

Modular Label-Free Electrochemical Biosensor Loading Nature-Inspired Peptide toward the Widespread Use of COVID-19 Antibody Tests

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Cite This: *ACS Nano* 2022, 16, 14239–14253



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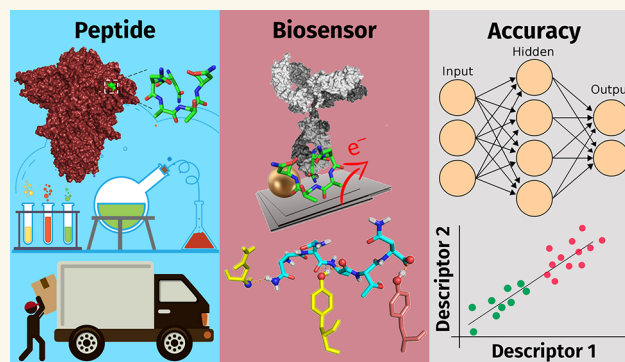
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ABSTRACT: Limitations of the recognition elements in terms of synthesis, cost, availability, and stability have impaired the translation of biosensors into practical use. Inspired by nature to mimic the molecular recognition of the anti-SARS-CoV-2 S protein antibody (Ab^S) by the S protein binding site, we synthesized the peptide sequence of Asn-Asn-Ala-Thr-Asn-COOH (abbreviated as PEP2003) to create COVID-19 screening label-free (LF) biosensors based on a carbon electrode, gold nanoparticles (AuNPs), and electrochemical impedance spectroscopy. The PEP2003 is easily obtained by chemical synthesis, and it can be adsorbed on electrodes while maintaining its ability for Ab^S recognition, further leading to a sensitivity 3.4-fold higher than the full-length S protein, which is in agreement with the increase in the target-to-receptor size ratio. Peptide-loaded LF devices based on noncovalent immobilization were developed by affording fast and simple analyses, along with a modular functionalization. From studies by molecular docking, the peptide–Ab^S binding was found to be driven by hydrogen bonds and hydrophobic interactions. Moreover, the peptide is not amenable to denaturation, thus addressing the trade-off between scalability, cost, and robustness. The biosensor preserves 95.1% of the initial signal for 20 days when stored dry at 4 °C. With the aid of two simple equations fitted by machine learning (ML), the method was able to make the COVID-19 screening of 39 biological samples into healthy and infected groups with 100.0% accuracy. By taking advantage of peptide-related merits combined with advances in surface chemistry and ML-aided accuracy, this platform is promising to bring COVID-19 biosensors into mainstream use toward straightforward, fast, and accurate analyses at the point of care, with social and economic impacts being achieved.

KEYWORDS: noncovalent immobilization, gold nanoparticle, electrochemical impedance spectroscopy, SARS-CoV-2, machine learning



Lessons from the 2002 severe acute respiratory syndrome virus (SARS-CoV) outbreak have driven the adoption of technological methods to combat the Coronavirus disease 2019 (COVID-19) pandemic, which has deeply impacted our health and lifestyle.¹ For instance, while the SARS-CoV was identified in 5 months using methods such as transmission electron microscopy (TEM) and genetic sequencing, these tools assured the identification of the new SARS-CoV-2 after only 3 weeks.² Conversely, the translation of testing technologies capable of delivering point-of-care (POC) and extensive diagnostics trials (many trials per

thousand people) into the real world remains a crucial challenge.^{2,3} On-field and mass testing kits play a pivotal role at the front line to combat epidemics and pandemics as they

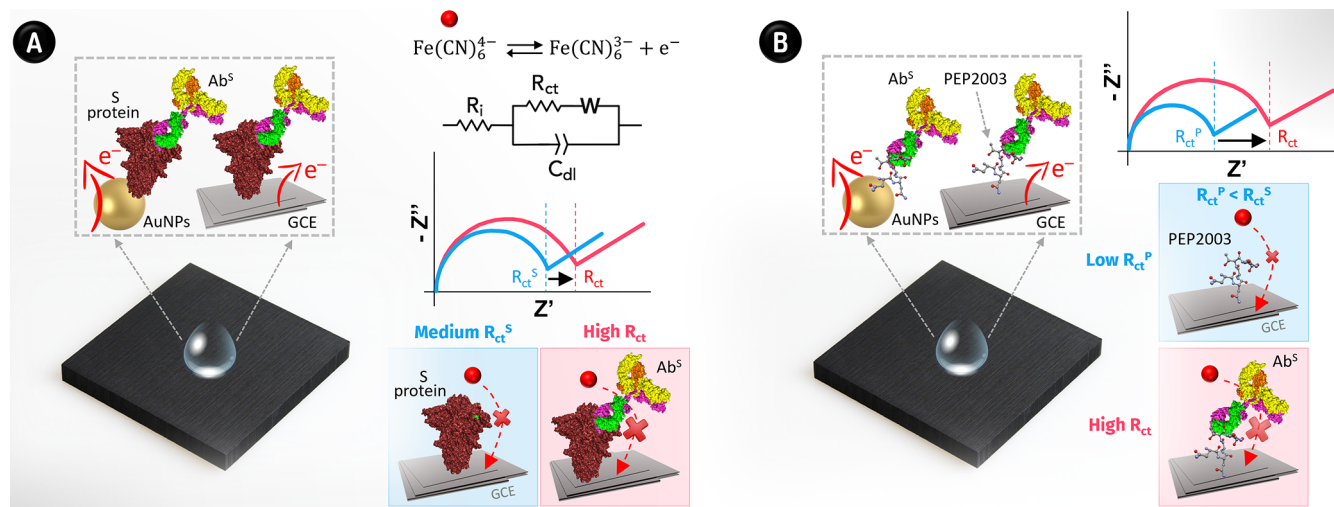
Received: May 4, 2022

Accepted: August 11, 2022

Published: August 15, 2022



Scheme 1. Increase in sensitivity by the peptide-loaded biosensor. (A) LF-EIS device with protein and (B) peptide adsorbed on negatively charged AuNPs and GCE to sense Ab^{S} by measuring the blocking of electron-transfer reactions between the redox probe ($\text{Fe}(\text{CN})_6^{4/3-}$) and electrode. The AuNPs are crucial to increase the electron-transfer kinetics. The peptide-loaded route leads to higher relative variations in R_{ct} (calculated from the Randles circuit as shown in (A)) before (blue) and after (red data) Ab^{S} with binding, hence affording a superior sensitivity. R_{p} , CPE, and W mean, respectively, ionic resistance of the solution, constant phase element, and Warburg element. R_{ct}^{S} and R_{ct}^{P} mean the signals in the absence of Ab^{S} using S protein and peptide as receptors, respectively, with $R_{\text{ct}}^{\text{S}} > R_{\text{ct}}^{\text{P}}$ as illustrated in the images (highlighted by blue/red rectangles) for GCE/receptor surfaces (the same principle is valid for AuNP/receptor surfaces). The illustrations are out of scale. In contrast to the S protein arrangement in (A), it is noteworthy that this protein is hypothesized here to be mainly adsorbed on AuNPs with steric hindrance of its binding domains as discussed later.



decentralize diagnoses from clinical laboratories and provide comprehensive feedback on the health of a large number of individuals, thus decreasing the population infection risks and assisting the decision-makers against virus spread (e.g., in actions such as the timely isolation of infected people and gradual relaxation).⁴ Additionally, these devices can afford in-home monitoring of chronic diseases and medical assessment in resource-poor settings, further contributing to life-saving procedures by remotely providing physicians with rapid physiological data, ultimately aiding clinical prognoses, early treatments, and personalized medicine.^{5,6}

The primary methods used for sensing the SARS-CoV-2 have contributed to slowing down the spread of COVID-19, with a significant global impact even after the economy opened.⁷ Although valuable, such approaches show key limitations that limit their usefulness, throughput, and massive applications. While polymerase chain reaction (PCR) and enzyme-linked immunosorbent assays (ELISA) typically suffer from time-consuming tests, high workload (multistep routines), and the requirement for skilled personnel, costly centralized laboratory settings, and special reagent kits, the serological assays generally face drawbacks such as relatively low sensitivity and long sample preparation time.^{2,5,8–10}

There is thus a critical need to develop hand-held and scalable methods for fast, field-deployable, accurate, and daily detection of COVID-19 infection.^{10–12} Label-free (LF) impedimetric biosensors have been devised as a promising strategy to cope with POC and mass testing. This detection is based on the effect of the receptor–target binding over the charge-transfer resistance (R_{ct}), which is usually measured utilizing electrochemical impedance spectroscopy (EIS) with a reversible redox probe in solution.^{7,13} As merits, this type of biosensor covers only one immunoaffinity step, dispensing secondary recognition elements or enzymes. Therefore, besides

the advantages originated from electrochemical detection (low cost, simplicity, high sensitivity, and compatibility for the miniaturization of assays and instrumentation), the LF-EIS biosensors can enable disease diagnoses/screening faster and simpler than the broadly used competitive and sandwich immunoassays.

Concerning the biomarker, while antibody assays struggle to provide early diagnostics due to the lag in their production, such analyses carry benefits over the nucleic acid and antigen tests that include, but are not confined to, a much longer detection window (2 to 3 months), higher stability of antibodies in comparison with viral RNA, and more uniform distribution of Ab in blood than virus in respiratory tract samples.¹⁴ These properties are helpful to translate biosensors into clinical practice.^{7–10} In addition to disease screening and diagnostics, the Ab tests play a key role in identifying convalescent plasma donors, supporting the deployment of vaccines and guiding decision making on social restrictions, contact tracing, and lockdown because they can detect prior infection and assess immunity.^{14,15}

Biological macromolecules have been widely used as recognition elements for specifically sensing analytes. For instance, the spike (S) protein has been applied to target binding antibodies, i.e., the immunoglobulins IgG and IgM, for COVID-19 diagnostics. As a limitation, similar to other biological molecules, the S protein is difficult to express in prokaryotic cells,⁹ therefore making its synthesis laborious and costly and limiting its large-scale production. In fact, the recognition reagents were in short supply during the COVID-19 pandemic,⁵ limiting the total testing capacity. Further, biological macromolecules can undergo denaturation that impairs their “lock and key” property, with the ensuing incidence of false negatives and positives.^{11,16} To date, the poor stability of big-molecule recognition elements (typically 3

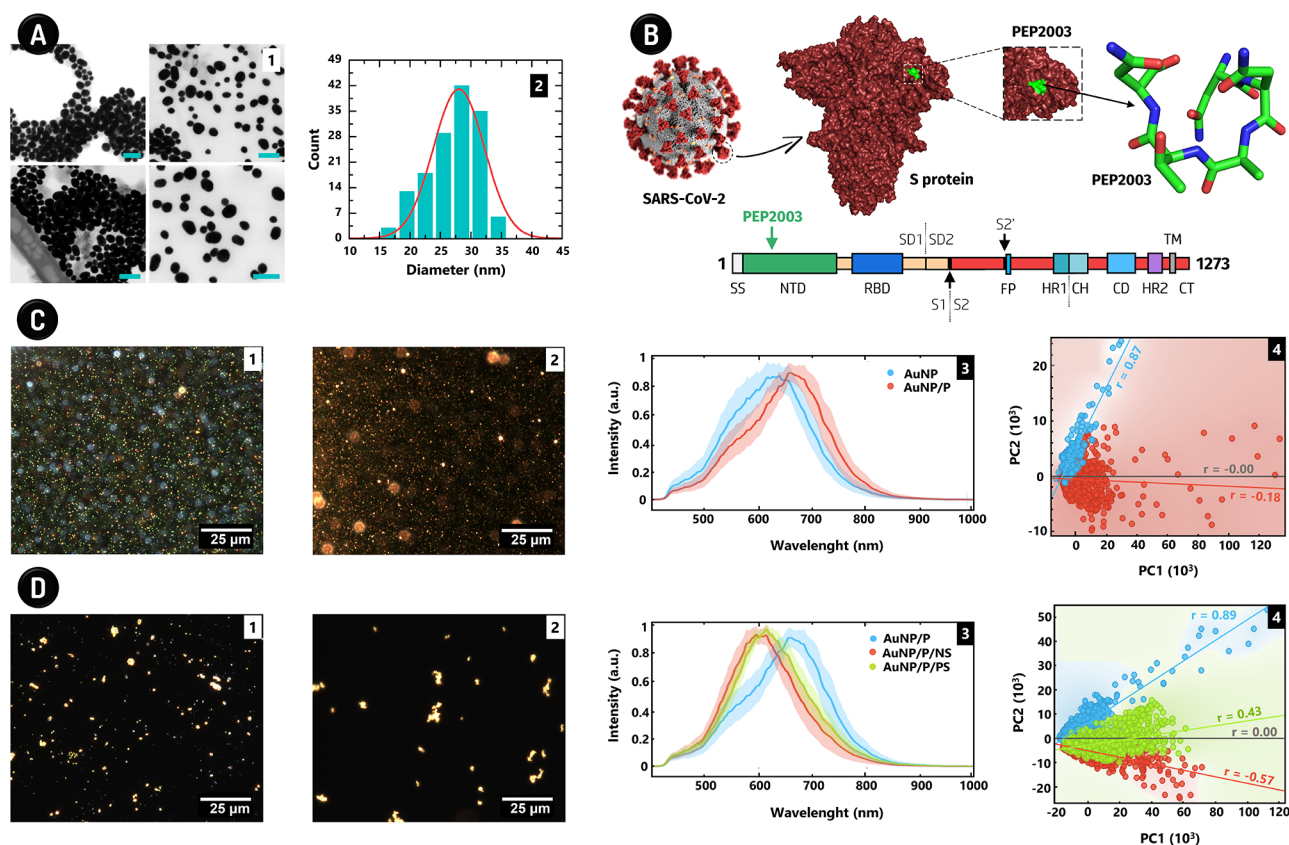


Figure 1. Nanoparticles, PEP2003, and studies on its adsorption on AuNPs and anti-Ab^S specificity. (A) STEM image (1) and average size distribution of the AuNPs (2). The scale bars represent 60 nm. (B) Structural illustrations of the SARS-CoV-2, S protein, and PEP2003 as indicated. In the latter case, the colors in red, blue, and green mean oxygen, nitrogen, and carbon, respectively. The bottom image shows the primary structure (amino acids 1 to 1273) of the SARS-CoV-2 S protein⁴⁵ with indication (green arrow) of the PEP2003 position in the NTD (between the amino acids 121 and 125: Asn-Asn-Ala-Thr-Asn-COOH). SS, S2', FP, HR1, CH, CD, HR2, TM, and CT mean, respectively, signal sequence, protease cleavage site, fusion peptide, heptad repeat 1, central helix, connector domain, heptad repeat 2, transmembrane domain, and cytoplasmic tail, whereas the black arrows indicate protease cleavage sites. (C) Enhanced dark-field microscopy–hyperspectral images of AuNPs (1) and AuNP/PEP2003 (AuNP/Pep) conjugates (2) and the resulting average spectra (3) and PCA plots (4). (D) Enhanced dark-field microscopy–hyperspectral images of AuNPs/Pep after interaction with control sample (CS; 1) and positive serum (PS; 2) and the resulting average spectra (3) and PCA plots (4). In (C) and (D), the data colors in (3) are valid in (4).

days when stored at 4 °C in electrolyte) along with their complex and low-yield synthesis have contributed to impair commercialization and clinical adaptation of biosensors due to obstacles such as improper handling, transportation and storage, and high cost.¹⁶

Protein subunits such as receptor-binding domain (RBD) and peptides (5 to 15 amino acids) have been devised as promising solutions to cope with the issues mentioned above of biosensors loading big-molecule bioreceptors. Cai et al.¹⁷ used a peptide-loaded assay for the chemiluminescence detection of COVID-19 IgG and IgM. The peptides can be synthetically synthesized via reproducible routines, and they are also not amenable to denaturation, showing mechanical and thermal stabilities. To date, these subunits can maintain their “one-lock, one-key” interaction ability with ligands even at high temperatures (e.g., >100 °C).¹⁸ Therefore, nature-inspired peptides identified from structural analyses and computational modeling to mimic biomolecule epitopes have garnered considerable interest in developing the next-generation class of POC biosensors.^{11,16}

To address the gaps in the current state-of-the-art biosensing technologies as discussed before, we here developed an LF-EIS immunosensor based on a peptide mimicking the N-terminal

domain (NTD) in S protein (it is located on the N-terminal subunit, S1)¹¹ for sensing anti-SARS-CoV-2 S protein Ab (abbreviated herein as Ab^S). Our goal is to generate a straightforward, fast, and accurate COVID-19 screening method loading a stable (long shelf life), easily synthesized, and cost-effective recognition element. Screening of patient sera and plasma was successfully reached by taking advantage of (1) bioreceptor-related features, (2) advances in surface chemistry, and (3) high accuracy, which are key to bring the devices into mainstream use. (1) Inspired by nature to mimic anti-Ab^S binding, the peptide (abbreviated as PEP2003) was used to produce a glassy carbon-based LF-EIS biosensor. Originated from a no template control (NTC) portion of the S protein NTD, such a peptide was gleaned from phagolysosomal proteolysis using a virtual proteolytic cleavage tool.^{19,20} PEP2003 avoids denaturation risks, and it can be chemically synthesized.²¹ (2) This peptide was physisorbed on a glassy carbon electrode (GCE) coated with gold nanoparticle (AuNPs) through a fast routine (30 min). In contrast to covalent functionalization, physical adsorptions avoid the sp²–sp³ rehybridization of carbon, thus preserving its conductivity.²¹ Such a process further can be easily tailored to sense other targets from a simple, fast, and universal drop-casting

step. (3) PEP2003 was impressively found to generate a sensitivity higher than the full-length S protein (Scheme 1), which is supported by the increase in the target-to-receptor size (TRS) ratio.¹³ This high sensitivity was key to ensure accurate COVID-19 screening by allowing a high dilution of the samples (500-fold). In addition to delivering the analysis of small-volume samples (12.0 nL), this step inhibits the nonspecific adsorption of proteins (biofouling) as the interferences present in the biofluids are diluted to negligible contents,⁸ preventing the occurrence of false positives.

We further used a recent shortcut to reach accurate diagnoses, that is, the amalgamation of biosensing advances with machine learning (ML) to treat the device data.^{22,23} The impedances were subjected to processing by methods such as the algorithm sure independence screening and sparsifying operator (SISSO). This supervised approach converts the recorded input features into low-dimensional and straightforward mathematical equations aiming at accurate qualitative and/or quantitative analyses even from a small number of training sets, thus meeting the trade-off between accuracy and simplicity/speed of computation.^{24–27} In this work, SISSO was capable of providing the classification of 39 samples into two groups, prepandemic individuals (15) and COVID-19 convalescent patients (24).

RESULTS AND DISCUSSION

Functionalization and Peptide. The method relied on noncovalent adsorption of the recognition element on GCE/AuNPs from simple drop-casting steps to make the immunoassays. The routines for recognition element immobilization and detection lasted roughly 125 and 35 min, respectively. Briefly, the AuNPs (6.0 μ L, 1 h to guarantee solvent evaporation) and receptor (6 μ L, 1 h; PEP2003 and S protein were tested as elements to recognize Ab^S) were subsequently dropped onto GCE followed by the protocols of incubation in diluted samples (6 μ L, 30 min), washing, and probe dropping (1.0 mmol L^{−1} [Fe(CN)₆]^{3/4−}) for EIS detection. The increase in R_{ct} with target binding was used as the transducer response to detect Ab^S (see Scheme 1).

Using citrate as a biocompatible reducer, the method described by Turkevich-Frens was adopted to synthesize the AuNPs by affording a simple, low-cost, and reproducible routine.²⁸ Spherical AuNPs (Figure S1) had an average diameter of 28.0 ± 0.3 nm based on images by scanning transmission electron microscope (STEM; Figure 1A). In accordance with analyses by dynamic light scattering (DLS), as expected, the AuNPs increased in size when dispersed in water, with the hydrodynamic radius (R_H) being 17.0 ± 0.1 nm (average diameter: 34 nm). From ultraviolet–visible (UV–vis) spectroscopy tests, these dispersed AuNPs led to an absorbance peak at 520 nm, which is in agreement with the DLS results, as it implies the incidence of AuNPs with an R_H of around 18 nm.^{29,30} The dispersed AuNPs had a zeta potential of -29.0 ± 1.6 mV because of the occurrence of citrate-adsorbed surfaces (see Scheme 1A), hence assuming a negative electron density that likely contributed to their electrostatic anchoring on the GCEs and the succeeding receptor adsorption as discussed later.

Located in the S protein NTD, the PEP2003 is comprised of five amino acids, namely, Asn-Asn-Ala-Thr-Asn-COOH (Figure 1B). The presence of amine and amide bonds in this peptide was confirmed through Fourier transform infrared (FTIR) spectroscopy, with a broad band assigned to C=O

out-of-plane bending (amide VI, 598.2 cm^{−1}) and characteristic peaks of N–H out-of-plane bending (721.8 cm^{−1}), amide II and elongation of the C–N bending (1531.8 cm^{−1}), and C=O (amide I, 1651.9 cm^{−1}) and N–H stretching (primary amine, 3287.3 cm^{−1}; Figure S2).^{31–36}

Colloidal Stability. The AuNPs were dispersed in water before dropping on GCE, while the recognition elements and samples were diluted in electrolyte to perform immunoassays. Hence, water was selected as dispersant medium to assess the colloidal stability, corona formation, and noncovalent modification of the AuNPs in dispersion. From experiments of UV–vis spectroscopy, these bare AuNPs showed a good colloidal stability. The absorbance data remained practically constant for 2 h, signaling the absence of any sedimentation over this period (Figure S3). This stability is likely owing to electrostatic repulsions between the negatively charged nanoparticles.³⁷

Confirming the Noncovalent Modifications. The adsorption of the receptors onto AuNPs was confirmed through bulk (DLS, hyperspectral imaging, UV–vis spectroscopy, and static light scattering, SLS) and surface approaches (X-ray photoelectron spectroscopy, XPS). The nanoparticles were diluted in water for the bulk characterization tests as aforesaid. In accordance with the electrostatic adsorption of the S protein and PEP2003, these bioreceptors caused the AuNP zeta potentials to change to more positive values (Table S1), being roughly -15.0 mV in both cases. Compared with the bare AuNPs, the conjugates of AuNP with S protein and PEP2003 showed a slightly superior R_H (20 nm; Table S1 and Figure S4). The existence of interactions between the AuNPs and bioreceptors was also revealed by UV–vis absorption analyses (Figure S5). The biomolecules led to a shift in the absorption peak (initially at 525 nm) of 4 nm, suggesting their adsorption, as the binding and proximity of the biomolecules on AuNP surfaces can alter the absorption wavelength.³⁰ This plasmon coupling effect can be used for colorimetric sensing and studies on molecular conformational modification.^{38–41}

Hyperspectral imaging analyses also indicated the noncovalent adsorption of the peptide onto AuNPs. Such measurements provided a multivariate signature of the dispersions (AuNP and AuNP/PEP2003) by combining dark-field imaging with spectrophotometry assays.^{42,43} Spectra ranging from 400 to 1000 nm were collected at each pixel, thus generating a robust analysis. The spectra from AuNPs and AuNP/peptide could be easily recognized as confirmed by the nonsupervised method of principal component analysis (PCA; Figure 1C). The resulting average angular coefficients (r) were found as $+0.87$ for AuNP and -0.18 for AuNP/peptide.

The formation of single-composition corona layers (protein or peptide) around the AuNPs was indicated by SLS. The values of gyration radius (R_G) and R_H increased by ± 5 nm with each PEP2003 additional layer (see Figure S4), meaning the peptide can cover the AuNPs and thus contribute to the stabilizing corona effect (Table S1). The corona thicknesses could be obtained by differential centrifugal sedimentation (DCS) using a correction model reported by Davidson et al.⁴⁴ The S protein-formed corona thickness was revealed to be 17.4 nm, whereas this value was 2.3-fold lower for the PEP2003, 7.6 nm. The corona layer created by this small-size peptide was only detected when doubling its concentration (Figure S6). While the S protein is about 18–23 nm long and comprises 1160 to 1400 amino acids,^{37,46} the peptide is composed of only five amino acids, thus supporting the prior discrepancies.

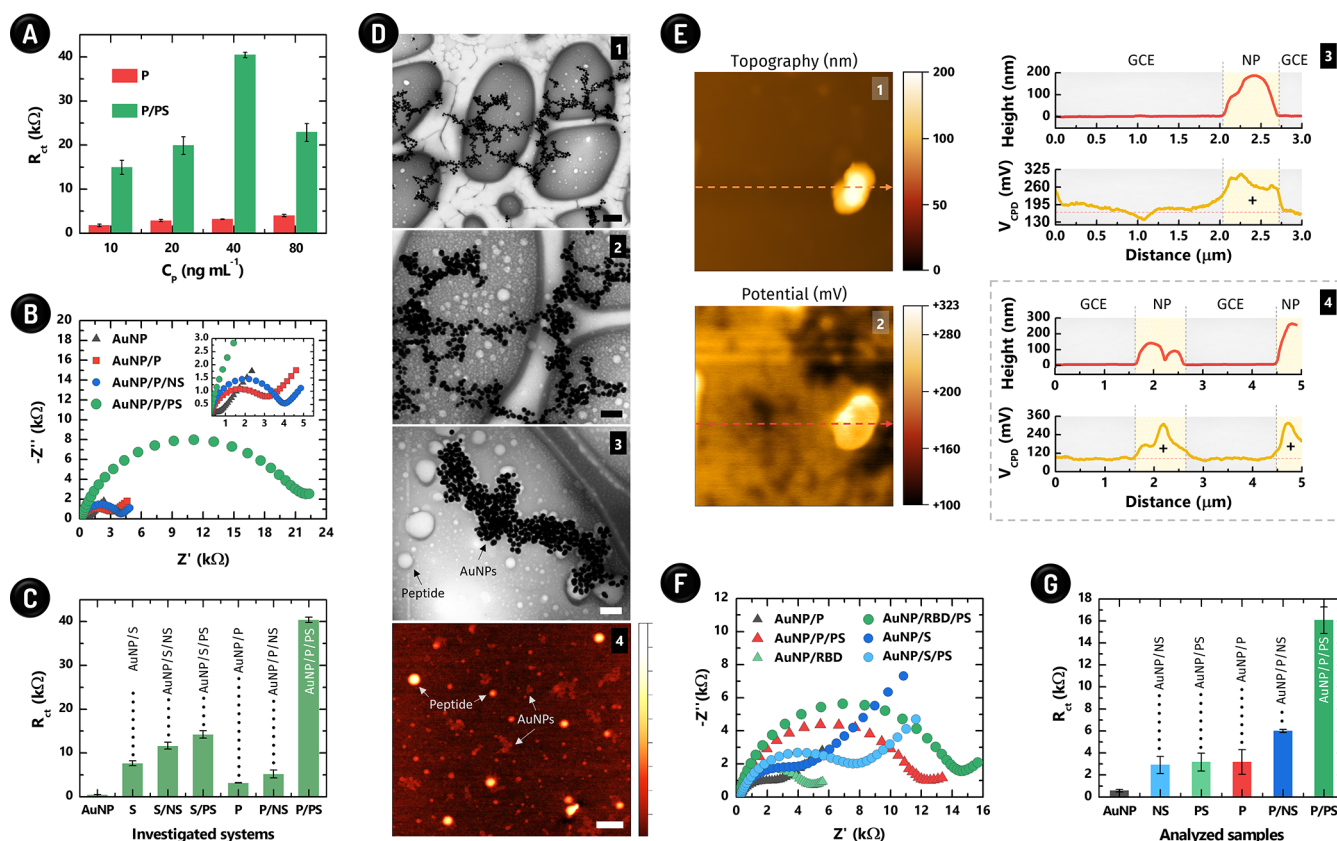


Figure 2. Immunoassays. (A) R_{ct} before and after exposures to a positive serum (1:500 v/v in electrolyte) to assess the peptide concentration (C_p). (B) Nyquist plots for GCE after exposures to AuNP, AuNP/peptide (AuNP/P), AuNP/peptide/negative sample (AuNP/P/NS), and AuNP/peptide/positive sample (AuNP/P/PS). Inset shows an enlarged view of the high-frequency data. Z'' and Z' mean imaginary and real impedances, respectively. (C) Resulting R_{ct} using PEP2003 or S protein (S) as bioreceptor. The investigated systems are further discriminated in the graphic (vertical texts). (D) STEM (bright-field signals; 1–3) and AFM (4) images of the peptide in the presence of AuNPs on a carbon grid (1–3) and GCE (4). The scale bars represent 100 (1), 250 (2, 3), and 300 nm (4). The height scale in (4) varies from 0 to 20 nm. (E) AFM-KPFM tests of an area ($4 \mu\text{m}^2$) covering AuNPs (NP) on GCE. 2D maps of topography (1) and V_{CPD} (2) and resulting profiles of height and V_{CPD} (3) along a line as underlined in (1) and (2). Further profiles are shown in (4), whose corresponding topography and V_{CPD} maps are shown in the Supporting Information (see Figure S15). (F) Nyquist plots before and after exposures to a positive serum with S protein, RBD, and peptide. (G) R_{ct} data from Nyquist plots in the absence and presence of the peptide.

The prior bulk optical techniques confirmed the non-covalent adsorption of the PEP2003 onto the AuNPs in dispersion. Next, we sought to chemically investigate the solid nanoparticles (after drying) by XPS (Figure S7). The incidence of the N 1s peaks proved the coating of the AuNPs by the S protein and PEP2003. In the latter case, a sensitive detection was only achieved when concentrating the PEP2003 by a factor of 8 times (Supporting Information).

Specificity of the PEP2003 against Ab^S . The peptide proved to be an effective bioreceptor for COVID-19 Ab sensing. From ELISA studies, the optical density responses (at 450 nm) mediated by enzyme-loaded anti-S protein IgG increased with the concentration of PEP2003 in the range from 0.4 to 50.0 ng L^{-1} (Figure S8). This enhancement occurred as a rate of 198.9×10^{-3} per ng L^{-1} ($R^2 = 0.86$). It is noteworthy that the specificity of IgG against SARS-CoV-2 has been reported to be higher than the values achieved by IgA and IgM.^{2,8–10}

Because the specific binds of the peptide to Ab^S lead to a partial electrical neutralization of the AuNP/peptide conjugates as mentioned above, they are expected to cause the aggregation of the nanoparticles,³⁷ as it was indeed observed in serum samples through hyperspectral imaging (Figure 1D).

The aggregation of the peptide-coated AuNPs was more prominent after exposure to a COVID-19 positive serum when compared with incubation in a negative serum, further agreeing with the anti- Ab^S reactivity of the PEP2003.

One should highlight that our immunoassay is not susceptible to bulk colloidal phenomena such as nanoparticle aggregation because only interface interactions occur between the electrode-coating recognition elements and Ab^S present in the samples. In addition, the rates for false negatives and positives could be inhibited as a function of the high sensitivity of the LF-EIS biosensor by allowing a relevant dilution of the patient sample. The prior ELISA and hyperspectral imaging results with AuNP/peptide conjugates in dispersion suggest that the peptide can act as a recognition element for COVID-19 Ab tests.

LF Biosensor. The interactions in the interfaces were monitored by square-wave voltammetry (SWV) or EIS analyses with the probe, i.e., $1.0 \text{ mmol L}^{-1} [\text{Fe}(\text{CN})_6]^{3/4-}$, in solution. In these label-free fashions, the signal-transduction mechanism is driven by the steric hindrance that originates from the physisorption of biomolecules on the electrode, slowing down the collisional (or approaching) dynamics of the redox probes with the surface electrode.^{5,6,13,47} Thus, the

probes can approach the underneath electrode for undergoing redox reactions at a slower rate, leading to a suppression of the faradic current and amplification of the R_{ct} when interrogating the sensors with SWV and EIS, respectively (see Scheme 1). Importantly, the variations in these signals are quantitatively related to the target concentration and size, with the biosensor responding significantly to the specific binding of the target to its recognition element. Further, the collisional dynamics of the redox probe is hampered by big-size recognition elements (e.g., proteins), while no significant blocking is noted when using small-size elements (e.g., peptides).¹³ In this regard, the signal relative changes in the absence and incidence of specific binding events are supposed to increase with the target-to-receptor size ratio, as it was indeed observed herein, as the peptide-loading devices led to a superior sensitivity compared with the S protein-based systems as discussed later. One should also emphasize that conformational changes of proteins acting as receptors in biological fluids can also modify the signals of steric hindrance-based immunosensing devices.⁴⁸

Peptide Concentration. LF-EIS analyses ($n = 3$) were made to optimize the PEP2003 concentration that ranged from 10.0 to 80.0 ng L⁻¹ (Figure 2A). Nyquist plots were obtained before and after exposures to a positive serum sample for COVID-19 (1:500 v/v in phosphate buffer), displaying semicircle areas and straight lines tied to charge-transfer- and diffusion-limited steps, respectively.⁴⁹ A similar profile was noted throughout this work. The relative alterations in R_{ct} (collected from the Nyquist plots), which happened at a rate of 0.9 k Ω g⁻¹ L, increased with the amount of peptide adsorbed on GCEs in the range from 10.0 to 40.0 ng mL⁻¹. Such a result is likely due to a gradual increase in the total number of targets bound at the sensor surface, with an enhanced peptide–Ab^S binding-induced steric hindrance being achieved.

With the PEP2003 at 80.0 ng mL⁻¹, conversely, we observed a decrease in the R_{ct} relative changes (see Figure 2A), revealing a drop in the binding-induced steric hindrance that is supposed to be caused by the blocking of the peptide binding sites. Hence, the PEP2003 concentration of 40.0 ng mL⁻¹ was selected for the subsequent assays. Concerning the S protein, its concentration (4.0 μ g mL⁻¹) was based on ELISA assays as recently described in the literature.⁴⁵

Transduction Approach. The spatial blocking effect created by the Ab^S (after binding to PEP2003 and S protein) against the probe collisional dynamics could be more sensitively monitored by EIS through the increase in R_{ct} compared with the SWV detection of the faradaic current suppression (Figure 2B and Figure S9). In fact, EIS is a sensitive method facing small changes at the interface.^{7,13} The relative variations in response after incubation of PEP2003-loaded biosensors in positive serum ($n = 3$) were measured as 25.2% (SWV) and 85.1% (EIS) over the data for a healthy person serum (control). Thus, LF-EIS devices were used in the following experiments.

Sensitivity: Peptide vs Protein. From the prior Nyquist plots (see Figure 2B) and Randles circuit, we collected the R_{ct} values for GCE after modifications with nanoparticles and the bioreceptors ($n = 3$; Figure 2C). In addition to assisting the noncovalent immobilization of the S protein and peptide, the AuNPs enhanced the charge-transfer kinetics as endorsed by the substantial decrease in R_{ct} from 1.0 (bare GCE) to 0.3 k Ω (GCE coated with AuNPs). The R_{ct} attained by the peptide-loaded electrodes after exposure to a negative serum (5.2 k Ω) was 2.3-fold lower than the resistance by the GCE loading the

full-length S protein (11.7 k Ω). This result is ascribed to the smaller size of the peptide, resulting in less prominent steric hindrance against the [Fe(CN)₆]^{4/3-} redox probe. Further, in agreement with the larger TRS ratio in the PEP2003 with immunosensor when compared with the S protein-based system¹³ as discussed before, the tests utilizing the PEP2003 resulted in a superior ability to discriminate the negative from positive serum (see Figure 2C). The relative increments in R_{ct} were around 1.2 (11.7 to 14.3 k Ω , data for negative and positive serum, respectively; ~22.2%) and 7.8 times (5.2 to 40.4 k Ω ; ~676.9%) when employing the S protein and PEP2003 as recognition elements, respectively.

While the peptide is expected to generate both (i) a lower R_{ct} after incubation in negative serum and (ii) higher relative variations in R_{ct} upon exposure to negative and positive samples as observed here, the S protein is supposed to provide a more pronounced increase in the final R_{ct} (after Ab^S with binding) by exhibiting a large number of epitopes, eight, with the probe steric hindrance and, therefore, the reactivity for IgG and IgM being boosted.⁵⁰ In contrast, after exposure to the positive serum, the peptide with biosensor led to an R_{ct} (40.4 k Ω) 2.8 times higher than the resistance attained by the S protein (14.3 k Ω). This data indicates that the load of Ab^S captured by the S protein-based electrode is lower than the number of targeted antibodies bound at the PEP2003-based surface. The steric hindrance of the protein binding sites after electrostatic adsorption on electrodes is hypothesized to trigger its poor anti-Ab^S reactivity, as demonstrated herein through electrostatic potential studies (these data are discussed later).

The higher sensitivity provided by the PEP2003 can thus be attributed to two phenomena, namely, its larger (i) TRS ratio in comparison with the S protein-based system⁵¹ and (ii) anti-Ab^S reactivity in our sensing mode. Both characteristics contribute to amplifying the changes in R_{ct} for negative and positive samples, thus increasing the clinical sensitivity of the impedimetric label-free immunosensor.

Surface Arrangement of the AuNPs and Peptide. The peptides can aggregate into different forms depending on the substrate, concentration, and medium conditions. For instance, according to a recent study,⁵² a SARS-CoV-2 S protein-based peptide could generate globular aggregates (30–40 nm), nanotapes (20–100 nm fibrillar structures), or even mesh-like networks on Cu/carbon grids. The fibril self-assemblies can create globular aggregates because of backbone hydrogen bonds that lead to the stacking of extended peptide structures. Here, the PEP2003 aggregates were found out to be mostly composed of 136.9 $\pm$ 16.6 nm globular structures ($n = 17$), with a few fibrils being verified as well according to AFM images (Figure S10).

From STEM and AFM analyses on a carbon grid and GCE (our electrode for the immunoassays), respectively, the prior globular structures were also observed in the presence of the AuNPs (Figure 2D and Figures S11–S14). Their diameters onto GCE were 112.2 $\pm$ 9.2 nm ($n = 9$). Such structures suffered from degradation under continued exposure to the STEM beam, indicating that they correspond to biological material, i.e., peptide aggregates. The latter aggregated into single spherical nanoassemblies on the AuNPs and, especially, the GCE surfaces, while the AuNPs created both single and aggregated forms. Nonetheless, it is worthy to emphasize that the partial AuNP aggregation on the electrodes did not compromise the reproducibility of the immunoassays that resulted in low standard deviations, as discussed later.

Regarding the S protein, it only led to the self-assembling of fibrillar structures on carbon surfaces according to SEM analyses (Figure S15).

In addition to the anchoring on AuNPs, the physisorption of the recognition elements on GCE experiences electrostatic attractions because the surface of this electrode was also revealed to be negatively charged. This charging is caused by electronic transfers from the AuNPs as demonstrated by analyses of AFM coupled to Kelvin probe force microscopy (KPFM), which provided the monitoring of the contact potential differences (V_{CPD}) of GCE ($V_{\text{CPD}}^{\text{GCE}}$) with AuNP ($V_{\text{CPD}}^{\text{NP}}$; Figure 2E and Figure S16). We found positive differences $V_{\text{CPD}}^{\text{NP}} - V_{\text{CPD}}^{\text{GCE}}$ ($+165.5 \pm 75.3$ mV, $n = 4$), thus revealing the spontaneous transfer of electrons from AuNPs to GCE surface. This phenomenon aims to equalize the Fermi level, and it arises from the distinct work functions (Φ) of the materials, with AuNP $\Phi < \text{GCE } \Phi$ in this case.^{49,53}

Immunoassays with Protein, RBD, and Peptide.

Analyses of another positive serum with RBD as receptor ($n = 3$), an immunogenic fragment from S protein,⁵⁴ also confirmed hypotheses addressed before, namely, the increase in the initial (before) and final (after binding to Ab^S) R_{ct} with the size and number of epitopes on the recognition element (Figure 2F), respectively. When using RBD, the initial and final R_{ct} values were, respectively, 36.6% and 17.3% higher than these same values reached by the peptide-loaded biosensor. Such variations agree with the higher size and anti-Ab^S reactivity of the RBD compared with the peptide.^{7,55,56} Nonetheless, the biosensors loading these bioreceptors led to a similar sensitivity in terms of the variations in R_{ct} which were roughly 9.7 k Ω , further supporting that the peptide can act as an Ab^S bioreceptor for COVID-19 screening.

Effect of the Electrode Biofouling on Clinical Accuracy. The R_{ct} by the peptide-based biosensor to a negative sample was 1.6-fold higher than the background R_{ct} (achieved after incubation in electrolyte; see Figure 2C), indicating the nonspecific adsorption of proteins in the serum onto the electrodes.²³ This biofouling was also indicated by hyperspectral imaging (see Figure 1D). In this case, the AuNP/PEP2003 conjugates showed a subtle aggregation in negative serum that was likely induced by the protein corona nonspecifically adsorbed around the AuNPs in dispersion.⁵⁷ While a blocking agent covering the electrode could decrease the R_{ct} signal in negative sera by preventing biofouling,²³ these protective layers are expected to create a steric hindrance of the small-sized surface-tethered peptides, hampering their ability for Ab^S recognition. Despite the absence of an antibiofouling layer, such a biofouling did not appear to disturb the clinical accuracy, as discussed next.

In the absence of the PEP2003, the protein corona originating from the nonspecific biofouling did not result in any differentiation between the samples of healthy individuals and infected patients. In practice, the R_{ct} achieved for these samples (without recognition element) were similar to each other (~ 3.0 k Ω ; Figure 2G and Figure S17), along with the background R_{ct} (peptide-based electrode in electrolyte). In contrast, the R_{ct} data for negative and positive samples in the presence of the PEP2003 were 1.8- and 5.0-fold higher than the background response, respectively, further supporting the capability of our biosensor in recognizing Ab^S.

Relationship with Concentration. To properly evaluate the sensitivity of the S protein- and PEP2003-loaded LF-EIS biosensors, further tests ($n = 3$) were made by changing the

dilution of a positive serum sample that covered a broad range, varying from 1:1000 to 1:100 v/v in electrolyte (Figure S18). The R_{ct} -to-dilution signals followed a linear model ($R^2 = 0.95$). In agreement with the prior findings, the peptide-based electrodes provided a sensitivity (angular coefficient of the regression curves) 3.7 times higher than the value attained by the biosensors with the S protein. The sensitivities were calculated as 2.1 (S protein) and 7.7×10^{-3} k Ω (v/v)⁻¹ (peptide). Analyses of standard solutions of anti-SARS-CoV-2 S protein IgG (IgG^S) at distinct concentrations were also accomplished (Figure 3). R_{ct} varied linearly at the range from

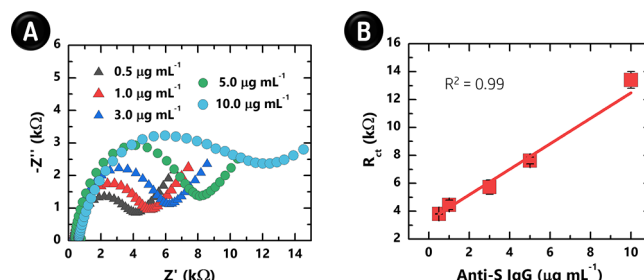


Figure 3. Relationship with concentration. (A) Nyquist plots to standards of IgG^S at different concentrations as indicated. (B) Resulting analytical curve as a function of the R_{ct} responses (k Ω).

0.5 to 10.0 $\mu\text{g mL}^{-1}$ ($R^2 = 0.99$), with the limit-of-detection (LOD) being 0.2 $\mu\text{g mL}^{-1}$. The use of graphene on carbon electrode is an alternative to improve this LOD, as recently described in the literature for IgG^S detection.⁵⁸ In this case, paper-based LF-EIS biosensors with S protein RBD as receptor achieved a LOD as low as ~ 1.0 ng mL⁻¹. One should also note that independent electrodes were used in all of the prior assays for each evaluated condition. The low standard deviations suggest satisfactory levels of reproducibility (see the error bars in Figure 2).

Shelf-Life Evaluation. The stability of the raw materials that are required to prototype the device and prepare the immunosensing interface is commonly a considerable drawback facing the commercialization and translation of biosensors into the real world.⁵⁹ This issue is particularly challenging for the biological macromolecules as described above. According to recent studies,⁶⁰ LF-SWV biosensors bearing the human angiotensin-converting enzyme 2 (ACE2) receptor covalently bound at graphite surfaces were stable for 1 day after being stored under dry conditions at 4 $^{\circ}\text{C}$. When stored at 4 $^{\circ}\text{C}$ in electrolyte, nonetheless, the biosensors preserved 100% of their initial signals for 3 days and a decrease of 23% was observed after 6 days in these conditions. The peptide-loaded devices are expected to show a higher shelf-life, as these small-sized biomolecules are not amenable to denaturation. In fact, our LF-EIS biosensors with the PEP2003 as bioreceptor preserved 95.1% and 83.6% of the initial R_{ct} after 20 and 30 days, respectively. The electrodes were stored dry at 4 $^{\circ}\text{C}$, and the EIS assays were made after exposure to a positive serum sample. Considering the assays since day 1, the global average R_{ct} was 4.2 ± 0.2 k Ω with the intraday confidence intervals ranging from 0.2 to 0.4 k Ω ($\alpha = 0.05$).

Poor Reactivity of S Protein. The electrostatic potentials of the full-length S protein were scrutinized to understand its poor anti-IgG^S reactivity (as previously observed), as these potentials provide information on the most likely orientations of this protein upon electrostatic adsorption on a nanoparticle

bearing a net negative charge. From the data (Figure 4A), the NTD and RDB domains show a surface mostly comprised of

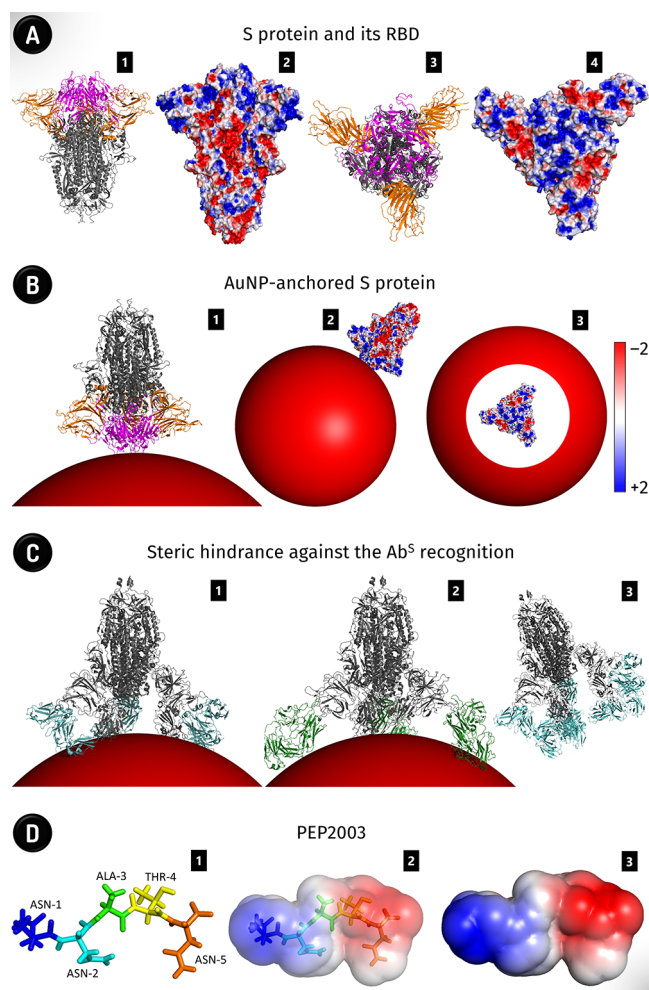


Figure 4. Electrostatic potential assessment. (A) Cartoon representations (1, 3) and electrostatic potentials (2, 4) of the trimeric S protein. (B) Electrostatically driven positioning of the S protein on AuNP (red sphere; 1) and potential images of this noncovalent interaction (2, 3). A bottom view makes it clear that this anchoring involves the protein RBD and NTD (3), with the ensuing blocking of these sites for Ab binding. In the prior cartoon representations, the colored regions highlight the NTD (orange) and RBD (pink) sites. (C) Representations illustrating the steric hindrance of the AuNP-adsorbed protein RBD and NTD, thus hindering the IgG^S capture. S protein with Fab domain of NTD antibodies (PDB ID: 7C2L)⁶² (1) and Fab domains of NTD and RBD antibodies (PDB ID: 7E8C)⁶³ in the presence (2) and absence of the AuNP (3). In these images, the S protein is displayed in gray, whereas the Ab Fab is colored. (D) Peptide structure (1) and its electrostatic potentials (2, 3) taking up the pose 01 docking conformation (see Table S3). The disposition of the PEP2003 amino acids is the same in the three cases. The blue/red scale in (B) represents the electrostatic potentials (-2 up to $+2$ kT e^{-1}), being valid in (A) and (D) as well.

positively charged regions, whereas the ACE2 anti-receptor binding site is dominantly negative.⁶¹ Based on charge complementarities, we can thus hypothesize that the non-covalent immobilization of the S protein onto AuNPs is subjected to electrostatic interactions involving the negatively charged nanoparticles and the NTD and RDB domains of the

S protein (Figure 4B), which are relevant anti-Ab binding regions.⁶⁴ Thereby, this immobilization is supposed to produce a partial steric hindrance of the S protein binding domains (Figure 4C and Figure S19), with the IgG^S recognition by this protein being compromised, as verified herein.

One should emphasize that the S protein binding sites are also supposed to be partially hidden upon physisorption on GCE because the AuNPs electronically charge its surface as discussed before (see Figure 2E and Figure S16). We have also achieved the electrostatic potentials of the peptide, and the result suggests that it behaves like a dipole, with its N-terminal end carrying a net positive charge (Figure 4D). Although this binding region is subjected to electrostatic interactions with the electrodes, the peptide exhibits distinct anti-IgG^S binding regions as discussed next, thus supporting its ability to recognize the targeted Ab.

Binding of PEP2003 to IgG^S. We used a molecular docking method to assess the PEP2003–IgG^S binding in terms of energy, bound poses, and interaction modes. The 10 most thermodynamically stable poses (Figure 5A and Figure S20)

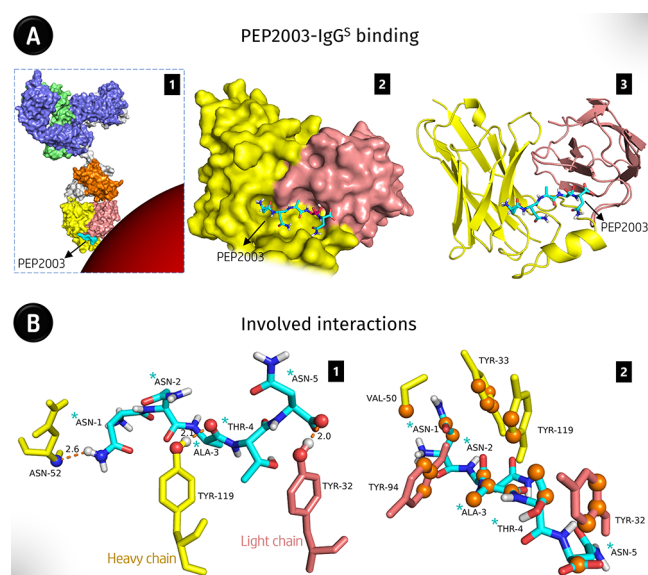


Figure 5. Studies on the PEP2003–IgG^S interactions. (A) Illustrations of the PEP2003–IgG^S interaction with the peptide in cyan (1), along with an enlarged view of this binding (2, 3). (B) Peptide–antibody binding through hydrophobic (1) and hydrogen bond interactions (2). The receptor backbone atoms were omitted, while the variable regions of the IgG^S heavy and light chains are colored in yellow and deep salmon, respectively. The amino acids of the PEP2003 and IgG^S that participate in these interactions are discriminated, with the peptide amino acids being highlighted by cyan asterisks.

showed energies from -6.7 to -7.2 kcal mol⁻¹ (Table S2). This investigation also revealed the amino acids involved in these specific interactions (Tables S3–S5), which were revealed to be driven by hydrogen bond and, especially, hydrophobic interactions (Figure 5B). In agreement with the maintenance of the Ab^S recognition ability by the peptide after its adsorption on the electrode, the above data indicate the incidence of various domains in the peptide with anti-IgG^S reactivity. Another point to be stressed is the conformational adaptability of the IgG^S loops present in the variable region of the fragment antigen binding (Fab),⁶⁵ which can undergo

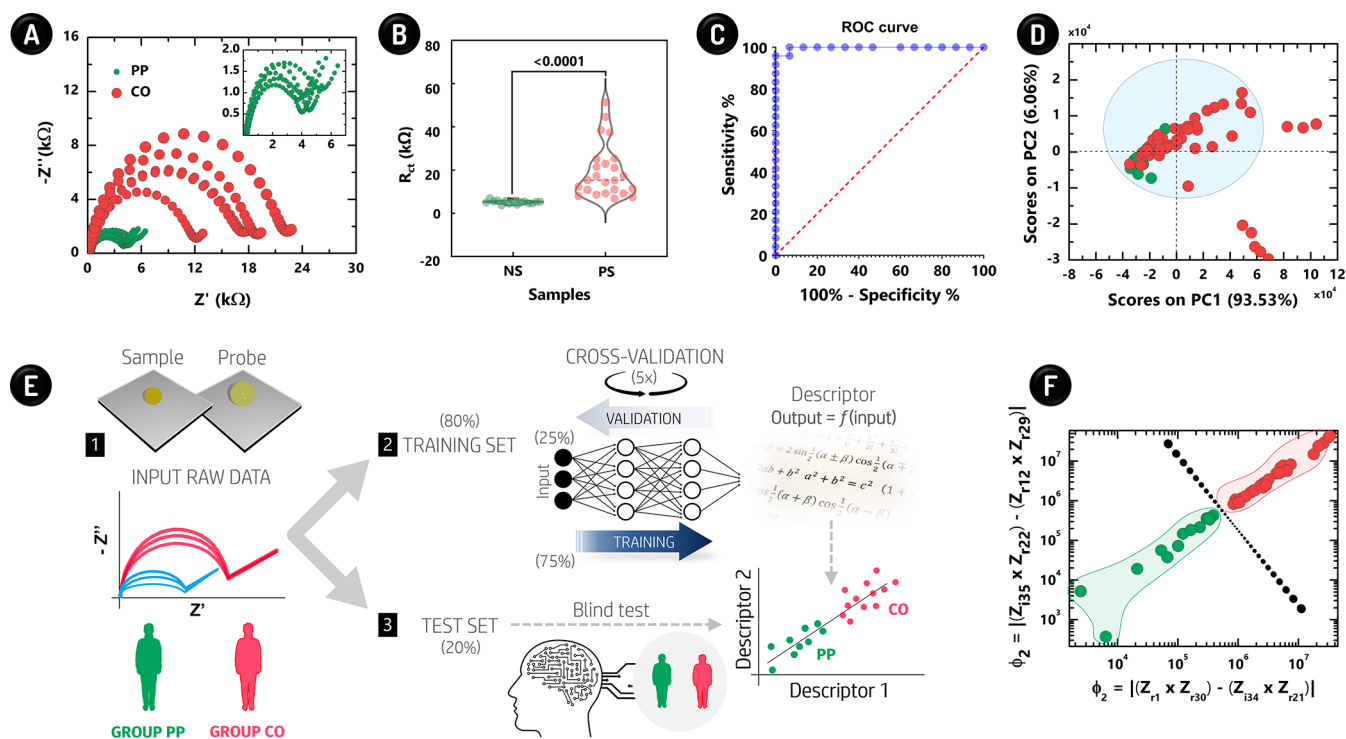


Figure 6. Application to 39 diluted human samples for COVID-19 screening. (A) Typical Nyquist plots of 1.0 mmol L⁻¹ [Fe(CN)₆]^{3/4-} after incubation of the electrodes in sample (1:500 v/v in electrolyte) from some prepandemic individuals (PP) and convalescent patients (CO). The color definitions of the samples are valid throughout this figure. (B) Violin, (C) ROC, and (D) PCA score plots obtained from the values of R_{ct} . The blue circle in the PCA plots highlights 90.0% of the Z data from all of the samples. (E) Treatment of the sensor features by the SISSO for classifying human samples into two groups, i.e., PP and CO. The R_{ct} values recorded of all samples were used as input data (1), which were separated at training and test sets. The training set was split at a ratio of 75/25 (training/validation sets) for optimizing the descriptor through 5-fold cross-validation (2). Then, the trained model was used in the classification of the test set (blind test) for accuracy evaluation (3). (F) Screening of the human samples into PP and CO using two simple SISSO descriptors (ϕ_1 and ϕ_2). In these cases, the superscripts “i” and “r” mean Z'' and Z' , respectively, whereas the numeric superscripts correspond to the frequencies 30.0 k Ω (1), 1.2 k Ω (12), 85.0 Ω (21), 63.4 Ω (22), 8.1 Ω (29), 6.1 Ω (30), 1.9 Ω (34), and 1.4 Ω (35).

conformational changes to accommodate the peptide upon adsorption on GCE/AuNP surfaces.

Effect of the AuNP Size. Analyses with AuNPs smaller than 30 nm also led to the differentiation of negative from positive serum. Such nanoparticles also had a spherical shape with an average diameter of 8.1 ± 0.1 nm based on STEM images (Figure S21). From DLS, the R_H was 10.0 ± 0.3 nm (average diameter: 20 nm) with a zeta potential of -35.0 mV. In this case, the best results in terms of reproducibility and sensitivity were attained by mixing AuNPs and recognition element (1:1 v/v) in the liquid phase for 1 h, followed by the dropping of this dispersion onto the electrode and the same succeeding steps as made with the 28 nm AuNPs. Compared with these nanoparticles, the 17 nm AuNPs promoted a higher charge-transfer kinetics (0.1 k Ω R_{ct}) likely due to their larger surface areas (Figure S22). We could distinguish the R_{ct} of negative from positive serum in the presence of the S protein on 17 nm AuNP-based electrodes, but even more clearly in the presence of the PEP2003 as observed before when using the 28 nm nanoparticles (Figure S23). Such a result reinforces the increase in sensitivity with the TRS ratio.

Although their higher electrocatalytic effect over the 28 nm AuNPs, the smaller ones lead to an increase in the response time (the time needed for a detectable number of target molecules to be captured by the biosensor) according to the first-order Langmuir kinetics (binding-limited regimes), along with a decrease in the total number, i.e., surface concentration,

of targets bound at the biosensor surface.⁶⁶ This decrease originates from the reduced surface concentration of recognition elements (this factor is less relevant for the small-size PEP2003) and the increase in the AuNP curvature, with a spatial blocking being noted against the target approaching. Such phenomena reduce the binding-induced steric hindrance and, thereby the sensitivity of the 17 nm AuNP with biosensor that was revealed to be only commensurate with the data using the 28 nm AuNPs. Specifically, the relative changes in R_{ct} were 3.8 (2.9 to 11.0 k Ω , data for negative and positive serum, respectively; 73.6%) and 3.9 times (4.6 to 18.1 k Ω ; 74.6%) when employing the 17 and 28 nm AuNPs, respectively (see Figure S23). Conversely, one should note that the smaller AuNPs remained stable in the dispersion for 1 week, while the 28 nm AuNPs sedimented after this period (see Figure S3). This higher stability of the 17 nm AuNPs is a relevant feature in terms of the commercial adaption of the method.

COVID-19 Screening. Using 28 nm AuNPs, the clinical applicability of the biosensor was investigated through the recognition of two types of diluted human samples: (i) prepandemic individuals (15) and (ii) COVID-19 convalescent patients (24). These samples were diluted 1:500 v/v in electrolyte (7.3 pH). Apart from the merits of Ab biosensors to deploy POC and mass tests as discussed above, the incidence of IgG in sera or plasma has delivered diagnostics with high accuracy.⁶⁷

From the achieved Bode (Figure S24), Nyquist (Figure 6A), and violin plots (Figure 6B), all of the prepandemic samples showed similar final R_{ct} responses, while the signals for the positive sera were found to be dispersed. This high heterogeneity is likely owing to factors such as age, sex, and genetics that make the tumoral responses of the patients different, modifying the IgG^S concentration and ultimately the data of R_{ct} . Overall, the R_{ct} derived from healthy individuals did not exceed 10.0 k Ω , whereas the responses from convalescent patients ranged from $\sim$ 10.0 up to $\sim$ 50.0 k Ω . Accordingly, some false negatives were noted as the signals from positive sera exceeded the cutoff line. We obtained the receiver operating characteristic curve (ROC) to quantitatively assess the screening performance (Figure 6C). The area under this curve (AUC) was 0.99, with the cutoff R_{ct} being 6.3 k Ω . The sensitivity and specificity were 93.0% and 100.0% (70.2% to 99.7% confidence interval, $n = 3$), respectively.

Following preliminary COVID-19 screening from cutoff R_{ct} values, we attempted to further improve the sensitivity by processing the biosensing data by ML methods, i.e., PCA, partial least-squares-based discriminant analysis (PLS-DA), and SISSO. The exploratory analysis of the data set was first made by PCA. From the score plots (Figure 6D), the two first principal components (PCs) explained 99.6% of the signal variance, with a substantial weight of the low-frequency region (0.1 to 114.0 Hz; Figure S25). This result reveals a higher relevance of the diffusion-limited steps for COVID-19 screening. Approximately 90.0% of the Z values from prepandemic individual samples remained grouped on the score plot negative quadrants (PC1 and PC2).

Next, the PLS-DA method was utilized for classification. We split the training/test samples at a 76/24 ratio. To attain a robust model of PLS-DA, the training set was processed through a randomized 10-fold cross-validation using the same prior ratio for this so-called training/validation set. The accuracies were scrutinized assuming the clinical condition of the samples (prepandemic individuals or convalescent patients) as the true data. The three first latent variables (LV) explained 99.8% of the variance on the X-block. The addition of the LV3 enabled a better separation between the two sample classes in the score plots (Figure S26). Concerning the threshold, the predicted y was 0.5964, below which any Z value was inferred as signaling convalescent patients (Figure S27). We reached an accuracy of 100.0% for the test set (Table S6).

The ability of the immunosensor to ensure accurate COVID-19 screening was further successfully assessed by a descriptor-based ML method, i.e., SISSO, with ensuing 100.0% accuracy as well (Figure 6E). The training/test and training/calibration sets were split at the ratios of 85/15 and 75/25, respectively, with a randomized 5-fold cross-validation to fit two mathematical descriptors. As an advantage over PLS-DA, the SISSO-based learning tasks deliver simple descriptors that meet the trade-off between accuracy and simplicity/velocity of computation. The method generated two equations each covering only four features from the entire Nyquist plots (44 values of Z vs frequency), with the sample classes being clearly classified (Figure 6F).

In contrast to PCA, Z values throughout the range of frequencies were selected by SISSO, meaning both charge-transfer and diffusion rates were important for the classification in this case. The easy-to-use SISSO descriptors benefit the development of ML-aided sample-to-answer analyses on

mobile phones, which would greatly facilitate detection at the point of care because no data treatment or result interpretation by the user is required.⁶⁸ One should also stress that these findings show the potentiality in using ML for smartly picking up specific features from whole spectra for improving the clinical accuracy compared with univariate data-based traditional approaches.^{69,70}

Selectivity of the PEP2003 against Ab^S. Importantly, the results above also support the selectivity of the PEP2003-loaded biosensor, as all of the 15 prepandemic samples tested positive (through ELISA) for the endemic human coronaviruses (hCoVs) 229E, NL43, OC43, and HKU1 (Table S7). Recently, these hCoVs were revealed to be responsible for 5% of the respiratory tract infections around the world,⁷¹ hence being potential interferents against the COVID-19 Ab tests. Cross-specificity of the PEP2003 with hCoVs was not noted by taking up the accurate screening of the samples from prepandemic individuals and COVID-19 convalescent patients as discussed above.

The method also showed a satisfactory specificity when challenged against individual nontarget antibodies (Figure S28), including monoclonal antibodies produced in vitro against the (i) glycoprotein RBD (IgGR1) and (ii) fusion protein containing the RBD and mouse IgG fragment crystallizable domain (mFc, IgGR2). Both these immunoglobulins were built to be specific for RBD, and, hence, they should not be specific for the PEP2003 since this peptide is located in the NTD (between amino acids 121 and 125), i.e., a domain that precedes the S protein RBD. Accordingly, 28 nm AuNP-loaded biosensors did not respond markedly to these anti-RBD nontargeted antibodies, with their signals being commensurate with the background R_{ct} . Last, no signal was verified for the other two antibodies with no direct relationship with the SARs-CoV-2 virus. Specifically, mouse monoclonal antibodies prepared against a (i) guinea pig T-Bet 12 amino acid peptide (IgGT) and (ii) guinea pig CD3 Epsilon recombinant form (IgGE) were scrutinized as nontargeted Ab as well (see Figure S28). The prior results reinforce the anti-Ab^S specificity of the PEP2003.

CONCLUSION

Combining a nature-inspired peptide with advances in surface chemistry, analytical performance, and ML-mediated accuracy, our ultimate goal was to generate a biosensing platform that can be helpful to translate field-deployable, modular, and accurate diagnostic systems into clinical practice. To meet these requirements, we developed LF-EIS biosensors with noncovalent adsorption of a peptide onto glassy-carbon electrodes for COVID-19 Ab testing. Notably, the PEP2003 can be easily obtained by chemical synthesis, and it is not prone to denaturation, thus addressing the trade-off between scalability, cost, and robustness. The biosensor preserved 95.1% of the initial R_{ct} data for 20 days when stored dry at 4 °C. Our findings further confirmed that the peptide can be adsorbed on an electrode while maintaining its ability for anti-SARS-CoV-2 Ab molecular recognition and leading to a sensitivity higher than the S protein.

Regarding the noncovalent functionalization of the recognition elements, it creates a modular architecture, as it can be easily extended to other targets from a simple and fast drop-casting step. One should also note that our method is not a reagentless strategy because of the use of the [Fe(CN)₆]^{4/3-} probe in solution.⁵ Nonetheless, our steric-

hindrance-assisted immunosensor required only one washing step (after preparing the biosensing interface as accomplished during the shelf-life assays).

The synergy between sensitivity and ML assured a good clinical accuracy. The sensitive responses afforded by the biosensor allowed for a large sample dilution, thus preventing cross-reactivity and biofouling interferences, whereas two simple ML-fitted descriptors were able to deliver the COVID-19 screening of 39 human samples (sera and plasma) with 100.0% accuracy.

Although the set of results above is an encouraging indicator, assays of large-cohort patient samples need to be conducted further to reliably scrutinize the (i) capacity of the platform for accurately providing quantitative readouts and (ii) its accuracy. In fact, the existence of different anti-Ab^S binding sites in the peptide may eventually lead to false positives. Thereby, analyses of a large number of bodily fluids are crucial to assess if the platform is effectively able to specifically detect the targeted Ab^S.

In addition, the use of a glassy-carbon electrode limits the commercial adaptation of the approach along with its application at the point of care. In this way, cost-effective and scalable carbon electrodes (e.g., pyrolyzed paper and screen-printed electrodes) should be utilized in the future.⁷² Further efforts should also be focused on creating PEP2003-loaded field-effect transistor biosensors toward sensitive assays. In this case, the distance of the target from the semiconductor surface is narrowed, diminishing the Debye screening effect (i.e., the partial screening of the surface charge of bound targets by highly saline fluids) and ultimately favoring the accumulation or depletion of charge carriers on the semiconductor channel,⁷³ with the sensitivity being improved.

MATERIALS AND METHODS

Chemicals. All reagents used were of analytical grade without prior purification. Ultra-high-purity water (Millipore Milli-Q system) was used in the preparation of the solutions. Potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium ferricyanide ($K_3[Fe(CN)_6]$), sodium citrate ($Na_3C_6H_5O_7$), and gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium chloride (KCl), dibasic sodium phosphate (Na_2HPO_4), and monobasic sodium phosphate (NaH_2PO_4) were acquired from Labsynth (Diadema, SP).

Biological Material. We use the recombinant S protein from SARS-CoV-2, provided by the Cell Culture Engineering Laboratory at COPPE/UFRJ, as a biological recognition element after being diluted in phosphate-buffered saline (PBS) solution ($4.0 \mu g mL^{-1}$).⁴⁵ The RBD protein was purchased from BioLinker (São Paulo, Brazil). The PEP2003 was synthesized according to the literature.²⁰ The lyophilized powder was resuspended in PBS at distinct concentrations (20.0, 40.0, and $80.0 ng mL^{-1}$). The biological samples and human serum were acquired from the Cell Bank of Rio de Janeiro (BCRJ, Brazil). Sera from SARS-CoV-2 positive donor patients were analyzed by an antibody test, i.e., the Medtest SARS-CoV-2 IgG/IgM rapid test cassette (lot COV20030081). Besides, plasma from SARS-CoV-2 negative donor patients were collected before the outbreak (CAAE: 43139921.2.0000.5594). These samples were stored at $-80^\circ C$. For use, they were kept at room temperature until complete thawing. Last, the four nontargeted antibodies (IgGR1, IgGR2, IgGT, and IgGE) were provided by the Biodefense and Emerging Infections Research Resources Repository (BEI Resources).

Gold Nanoparticles. The AuNPs were synthesized according to Turkevich-Frens.²⁸ To synthesize 100 mL AuNP dispersions (final concentration of $0.8 mmol L^{-1}$), 23.0 mg of $HAuCl_4 \cdot 3H_2O$ in 95.0 mL of ultrapure deionized water was heated at $100^\circ C$. Then, 5.0 mL of 1% v/v sodium citrate solution at $100^\circ C$ as well as added into

the gold dispersion under stirring. The mixture was left under stirring until it reached the characteristic red color (approximately 20 min), and then it was allowed to cool naturally. The colloidal dispersions were stored at $18^\circ C$.

Characterization. The AuNPs in the dispersion were characterized by DLS, electrophoretic light scattering (ELS), UV-vis spectroscopy, hyperspectral imaging, and DCS. Further techniques used to obtain information on the nanoparticles, receptors, and interactions were SEM, TEM, STEM AFM, AFM-KPFM, XPS, and FTIR. Details about these measurements are available in the [Supporting Information](#).

Biosensor Preparation. To perform the immunoassays, $6.0 \mu L$ of AuNP dispersion was dropped on GCE. After dispersion evaporation (60 min) with the ensuing AuNP deposition, $6.0 \mu L$ of the biomolecule solution was dropped and left for 60 min as well. Then, the functionalized electrodes were placed in contact with $6.0 \mu L$ of patient or standard samples (diluted 1:500 v/v in phosphate buffer: $0.1 mol L^{-1}$, pH 7.4) for 30 min, followed by the protocols of washing for 10 s under agitation in phosphate buffer and probe dropping ($1.0 mmol L^{-1}$ $[Fe(CN)_6]^{3/4-}$) for electrochemical detection at room temperature. The molecules investigated were the S protein, RBD, and PEP2003. The concentration of S protein and RBD was $4.0 \mu g mL^{-1}$.² For the assays using the peptide, the concentrations ranged from 10.0 to $80.0 ng mL^{-1}$.

Electrochemical Analyses. The GCE (disk with 3.0 mm diameter) was used as the working electrode, whereas Ag/AgCl and platinum wire were employed as reference and counter electrodes, respectively. To ensure surface homogeneity, the GCE was polished in an alumina suspension on a polishing fabric and then placed in an ultrasound bath for 15 min and washed with high-purity water. The assays were done on the Metrohm Autolab PGSTAT 302N system equipped with FRA2 using NOVA 2.1.3 software. The probe ($1.0 mmol L^{-1}$ $[Fe(CN)_6]^{3/4-}$) was diluted in $0.1 mol L^{-1}$ KCl. The EIS tests were conducted at the half-wave potential (roughly 240.0 mV) and the frequency range from 1.0×10^{-1} up to 3.0×10^4 Hz. The SWV tests were performed at a potential window from -0.2 to $+0.7$ mV, modulation amplitude of 20 mV, step potential of 1 mV, and frequency of 20 Hz. The analyses were made in triplicate and at room temperature. Details about ELISA are described in the [Supporting Information](#).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsnano.2c04364>.

Characterization of the biosensor, synthesis of the smaller nanoparticles, electrostatic potential calculation, molecular docking, data arrangement and analysis, enzyme-linked immunosorbent assays, cross-specificity evaluation against endemic human coronaviruses, specificity tests to nontargeted antibodies, supporting tables, and supporting figures ([PDF](#))

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<https://pubs.acs.org/10.1021/acsnano.2c04364>

Notes

The authors declare the following competing financial interest(s): R.S.L, W.A.A., A.C.H.C., and I.R.S.B. are listed as inventors on a patent filing application describing this technology.

ACKNOWLEDGMENTS

We wish to acknowledge Carlos A. R. Costa, Cleyton A. Biffe, Elcio L. Pires, João M. da Silva, Caroline Y. N. Nicoliche, and Otavio Berenguel for helping us with the characterization analyses. All these professionals are from the Brazilian Center for Research in Energy and Materials. This work was sponsored by the Brazilian Coordination for Improvement of Higher Education Personnel (CAPES; grant 88881.504639/2020-01), the Brazilian National Council for Scientific and Technological Development (CNPq; grants 304389/2019-6 and 407951/2021-0), the São Paulo Research Foundation (FAPESP; grants 2017/02317-2, 2019/19939-1, and 2020/05761-3), the National Institute of Science and Technology in Bioanalytics (grant FAPESP 2014/50867-3 and grants CNPq 465389/2014-7 and 401256/2020-0), and the Medical School Foundation (grant CG19110). We would also like to thank the Cell Culture Engineering Laboratory at COPPE/UFRJ for providing the recombinant SARS-CoV-2 S protein and the Biodefense and Emerging Infections Research Resources Repository (BEI Resources Repository), National Institute of Health (NIH), and National Institute of Allergy and Infectious Diseases (NIAID) for providing the monoclonal antibodies used in this work.

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