing platforms containing SARS-CoV-2 spike protein subunit 1 as a receptor. (A) Representative scanning electron microscopy images of ~ 54 nm edge length glass substrate-bound Au TNPs. (B) UV-vis extinction spectra of Au TNPs adsorbed onto a silanized glass substrate before surface modification (black curve, 857.9 nm), after formation of mixed SAM MOA and PEG₆-SH (red curve, 887.2 nm), after covalent attachment of 1.0 $\mu\text{g/mL}$ Sub1 via amide coupling (blue curve, 922.9 nm), and finally after incubation in a 100 nM SARS-CoV-2 neutralizing antibody IgG solution (green curve, 934.7 nm). (C) Shift in the λ_{LSPR} peak position ($\Delta\lambda_{\text{LSPR}}$) of the nanoplasmonic biosensing platform as a function of SARS-CoV-2 neutralizing antibody IgG concentrations (100 nM to 1 μM) in PBS buffer. Concentrations were plotted in a logarithmic scale to determine non-specific adsorption at a lower concentration range. The red dotted line represents the blank value obtained from the average of six measurements. The blue dotted line represents the z value, where $z = \text{mean of blank} + 3 \times (\text{standard deviation of blank})$. (D) Graphical representation of the steady-state LSPR response as a function of IgG concentration for the determination of K_d value using Sub1 as a receptor molecule. Langmuir isotherm fitting was used to determine the K_d value. All data points were established by taking the measurement of six individual biosensors.

Review Board and approved under Protocol number 10100. COVID-19 positive patients are classified as SARS-CoV-2 positive by nasopharyngeal qRT-PCR testing. Control plasma samples were collected during the pre-COVID-19 era (2018 or earlier, prior to COVID-19 being present in the USA) by the Indiana University BioBank. Individual nanoplasmonic biosensors in a 96-well format were incubated in a solution containing 10 μL of COVID-19 positive patient plasma (or healthy individual, normal control, COVID-19 negative) and 290 μL of PBS buffer overnight. The following day, the biosensors were rinsed with PBS buffer to remove any loosely bound biomolecules. LSPR extinction spectra were recorded, and the LSPR dipole peak, λ_{LSPR} , was determined for each well.

Spectroscopy and Microscopy Characterizations. All absorption and extinction spectra were collected utilizing a SpectraMax M5 microplate reader from Molecular Devices, LLC in the range of 400–1050 nm. All spectra were collected in PBS buffer (pH 7.2) to keep the bulk refractive index constant. The “background” was a blank coverslip immersed in PBS buffer. The reference (blank) was a biosensor incubated in PBS buffer (no analyte present). Scanning electron microscopy (SEM) images of Au TNPs were obtained using a JEOL 7800F SEM.

Data Processing and Statistical Analysis. *Determining the Size of Au TNPs.* The 53.8 ± 4.9 nm edge length Au TNP size was determined through Image J software and was averaged from 300 individual Au TNP size measurements.

Processing UV-Vis Extinction Spectra. For all extinction spectra, λ_{LSPR} was determined through curve fitting using Origin software, and $\Delta\lambda_{\text{LSPR}}$ was calculated by taking the difference between λ_{LSPR} before and after each fabrication step.

Processing the Calibration Curves, Limits of Detection, and Concentrations of Target Antibodies in Patient Plasma. Calibration curves were developed by plotting $\Delta\lambda_{\text{LSPR}}$ versus the SARS-CoV-2 neutralizing antibody IgG concentration. The concentration was plotted in the logarithm scale in order to investigate non-specific adsorption at a lower concentration range. All calibration curve equations were determined through linear regression using Origin software. Limits of detection (LOD) were determined by (1) calculating the “ Z value” of the blank, where $Z = \text{mean} + 3\sigma$ (σ is the standard deviation of a blank, and mean and σ were determined through six measurements), (2) plugging the Z value into the calibration curve equation as “ Y ”, and (3) solving the equation for “ X ”, where X equals the LOD. All values for calibration curves were determined through the average of six measurements obtained from three different batches of Au TNP-bound coverslips (two sensors from each batch), and each calibration curve was independently analyzed twice (1 week apart from each other) in order to avoid batch-to-batch variation of Au TNP size and uniformity.

Statistical Analysis of Patient Data. Mann-Whitney non-parametric test and area under the curve (AUC) of the receiver operating characteristic (ROC) graphs were plotted using GraphPad Prism at the 95% confidence interval. P -values represent the

following: 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), and <0.0001 (****). The calculated specificity (true negative rate) and sensitivity (true positive rate) were determined based on the literature procedure using eqs 3 and 4, respectively.^{10,19,33}

$$\text{specificity} = [\text{true negative}/(\text{false positive} + \text{true negative})] \times 100 \quad (3)$$

$$\text{sensitivity} = [\text{true positive}/(\text{false negative} + \text{true positive})] \times 100 \quad (4)$$

RESULTS AND DISCUSSION

Fabrication and Characterization of High-Throughput Nanoplasmonic Biosensing Platforms for SARS-CoV-2 Neutralizing Antibody Assays. There are three important structural parameters that control the overall sensitivity and selectivity of our nanoplasmonic biosensing platforms, as shown in Scheme 1: (1) the shape of plasmonic nanostructures as signal transducers; (2) the length of the linker molecules connecting the nanostructure and receptor; and (3) the choice of receptor biomolecules. (1) We selected Au TNPs as LSPR-based nanoantennas because of their high electromagnetic-field enhancement at the sharp tips (Scheme 1C,a).^{28,29} Therefore, any minute changes that occur in the local refractive index upon receptor–analyte interaction substantially influence the LSPR response. Moreover, depending on their edge length, Au TNPs display λ_{LSPR} in the region of 700–1000 nm, where human biofluids have low background scattering and absorption of endogenous biomolecules.^{21,28,34,35} We have previously demonstrated that Au TNPs are extremely stable in human biofluids such as plasma.^{36,37} Finally, their atomically flat and smooth surfaces allow homogeneous linker ligand packing that will reduce non-specific binding in real-life clinical sample analysis.^{36,38,39} (2) We used MOA as a linker molecule because the thiol group forms a strong Au–S bond, leaving –COOH as an end terminal group that can be activated via amide chemistry to covalently attach receptor molecules (Scheme 1C,b–d). Additionally, seven –CH₂ units in the linker bring the receptor in close vicinity to the TNP surface to observe the highest LSPR response while maintaining a closed-packed self-assembled monolayer (SAM) of the linker. We used PEG₆-SH as a spacer to enhance the non-fouling effect (Scheme 1C,b).^{40,41} (3) We studied two different biomolecules as receptors, spike protein sub-unit 1 (Sub1) (Scheme 1C,c) and two epitopes (S14P5 and S21P2) (Scheme 1C,d) for COVID-19 serology IgG assays (Scheme 1C,e–f. Sub1 has been widely used as a receptor molecule for COVID-19 serology IgG assays.^{13,17,18} Previous studies have shown that S14P5 and S21P2 epitopes are highly selective for the detection of SARS-CoV-2 spike protein neutralizing IgG. Herein, we sought to investigate the effects of receptor binding biomolecules on the overall sensitivity and selectivity of our nanoplasmonic biosensing platforms.

The as-synthesized colloidal dispersion of Au TNPs in acetonitrile displays λ_{LSPR} at ~850 nm (Figure S2). Figure 1A illustrates the representative scanning electron microscopy image of the glass substrate-bound Au TNPs with 53.8 ± 4.9 nm edge lengths. We initiated the construction of nanoplasmonic biosensing platforms for COVID-19 serology IgG assays using 53 nm edge-length TNPs as nanoantennas and Sub1 as a receptor molecule. The surface coverage of Au TNPs

onto glass substrates is 73 Au TNPs/ μm^2 . TNPs show λ_{LSPR} at ~860 nm in PBS buffer (Figure 1B, black curve). Upon functionalization of TNPs with a MOA SAM, 30.3 ± 1.5 nm red-shifting (shift to a higher wavelength, lower in energy) of λ_{LSPR} along with peak broadening and reduction of peak intensity is observed (Figure 1B, red curve). There are three potential reasons for these changes in the LSPR property: (1) increase of the local refractive index by the aliphatic backbone of the MOA ligands; (2) interfacial electron transfer between Au–S bonds;⁴² and (3) chemical interface damping of the plasmon.⁴³ Further attachment of Sub1 to MOA SAM-modified TNPs via amide coupling chemistry results in $+\Delta\lambda_{\text{LSPR}}$ of 35.8 ± 3.3 nm without any noticeable differences in peak broadening and decrease in peak intensity (Figure 1B, blue curve). Therefore, further red-shifting of λ_{LSPR} upon Sub1 attachment is caused by the local refractive index change and is directly related to the molecular weight of the protein (the higher the molecular weight, the higher the $\Delta\lambda_{\text{LSPR}}$).^{20,21} Utilizing the nanoplasmonic biosensors, we developed a calibration curve for the IgG assay by incubating wells in different concentrations (100 nM to 1.0 aM) of IgG solution. Measured $\Delta\lambda_{\text{LSPR}}$ values of 11.7 ± 1.0 (Figure 1B, green curve) and $1.5 \text{ nm} \pm 0.5 \text{ nm}$ are determined for 100 nM and 1.0 aM IgG concentrations, respectively. The $\Delta\lambda_{\text{LSPR}}$ versus IgG concentration produces a linear range from 100 nM to 1.0 pM, and the LOD is determined to be 236.6 aM (Figure 1C and Table S2). We believe that this low LOD could be due to the high binding interaction of SARS-CoV-2 Sub1 to SARS-CoV-2 spike protein neutralizing antibody IgG. To evaluate this, we calculated the equilibrium dissociation constant (K_d) using a 1:1 Langmuir model. The K_d value in the steady-state analysis was found to be 862.1 pM in PBS buffer (Figure 1D and Table S4), overall indicating a strong interaction between the Sub1 and SARS-CoV-2 specific IgG.^{9,11,19,31,32}

Our experimental results based on Sub1 as a receptor molecule in the biosensing platform show good sensitivity for SARS-CoV-2 spike protein neutralizing antibody IgG. However, such a large protein of ~8 and ~10 nm in width and height, respectively,^{44,45} is expected to reduce the sensitivity of the assay by limiting the amount of receptor molecules that can be attached to the SAM on the Au TNP, overall decreasing the amount of binding IgG.^{17,21,46,47} Moreover, the distance between the TNP surface and Sub1 is more than 12 nm. It is known that the electromagnetic field of nanostructures decays exponentially with the distance (“decay length”).^{24,48,49} Therefore, the biosensing sensitivity decreases as the analyte molecules bind further away from the nanostructure. Furthermore, using such a large protein could overall decrease the specificity of the assay due to the interaction between unwanted antibodies with the receptor rather than the antibody of interest or different antibodies produced from other viral infections.^{9,25,26} An innovative biosensor designer approach would be the functionalization of nanoantennas with small size receptors (bringing them closer to the nanostructure surface), which also display an extremely high selectivity toward SARS-CoV-2 IgG. A recent study showed that ~18 amino acid length peptides S14P5 and S21P2 (Scheme 1 and Table S1) displayed a stronger recognition for SARS-CoV-2 spike protein neutralizing antibody IgG than SARS-CoV specific IgG. Such an unique SARS-CoV-2 IgG detection approach has not been demonstrated in a solid-state, nanoplasmonic biosensing construct. Therefore, we sought to investigate whether the linear epitopes (S14P5 and S21P2), in

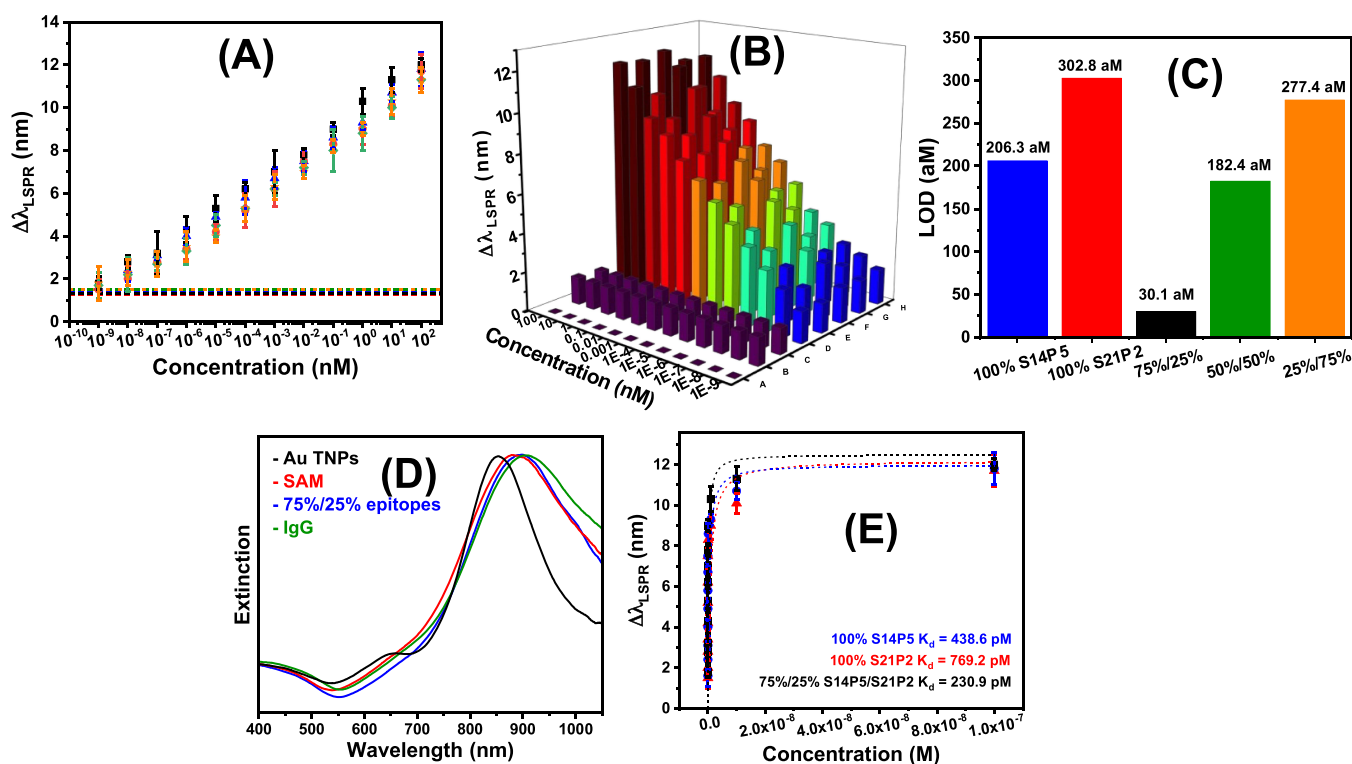


Figure 2. Determination of nanoplasmonic biosensing assay performance using epitopes as receptors. (A) Shift in the λ_{LSPR} peak position ($\Delta\lambda_{LSPR}$) of epitope-functionalized nanoplasmonic biosensing platforms as a function of different SARS-CoV-2 neutralizing antibody (IgG) concentrations (100 nM to 1 aM) in PBS buffer: 100% S14P5 (blue triangles), 100% S21P2 (red circles), 75%/25% S14P5/S21P2 (black squares), 50%/50% S14P5/S21P2 (green diamonds), and 25%/75% S14P5/S21P2 (orange stars). Concentrations were plotted in a logarithmic scale to determine non-specific adsorption at a lower concentration range. Dotted lines represent blank values obtained from the average of six measurements (100% S14P5 = 1.42 nm, 100% S21P2 = 1.25 nm, 75%/25% S14P5/S21P2 = 1.33 nm, 50%/50% S14P5/S21P2 = 1.50 nm, and 25%/75% S14P5/S21P2 = 1.50 nm). (B) Three-dimensional representation of $\Delta\lambda_{LSPR}$ values for epitope calibration curves shown in panel (A) in a multi-well format and in a single instrument run (A = blank, B = false positive, C = false negative, D = 100% S14P5, E = 100% S21P2, F = 75%/25% S14P5/S21P2, G = 50%/50% S14P5/S21P2, and H = 25%/75% S14P5/S21P2). False negative analysis was conducted by incubating the SAM-functionalized Au TNPs (without any receptors) in a 100 nM IgG solution. False positive analysis was conducted by incubating the epitope-functionalized biosensors in PBS buffer without IgG present. (C) Bar graph representing the calculated limit of detections for different epitope ratios. (D) UV-vis extinction spectra of glass substrate-bound TEA-passivated Au TNPs (before surface modification, black curve, 856.9 nm), after functionalization with a self-assembled monolayer (SAM) of 1.0 mM MOA: 1.0 μ M PEG6-SH (red curve, 887.0 nm), after covalent attachment of 75%/25% 1.0 μ g/mL S14P5/S21P2 via amide coupling (blue curve, 896.2 nm), and after adsorption of 100 nM SARS-CoV-2 neutralizing antibody IgG (green curve, 908.3 nm). (E) Graphical representation of steady-state LSPR response as a function of IgG concentration for the determination of K_d value for different receptors: 100% S14P5 (blue), 100% S21P2 (red), and 75%/25% S14P5/S21P2 (black). Langmuir isotherm fitting was used to determine the K_d value. All data points were established by taking the measurement of six individual biosensors.

place of Sub1 as receptors in our biosensor construct would enhance both the sensitivity and specificity of our serological COVID-19 IgG assay.

Highly Selective SARS-CoV-2 Neutralizing Antibody IgG Assays. Epitope S14P5 is located close to the RBD region of SARS-CoV-2 Sub1, whereas S21P2 resides close to the fusion peptide (where virus fusion occurs) of SARS-CoV-2 Sub2.²⁶ To further enhance the sensitivity of our nanoplasmonic biosensor-based IgG assay, the first parameter that we studied was to optimize the concentration ratio of the epitopes. This is because epitopes S14P5 and S21P2 are present in different regions of the SARS-CoV-2 spike protein. We functionalized MOA SAM-modified Au TNPs with 100% (1 μ g/mL) S14P5, 100% (1 μ g/mL) S21P2, 75%/25% S14P5/S21P2, 50%/50% S14P5/S21P2, and 25%/75% S14P5/S21P2 solutions in PBS buffer. Upon attachment of these epitope receptors, a $\Delta\lambda_{LSPR}$ between 9.8 ± 0.8 nm to 10.4 ± 0.3 nm is detected (Figure 2D, blue curve, Figure S3). The magnitude of red shifts is significantly smaller in comparison to the full spike subunit 1 ($\Delta\lambda_{LSPR} = 35.8 \pm 3.3$ nm). We believe that this is

due to the much smaller size of epitopes compared to Sub1, and therefore, the change in the refractive index around the TNP is substantially lower upon the epitope attachment. Our observation is in agreement with the literature for LSPR-based biosensing.^{17,21,46,47} Next, we developed calibration plots for epitope-functionalized biosensors by varying the concentration of SARS-CoV-2 neutralizing antibody IgG (100 nM to 1.0 aM) and then determined $\Delta\lambda_{LSPR}$ from the extinction spectra (Figure 2A,B,D and Figure S3). 75%/25% S14P5/S21P2 produced the highest $\Delta\lambda_{LSPR}$ for 100 nM IgG ($\Delta\lambda_{LSPR} = 12.0 \pm 0.3$ nm), and a $\Delta\lambda_{LSPR}$ between 11.3 to 11.8 ± 0.8 nm was detected for the other epitope ratios. Interestingly, different epitope-functionalized nanoplasmonic biosensors display a dynamic range over a concentration value of 10 orders of magnitude (100 nM to 10 aM). The dynamic range is further extended to 1.0 aM for 75%/25% S14P5/S21P2-functionalized biosensing platforms. This wide dynamic range is specifically important because antibody levels can significantly vary between person-to-person and during different stages of infection for the same individual. Therefore, it is highly

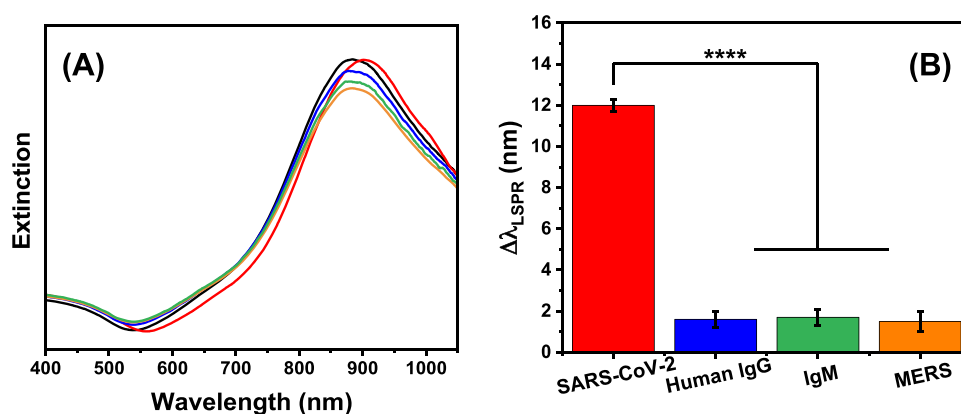


Figure 3. Specificity and cross reactivity test of epitope-functionalized nanoplasmonic biosensing platforms. (A) UV-vis extinction spectra and (B) representative shift in the λ_{LSPR} peak position ($\Delta\lambda_{\text{LSPR}}$) of 75%/25% S14P5/S21P2-functionalized nanoplasmonic biosensors (black line) after incubation in a solution of 100 nM SARS-CoV-2 neutralizing antibody IgG (red line/red bar), 100 nM human IgG (blue line/blue bar), 100 nM IgM (green line/green bar), and 100 nM MERS-CoV IgG (orange line/orange bar) in PBS buffer. Two-tailed unpaired *t*-tests, *****p* < 0.0001. All data points were established by taking the measurement of six individual biosensors.

advantageous for a single instrument, high-throughput assay to precisely measure the level of SARS-CoV-2 spike protein neutralizing antibody IgG over a concentration range spanning 11 orders of magnitude. To further confirm the attachment of IgG onto epitope-functionalized biosensors, we performed SEM analysis before and after the introduction of 10 nM IgG to the nanoplasmonic biosensing platform. As shown in Figure S4, we visually observed bright white spots around the edges and corners of Au TNPs after incubating the nanoplasmonic biosensing platform in a 10 nM concentration of IgG. Those bright white spots are not present in the “after epitope” SEM image, confirming that IgG is only present after it has been introduced.

Next, we calculated LODs for different epitope ratios and obtained a variation between 30.1 aM (75%/25% S14P5/S21P2) and 302.8 aM (100% S21P2) (Figure 2C and Table S2). Based on the results, we believe that mono-epitope S14P5 possesses a stronger affinity to SARS-CoV-2 IgG than mono-epitope S21P2. Interestingly, dual-epitope S14P5/S21P2 (75%/25%)-functionalized biosensors display the lowest LOD for SARS-CoV-2 IgG detection. It is reported in the literature that SARS-CoV-2 Sub1 has a higher affinity to produce more IgG antibodies than Sub2. This is due to the fact that Sub1 contains the RBD region, and thus in COVID-19, the maximum IgG is produced against the RBD region.^{10,11} Because S14P5 is in close proximity to the RBD region,²⁶ it may explain why having the higher percentage of S14P5 in dual-epitope-functionalized nanoplasmonic biosensors produces a better sensitivity. However, IgG is also produced against Sub2 of SARS-CoV-2.^{12,25,26} Therefore, the nanoplasmonic biosensing platform containing a small concentration of the linear epitope corresponding to Sub2, i.e., S21P2, is required to capture all the IgG produced against SARS-CoV-2. This could be the reason that an improved LOD is observed when the nanoplasmonic biosensing platform was fabricated with dual epitopes (75%/25% of S21P2/ S14P5) compared to only using 100% of the linear epitope corresponding to Sub1 (S14P5). The result is in agreement with the sensitivity trend observed under our experimental data (75%/25% < 50%/50% < 25%/75% < 100% S21P2). This could be due to the amount of IgG produced against S14P5 being greater than the amount produced against S21P2, meaning that when a lower percentage of S14P5 compared to S21P2 is present (25%

compared to 75%), not enough IgG is detected and excess receptors for S21P2 are present.

The calculated LOD of 30.1 aM for SARS-CoV-2 IgG detection using dual-epitope-functionalized nanoplasmonic biosensing platforms is more than 7-fold better than the biosensor fabricated with SARS-CoV-2 Sub1 as receptor molecules. Additionally, the calculated LOD is 10^3 – 10^7 times better than those from other LSPR-based SARS-CoV-2 IgG assays that utilize SARS-CoV-2 Sub1 as the receptor molecule (e.g., 0.5 pM and 9.5 nM)^{17,18} and 10^9 -fold improved LOD for IgG assay compared to the solution-phase colorimetric assay consisting of identical epitope-functionalized Au nanoparticles (LOD = 3.2 nM).⁹ Furthermore, the current LOD for IgG detection is more than five-fold better than our previously reported nanoplasmonic biosensing platforms, which was constructed using anti-IgG as the receptor.¹⁹ Anti-IgG is a bulky biomolecule, and thus, IgG would bind to anti-IgG nearly 10 nm away from the TNP surface. The result presented herein further supports our argument of distance-dependent receptor–analyte interaction, the overall influence on the local refractive index change, and LSPR-based biosensing sensitivity. Steady-state LSPR extinction measurements as a function of IgG concentrations show that SARS-CoV-2 IgGs display the strongest binding affinity for dual epitopes of S14P5/S21P2 (75%/25%) with a calculated K_d value of 230.9 pM, in comparison to mono epitope-functionalized biosensing platforms (Figure 2E, Figure S3, and Table S4). Our calculated K_d values for different epitopes, with varying percentages, are in agreement with the literature where K_d values were determined from kinetic analysis.⁹ Nevertheless, the abovementioned results suggest that dual-epitope S14P5/S21P2 (75%/25%)-functionalized nanoplasmonic biosensors would be the most ideal sensing platform for further investigation such as selectivity and clinical sample COVID-19 serology IgG assays, and therefore, the remaining part of the current work utilizes S14P5/S21P2 (75%/25%)-functionalized nanoplasmonic biosensing platforms.

Selectivity and Cross Reactivity Signature of Nanoplasmonic Biosensing Platforms. After establishing the right surface modification chemistry of plasmonic nano-antennas with dual-epitope functionalization to achieve the highest sensitivity, we next focused on investigating the selectivity of our nanoplasmonic biosensing platforms for

SARS-CoV-2 neutralizing antibody IgG assays. We performed selectivity tests by incubating the biosensors described above in 100 nM human IgG (common, non-virus specific IgG) and 100 nM IgM solutions (Figure 3). The $\Delta\lambda_{\text{LSPR}}$ values are 1.6 ± 0.4 and 1.7 ± 0.4 nm, respectively (Figure 3 blue curve/bar, Figure 3 green curve/bar). These values are negligible in comparison to the $\Delta\lambda_{\text{LSPR}}$ value of 12.0 ± 0.3 nm determined for 100 nM SARS-CoV-2 neutralizing IgG (Figure 3, red curve/bar). We believe that some transient interactions between S14P5/S21P2 epitopes with non-virus specific IgGs led to <2.0 $\Delta\lambda_{\text{LSPR}}$ and this argument is also applicable for IgM. We also performed a cross reactivity test by incubating our standardized biosensors in a solution of 100 nM Middle East respiratory syndrome (MERS)-CoV IgG. MERS-CoV is a coronavirus related to SARS-CoV-2 with many similarities in their nucleic acid genome and viral proteins.^{4,5,7,12} As shown in Figure 3 (orange curve/bar), the biosensors produced a $\Delta\lambda_{\text{LSPR}}$ of 1.5 ± 0.5 nm in MERS-CoV IgG. This value is even smaller than the lowest concentration-dependent $\Delta\lambda_{\text{LSPR}}$ shifts of SARS-CoV-2 neutralizing antibody IgG (1 aM, $\Delta\lambda_{\text{LSPR}} = 1.8$ nm). Finally, the two-tailed unpaired *t*-test provides a *p*-value of <0.0001 . Taken together, the results of nanoplasmonic biosensor selectivity and cross reactivity tests are highly encouraging in the context of utilizing this technology for COVID-19 serology IgG assays in clinical samples.

Performance of Nanoplasmonic Biosensing Platforms in SARS-CoV-2 IgG Detection from Clinical Plasma Samples. To demonstrate the clinical usefulness of our dual-epitope-functionalized nanoplasmonic biosensing platforms, we assayed SARS-CoV-2 IgG in a cohort of 216 patient plasma samples separated by training and validation cohorts (Figure 4). Before performing the serological IgG

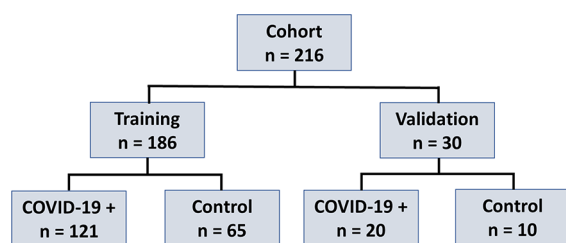


Figure 4. Study population ($n = 216$). COVID-19+ patients are classified as SARS-CoV-2 positive by nasopharyngeal qRT-PCR testing. Control plasma samples were collected during the pre-COVID-19 era (2018 or earlier, prior to COVID-19 being present in the United States).

assays, we developed a calibration curve utilizing the biosensing platform for SARS-CoV-2 IgG in 10% human plasma (Figure S5). We determined an LOD of 63.6 aM (Table S3). First, we validated our sensing approach using a training cohort of 186 plasma samples that included (1) adults who tested positive for COVID-19 by nasopharyngeal RT-PCR and (2) pre-pandemic plasma samples, designated as normal control (NC), from adults who have no records of respiratory infection. Importantly, the high-throughput assay capability of our biosensing platforms allowed measurements of 92 patient samples in a single instrument run, and each plasma sample was analyzed in duplicate to determine the average IgG concentration. Because of the ultrasensitivity of our assay, patient plasma samples were diluted 30X with buffer to reduce non-specific binding of unwanted biomolecules

present in plasma. As shown in Figure 5A, the concentration of IgG varies from 450 to 7900 fg/ μL , which is in agreement with literature reports that suggest that symptomatic patients produce a higher amount of IgG than asymptomatic individuals, and furthermore, the level of IgG can vary based on variations and mutations of SARS-CoV-2.^{9,10,13,14} We determined a *p*-value < 0.0001 through Mann–Whitney *t*-tests (Figure 5A) with a receiving operating characteristic area under the curve (ROC-AUC) equal to 1.00 (Figure 5B), suggesting that our biosensing platform can differentiate between a disease group and a control group with high statistical significance. In addition, the training cohort yields a calculated 100% sensitivity and 100% specificity, with the signal threshold for a positive test result equal to the mean plus three times the standard deviation of healthy individuals. Therefore, we show that our assay can obviate false positive and false negative responses.

To further evaluate the accuracy of our nanoplasmonic biosensing platforms, we analyzed an independent set of blinded validation cohorts. This validation cohort consisted of 30 plasma samples, including 20 individuals who tested positive for SARS-CoV-2 by RT-PCR and 10 healthy individuals (NC). Doubly randomized samples were provided to the researcher without any prior knowledge about the sample identity. As shown in Figure 5C, our biosensing platform can correctly identify 20/20 COVID-19 positive and 9/10 NC samples with calculated specificity and sensitivity of 90% and 100%, respectively. We are not certain why the patient 5 (NC 5) sample provides a false positive response, but we believe it could simply be an assay error. Another possible explanation includes non-specific binding from high levels of pre-existing antibodies in blood¹⁴ or cross reactivity of the IgG corresponding to the SARS-CoV-2 S and N proteins, which has previously been detected in $\sim 10\%$ of healthy individuals.⁵⁰ Moreover, this validation cohort resulted in an ROC-AUC equal to 0.997 (Figure 5D). Taken together, due to the demonstrated high specificity and sensitivity and the capability of assaying IgG directly from a low volume of clinical plasma samples via a high-throughput assay, we believe that our biosensor platform demonstrates the potential to serve as a promising alternative to gold standard immunoassays for COVID-19 serology IgG antibody detection and quantification.

CONCLUSIONS

Sensitive, selective, and quantitative COVID-19 serology IgG assays with high-throughput capability are an unmet need for epidemiological studies and monitoring immune response and durability of immunity as part of the global immunization programs. Utilizing the unique LSPR properties of noble metal nanostructures, we have developed a nanoplasmonic biosensing platform for the detection of SARS-CoV-2 neutralizing antibody IgG in a high-throughput format. By utilizing the unique SARS-CoV-2 linear epitope ratio (S14P5/S21P2) as receptor molecules in the biosensor construct, we have achieved an LOD as low as 30.1 aM. Based on binding affinity calculations (K_d in pM) and selectivity (human IgG and IgG analytes) and cross reactivity (MERS-CoV IgG) tests, we believe that our detection approach is highly selective for the detection of IgG in patients with COVID-19. The ultra-sensitivity allows the assay to be performed in 30X diluted patient plasma samples, thus improving the selectivity by reducing the adsorption of other circulating immunoglobulin.

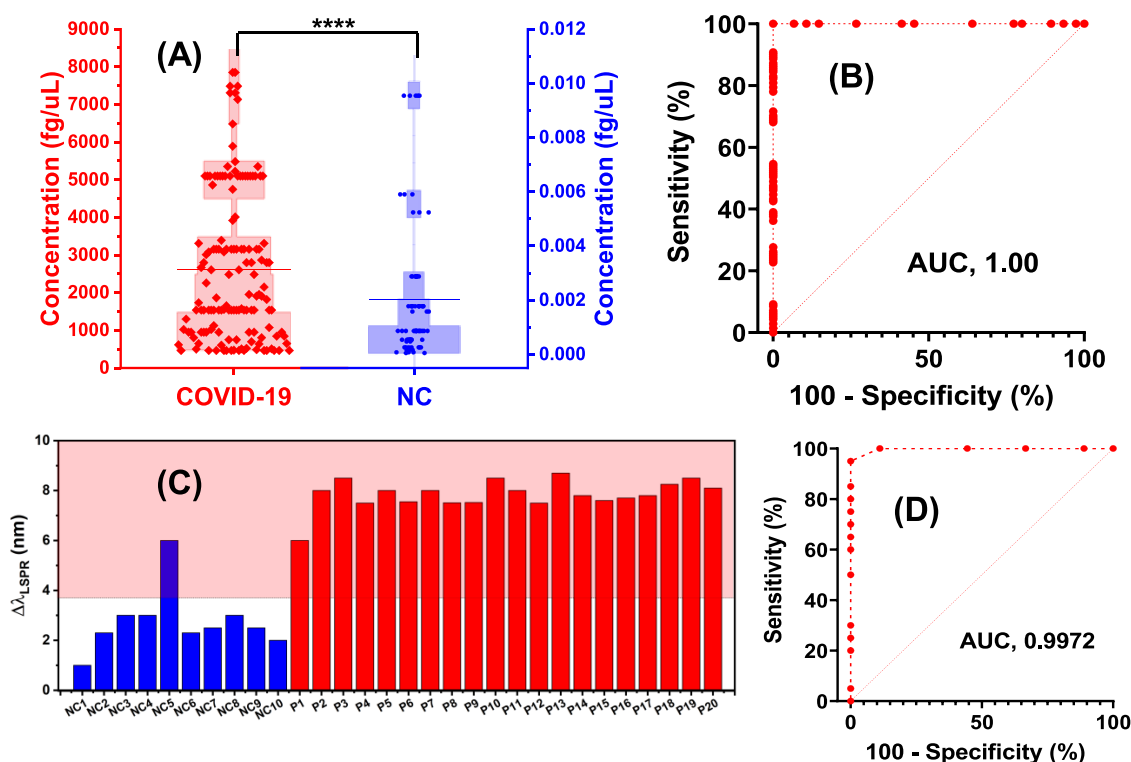


Figure 5. Clinical sample assay using nanoplasmonic biosensing platforms and the validation test. (A) SARS-CoV-2 neutralizing antibody IgG assay in 121 COVID-19 positive patient plasma samples versus 65 healthy individual plasma samples (normal control, NC). Mann–Whitney nonparametric *t*-test, *****p* < 0.0001. (B) ROC curve of NC versus COVID-19 positive patient samples of IgG. (C) Validation test results for the quantification of SARS-CoV-2 neutralizing antibody IgG in 20 COVID-19 positive patient plasma samples and 10 NC plasma samples. Samples were randomized during the assay. Transparent red box represents the threshold cutoff obtained from the mean + 3(standard deviations) of NC patient samples in the training cohort (*n* = 65). (D) ROC curve of validation test results for NC versus COVID-19 positive patient samples of IgG.

The detection dynamic range spans 11 orders of magnitude, allowing for the SARS-CoV-2 neutralizing antibody IgG assay from 10 μ L of plasma of real-world samples. The clinical applicability of our assay has been further demonstrated by detecting and quantifying SARS-CoV-2 IgG in 141 COVID-19 positive and 75 healthy individuals' plasma samples. Through a blind assay, we have achieved 90% specificity and 100% sensitivity.

Clearly, antibody testing will be the key for future epidemiological study and monitoring immunity against vaccination for COVID-19. We believe that our label-free assay provides a powerful analytical tool for serology testing that would be useful for understanding the immune response and durability during the active infection or re-infection with different SARS-CoV-2 variants. The biosensing platform is capable of analyzing multiple patient samples in one instrumental run, enabling it a high-throughput capability, which is important to analyze identical samples in multiple sets. Although our COVID-19 serology IgG assay demonstrates great promise, it requires human plasma; perhaps, further optimization of this biosensing platform in non-invasive human biofluids, such as saliva, can be pursued to develop a user-friendly, noninvasive serological test. Another area of our current nanoplasmonic biosensor construct that can be improved is transforming the platform to a 384-well format without compromising the sensitivity and selectivity. Taken together, keeping in mind that epitopes are conserved within SARS-CoV-2 variants, we believe that the potential utility of our high-throughput nanoplasmonic biosensing platform would be significant with the consideration that new SARS-

CoV-2 variants with growing number of mutations have been constantly emerging, and thus it will be important to determine specific antigen–antibody interactions and their consequences on long-term immune response in various population-based studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsami.2c06599>.

Tables for peptide sequences, different calibration data, and different binding dissociation data, photograph of the nanoplasmonic biosensing platform, UV–visible absorption spectrum, UV–visible extinction spectra, binding dissociation curves, and calibration plots in human biofluids (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Rajesh Sardar – Department of Chemistry and Chemical Biology, Indiana University–Purdue University Indianapolis, Indianapolis, Indiana 46202, United States; orcid.org/0000-0001-9680-1301; Email: rsardar@iupui.edu

Author

Adrianna N. Masterson – Department of Chemistry and Chemical Biology, Indiana University–Purdue University Indianapolis, Indianapolis, Indiana 46202, United States

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acsami.2c06599>

Notes

The authors declare no competing financial interest.

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