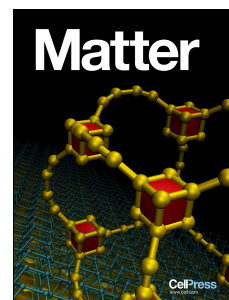


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Low-cost Biosensor for Rapid Detection of SARS-CoV-2 at the Point-of-Care

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Summary:

SARS-CoV-2, the virus that causes COVID-19, has killed over 3 million people worldwide. Despite the urgency of the current pandemic, most available diagnostic methods for COVID-19 use RT-PCR to detect nucleic acid sequences specific to SARS-CoV-2. These tests are limited by their requirement of large laboratory space, high reagent costs, multistep sample preparations, and the potential for cross-contamination. Moreover, results usually take hours to days to become available. Therefore, fast, reliable, inexpensive, and scalable point-of-care diagnostics are urgently needed. Here, we describe RAPID 1.0, a simple, handheld, and highly sensitive miniaturized biosensor modified with human receptor angiotensin-converting enzyme-2 (ACE2) that can detect SARS-CoV-2 using 10 μ L of sample within 4 minutes through its increased resistance to charge transfer of a redox probe measured by electrochemical impedance spectroscopy. The sensitivity and specificity of RAPID for nasopharyngeal/oropharyngeal swab and saliva samples are 85.3% and 100% and 100% and 86.5%, respectively.

Main Text:

SARS-CoV-2, the virus that causes COVID-19, continues to kill people at a staggering pace ¹, threatening the global economy, healthcare systems, and our lives. Despite the urgency of the pandemic, the high cost of production, lack of scalable technologies, and slow detection time hinder the widespread use of currently available diagnostics. Therefore, low-complexity point-of-care diagnostic tests with a rapid turnaround time are urgently needed.

Here, we describe a simple, inexpensive, and rapid test for the detection of SARS-CoV-2 (Figure 1a). RAPID 1.0 (Real-time Accurate Portable Impedimetric Detection prototype 1.0) transforms biochemical information from a specific molecular binding event between the SARS-CoV-2 spike protein (SP) and ACE2 into an electrical signal that can easily be detected.

RAPID 1.0 (henceforth referred to as RAPID) uses electrochemical impedance spectroscopy (EIS), an electrochemical technique extensively utilized for the characterization of functionalized electrode surfaces and the transduction of biosensors ^{2,3}. In our test, the EIS transducer signal reports the selective interaction/binding between the biological receptor immobilized on the electrode surface (i.e., ACE2) and its binding element (i.e., spike protein) ⁴. The binding between these two molecules causes a change in interfacial electron transfer kinetics between the redox probe, ferricyanide/ferrocyanide in solution and the conducting electrode sites. This electrochemical change is then detectable by monitoring the charge-transfer resistance (R_{CT}), the diameter of the semi-arc on the Nyquist plot, which correlates with the number of targets bound to the receptive surface ². The selectivity of an EIS biosensor mostly relies on the specificity between the target and the recognizing bioelement immobilized on the electrode surface and its robustness through the designed architecture surfaces to minimize non-specific binding of the analyte or adsorption of other biomolecules in solution.

We designed the electrochemical device to explore the remarkable binding affinity of SARS-CoV-2 spike protein (SP) to ACE2, its receptor in the human body ^{4,5} (Figure 2a). The working electrode (WE), where the (electro)chemical reaction/interaction takes place and is converted to a detectable analytical signal, was functionalized by a drop-casting method. Enzyme immobilization was achieved by cross-linking ACE2 using the bifunctional chemical cross-linker glutaraldehyde (GA) ⁶. This dialdehyde reacts mainly with the primary amino groups of proteins, for example, the ϵ -amino group of lysine residues or the N-terminal group of the protein chain ⁷.

We used bovine serum albumin (BSA) to block the electrode's surface after immobilization of ACE2. BSA is a functionally inert protein with a high density of superficial lysine residues that is commonly used for biosensor development ⁷.

Using these well-established protocols for bioelectrode development, we first added GA for 1 hour at 37 °C to fully cover the carbon electrode surface generating a cross-linked polymer that enables the covalent anchoring of ACE2 at 37 °C for 1.5 hours (Figure 1b). Next, BSA was added to the surface of the electrode for 30 minutes at 37 °C to block possible remaining active sites (i.e., working electrode's surface areas that were not functionalized with ACE2) thus preventing nonspecific adsorption to the GA layer by other proteins. We also incorporated an additional functionalization step using a 1.0% Nafion solution (Figure S2) to create a protective polymeric membrane enhancing the robustness of the biosensor ⁸. Interestingly, Nafion increased up to 2-fold the sensitivity of the biosensor, particularly when used at a concentration ranging between 1.0% and 1.5% (Figure S2). Given these results, we selected 1.0% Nafion (wt%) for subsequent optimization steps because of its optimal analytical response to low reagent usage ratio ⁸. This anionic membrane enables small positively charged species to cross and preconcentrate close to the biosensing surface. The Nafion layer also enhanced the robustness of RAPID by protecting against biofouling of the electrodic surface when exposed to the sample's complex matrix (e.g., proteins, lipids, and other macromolecules present in biological samples) that may interfere with the detection ^{8,9}.

To ensure that the biological activity of ACE2 was preserved upon immobilization onto the electrode's surface, we evaluated the response of the sensor when exposed to its natural substrate angiotensin II (Figure S1). A sensitive linear response was observed in the range of 1 pg mL⁻¹ to 10 µg mL⁻¹ of angiotensin II, demonstrating that our anchoring and stabilization strategies maintained the functionality of ACE2's active sites and revealing that the biosensor architecture did not obstruct ACE2.

Next, we characterized the effect of each modifier layer (GA, ACE2, BSA, and Nafion) on the electrochemical response of our modified electrode, recording cyclic voltammetry (CV) and EIS measurements in the presence of 5 mmol L⁻¹ of the redox probe potassium ferricyanide/ferrocyanide (Figures 2b and 2c). These results demonstrated that the peak current signal of the redox probe decreased when using CV and the resistance to charge transfer

increased after each functionalization step. The decrease in the peak current signal occurs due to the addition of nonconductive materials (*e.g.*, proteins) that block the active sites of the electrodic surface, hindering the kinetics of charge transfer of the redox probe. Our optimized protocol generated the best analytical signal for the detection of SARS-CoV-2 in human biofluid samples (Figure 1a). It consists of the following 4-steps: 1) modifying the working electrode with the immobilizing agent (GA); 2) covalent attachment of the recognition agent ACE2; 3) addition of the stabilization and active site blocking agent BSA; and 4) incorporating the permselective membrane (Nafion). A detailed protocol describing biosensor preparation, including the production of the screen-printed devices and functionalization, is provided in the Supplemental Appendix.

Our test can be performed at room temperature with minimal equipment and reagents, and costs \$4.67 to produce [\$0.07 to produce the bare electrode, \$4.50 to functionalize the electrode with the recognition agent ACE2, and \$0.10 to coat the electrode with GA, BSA, and Nafion used (Figure 1c)]. The overall cost of RAPID may be further reduced through recombinant production of ACE2 and ACE2 variants¹⁰. Our technology is also highly scalable, as the electrodes can be rapidly mass-produced by using commercially available screen-printers. One laboratory-sized unit is able to produce 35,000 electrodes daily (1.05 M electrodes/month) and this could scaled-up to 10.5 billion electrodes monthly with only 10,000 screen-printers (Table S1). These estimates take into account both the time needed to print the electrodes and all functionalization steps (*i.e.*, 1 hour for GA functionalization, 1.5 hours to incorporate ACE2, 0.5 hours for BSA, and 1 hour for Nafion; total of 4 hours). However, it must be noted that these steps can be fully automated into a production line for industrial purposes, drastically reducing time requirements.

We evaluated the incubation time (*i.e.* time of exposure of the sample to the biosensor to enable sensitive detection), and whether a centrifugation/dilution step was needed to detect SARS-CoV-2 in complex biological samples such as saliva¹¹. Next, we built dose-response curves using free spike protein to assess the effect of incubation time (Figure S4), centrifugation (Figure S3), and sample dilution (Figure S3) on the performance of RAPID. Our optimization steps revealed that an additional centrifugation step was not needed (Figure S3) since the use of neat saliva yielded similar results to those obtained using centrifuged samples. These results demonstrated that our approach is robust and can directly use human samples (NP/OP or saliva) without a prior pretreatment step, thus allowing the application of RAPID for streamlined and rapid point-of-

care diagnosis. We selected 2 minutes as the optimal incubation period of the sample on the working electrode's surface for sensitive SARS-CoV-2 detection in samples considering the detectability and analytical frequency of the tests (Figure S4). Our very minimal incubation time requirement (2 minutes) confirms the favorable configuration of the modified electrode that allows rapid interaction kinetics between the SP and immobilized ACE2 [kinetics constant rate of $10^4 \text{ M}^{-1} \text{ s}^{-1}$ in its natural environment ⁴]. Overall, RAPID provides a result in 4 minutes (2 minutes of sample incubation + 2 minutes to perform the EIS analysis), which is vastly faster than methods currently available for diagnosing COVID-19 (Figure 1c). It is important to note that the total time required to run each blank is an additional 4 minutes. However, we did not take this into account in our testing time calculations because the blanking step can be done before analyzing clinical samples, and we can use the R_{CT} values obtained for the blanks (PBS or VTM) to compare with the patient sample values.

Taking into account the optimal analytical conditions evaluated (Table 1), we built calibration curves for free SP (Figure 2d, S5a, S5b) and heat-inactivated virus (Figure 2e) using the normalized R_{CT} response, defined by the following equation:

$$\text{normalized } R_{CT} = \frac{Z - Z_0}{Z_0}$$

where Z is the R_{CT} of the sample and Z_0 is the R_{CT} of the respective blank solution: phosphate buffer saline (PBS), virus transportation medium (VTM), or healthy saliva. The normalization process of R_{CT} aims to correct eventual fluctuations in the sensor operation, such as the temperature at the testing point or variations due to analyst operation. The dose-response curve for the free SP in PBS solution ranged from 1 fg mL^{-1} to $10 \text{ } \mu\text{g mL}^{-1}$ (Figure S5a). A linear concentration range from 10 fg mL^{-1} to 100 ng mL^{-1} was obtained ($R^2 = 0.993$) and limits of detection (LOD) and quantification (LOQ) were calculated as 2.18 fg mL^{-1} and 7.26 fg mL^{-1} SP based on signal to noise ratios ($S/N=3$) and ($S/N=10$), respectively. We built a dose-response for the free SP in VTM medium at a concentration range from 10 fg mL^{-1} to 100 pg mL^{-1} (Figure S5b). A linear concentration range from 10 fg mL^{-1} to 1 ng mL^{-1} was obtained ($R^2 = 0.995$) and limits of detection (LOD) and quantification (LOQ) were calculated as 6.29 fg mL^{-1} and 20.96 fg mL^{-1} SP based on the signal to noise ratio ($S/N=3$) and ($S/N=10$), respectively. When performed in neat saliva, the calibration curve was built at a concentration ranging from 100 fg mL^{-1} to 100 ng mL^{-1} (Figure 2d). The calculated LOD and LOQ were 1.39 pg mL^{-1} and 4.63 pg mL^{-1} ,

respectively. The higher LODs obtained in saliva and VTM are consistent with the increased sample complexity compared to PBS solution.

The R_{CT} values of Nyquist plots were extracted by the application of an equivalent circuit (Figure S6). The equivalent circuit comprises two semi-arc regions observed in the Nyquist plots, where the first is a non-defined semi-arc at a high-frequency range due to inhomogeneity or defects in the electrode modification step (during drop-casting functionalization) and considerably small ($R_{CT} \sim 10 \Omega$)^{12,13}. The second parallel component of the equivalent circuit comprises an R_{CT} , whose signal intensity was proportional to the logarithm of the SP/virus concentration, and also presented a Warburg element to describe the mass transport (diffusional control).

The concentration range of SP detected by our device was 10-1,000 times lower than that reported in previous studies^{14,15}, thus underscoring the sensitivity of our approach. To assess the diagnostic capability of RAPID, we calibrated our biosensor using titrated solutions of inactivated SARS-CoV-2 ranging from 10^1 to 10^6 PFU mL⁻¹ (Figure 2e). RAPID exhibited high sensitivity presenting a limit of detection (LOD) of 1.16 PFU mL⁻¹, which corresponds to the order of 10^0 RNA copies μL^{-1} ^{16,17}, a viral load that correlates with the initial stages of COVID-19 (i.e., 2 to 3 days after onset of symptoms)¹⁸. Thus, RAPID's LOD and LOQ values are comparable to those of gold-standard approaches such as RealStar[®] SARS-CoV-2, CDC COVID-19, and e-Plex[®] SARS-CoV-2¹⁶ with the advantage of detecting symptomatic and asymptomatic individuals at the earliest stages of the infection allowing for rapid decision-making and the subsequent use of more appropriate and effective countermeasures. To ensure the repeatability, stability, and reproducibility of the results, we carried out three different experiments. First, 21 successive EIS measurements of the medium (PBS) were performed using the same device to verify the drift of the EIS response, yielding an RSD value of 5.3% (Figure S7). These results demonstrated that the device exhibits a repeatable and stable response. Next, the reproducibility of RAPID was evaluated by analytical curves in the range of 1 pg mL⁻¹ to 1 ng mL⁻¹ of SP and the analytical sensitivity of 10 electrodes from different batches was assessed (Figure S8). An RSD value of 6.8% was obtained, indicating that the electrochemical device fabrication and functionalization protocols display high reproducibility.

Next, we evaluated the stability of RAPID at different temperature storage conditions (25 °C, 8 °C, and -20 °C) over 10 days (Figure S9). Analytical curves were generated with SP at a concentration ranging from 1 pg mL⁻¹ to 1 ng mL⁻¹ and the sensitivity was normalized by the mean value of the three different biosensors used immediately after the functionalization steps.

5 The biosensors stored at room temperature did not detect the SP after 24 hours due to loss of enzymatic activity (Figure S9). The sensors stored at 8 °C were stable after 24 hours, but after 48 hours presented decreased sensitivity (around 50% of the initial response) keeping this low sensitivity for 7 days (Figure S9). Biosensors stored at -20 °C exhibited the most promising results since they were as sensitive as those used right after functionalization even after 96 hours

10 and retained 50% of their sensitivity after 10 days of storage (Figure S9).

Next, the performance of RAPID was assessed using both SARS-CoV-2-positive and negative clinical samples from the Hospital of the University of Pennsylvania (HUP) (Table S2 and S3), including a highly contagious SARS-CoV-2 UK B.1.1.7 variant (Tables 2 and S2). All samples were heat-inactivated at 56 °C for 1 hour. The effect of heat inactivation of SARS-CoV-2

15 samples on the analytical response of our biosensor was evaluated through measurements taken before and after sample inactivation at 56 °C for 1 hour (Figure S10). The results indicated that thermal inactivation affected the ability of SP to bind to ACE2, since a decrease of up to 60% was detected in the analytical response for sample 2 after heat inactivation (Figure S10). These results indicate that heat-inactivated clinical samples with very low viral titers may fall below

20 our current limit of detection. Rath and Kumar¹⁹ demonstrated using molecular dynamics simulations that temperatures >50 °C trigger the closing of the spike receptor binding motif (RBM), which buries the receptor binding residues preventing contacts between the SP and the ACE2 receptor. These insights may help explain the results obtained upon thermal inactivation of our biosensor (Figure S10). However, despite this decrease in SP binding to ACE2 upon heat

25 inactivation, the sensitivity of our method still enabled accurate viral detection in clinical samples containing a range of viral titers (Figure 2e).

We also observed that centrifuging the samples did not lead to increased impedimetric detection of the SP (Figure S3). Therefore, the NP/OP and saliva samples were used in VTM and PBS, respectively, following the Food and Drug Administration (FDA) recommendation for regulatory

30 applications. Of note, the detectability of impedimetric measurements after 2 minutes of incubation of the sample on the working electrode's surface was as high as longer incubation

times of 5 and 10 minutes (Figure S4), thus demonstrating RAPID's fast interaction kinetics between the SP and functionalized WE, as discussed above. Thus, we selected 2 minutes of incubation and set as a cut-off value a 10% change in the R_{CT} when compared to the blank solution. Such a cut-off threshold takes into account the LOQ obtained for inactivated virus analysis (Figure 2e), thus allowing discrimination between SARS-CoV-2 negative and SARS-CoV-2 positive samples (Tables S2 and S3).

In blinded tests, we analyzed 139 NP/OP swab samples (in VTM) obtained from patients after heat-inactivation, 109 of which were COVID-19 positive and 30 COVID-19 negative as determined by RT-qPCR and clinical assessment (Table S2). RAPID demonstrated high sensitivity, specificity and accuracy for NP/OP (83.5%, 100% and 87.1%, respectively; Table 2) and saliva (100%, 86.5% and 90.0%, respectively; Table 2) samples. RAPID missed a single sample, which presented a viral count lower than its LOD (10^{-1} RNA copies μL^{-1}). It is worth noting that although the heat inactivation protocol decreased the response of our biosensor due to the inactivation of SP (Figure S10), the outstanding sensitivity of RAPID (Table 1) enabled high detectability (Table 1) and hit rate (Table 2). Out of the 12 negative NP/OP swab samples present in our sample set, 100% were confirmed as SARS-CoV-2 negative by RAPID (Table 2). In addition, the highly contagious SARS-CoV-2 UK variant B.1.1.7 was obtained from a government testing site in Philadelphia (Tables 2 and S3). RAPID successfully identified this sample as positive with a normalized R_{CT} value of 1.10 (Table S2), thus underscoring its ability to detect emerging mutant variants of SARS-CoV-2.

To evaluate RAPID's diagnostic efficacy in a more complex biological environment, we tested saliva samples from 50 patients (Table S3) under the same conditions used for the NP/OP swab samples. The greater complexity of saliva, compared to swab samples, is known to hinder the accurate detection of infectious agents^{11,18}. Saliva is a biofluid that is susceptible to large variations in composition depending on different factors such as the ingestion of food and drinks prior (30-60 minutes) to sample collection, which can lead to the dilution of the saliva matrix, and the insertion of exogenous molecular species that may interfere with accurate detection. Even using highly heterogenous saliva samples, the sensitivity of RAPID remained high (100%), however false positives led to decreased specificity (86.5%), and an accuracy of 90.0% (Table 2). The latter results may be explained by potential interactions between ACE2, which is a

carboxypeptidase and amino acid transporter, and other biomolecules that can be found in neat biofluids, such as regulatory peptides and peptide hormones (e.g., angiotensin, bradykinin, ghrelin, apelin, neurotensin, and dynorphin)²⁰. Thus, we believe the performance of RAPID will improve when using fresh saliva samples at the point-of-care. It is worth noting that among the SARS-CoV-2-positive saliva samples, our test identified as positive two samples that had been previously erroneously detected as negative by RT-qPCR, therefore indicating that RAPID may help correctly diagnose COVID-19 in samples previously misdiagnosed by other methods.

Several key analytical features were used to compare the performance of RAPID with respect to other electrochemical methods reported in the literature (Table 3). Our method provides the highest sensitivity (LOD of 2.8 fg mL⁻¹) for the detection of SARS-CoV-2 spike protein with excellent time of detection and overall cost (Table 3). Additionally, the robustness of RAPID was evaluated in a large clinical sample set (Table S2 and S3), and all results were compared with those obtained by RT-qPCR (Table 2), thus highlighting the reliability of our method. All experiments described thus far (e.g., detection of SARS-CoV-2 spike protein and clinical samples) were performed using the eChip version of the electrode (e.g., Figure 2, Table 2, Figures S2 and S3). After successfully applying the eChip (composed of printed circuit board) to clinical samples (Tables S2 and S3) and obtaining robust and sensitive results (Table 1), we sought to construct an optimized electrode composed of a material that was more accessible and inexpensive to enable scale-up production of RAPID. We selected filter paper as the main component of electrochemical paper-based analytical device (ePAD) as it is easy to handle (maleable), accessible, and inexpensive [paper filter costs \$0.50 per 1 m² whereas printed circuit board (PCB) costs \$40.00 per 1 m²]^{21,22}. We adapted and demonstrated the applicability of ePAD in a portable potentiostat connected to a smart device (Figure 3a). We used the screen-printing method to fabricate the electrodes and combined wax-printing technology to pattern the electrochemical cell onto the paper filter. Thus, the ePAD is composed of more accessible and low-cost material, enabling scalable manufacturing and on-demand testing at the point-of-care^{21,22}.

In order to enhance the detectability (*i.e.*, the LOD) of RAPID, we added 2.5-fold increased volumes of the modifiers (GA, ACE2, BSA, and Nafion) on the surface of the WE during the fabrication process. This approach allowed higher sensitivity towards the detection of SP, which was used to generate a calibration curve (Figure 3b). We attribute the enhanced detection (7-fold

increase) of the paper-based version of RAPID compared to the phenolic-based electrode (eChip) to the higher amount of recognition element (ACE2) used on the working electrode's surface. However, it is worth noting that the eChip version already demonstrated excellent performance at detecting SARS-CoV-2 (Tables 1 and 2) and, although its sensitivity can be further increased by using a higher concentration of ACE2 (Figure 3b), this would increase the cost of the test since recombinant ACE2 accounts for 95% of the final cost of RAPID 1.0 (Figure 1c).

RAPID presented higher accuracy, specificity, and selectivity than most existing electrochemical methods available for SARS-CoV-2 detection (Table 3)¹⁶. We also assessed RAPID's specificity in cross-reactivity assays by exposing our sensor to the following seven different viruses: three coronaviruses (MHV – murine hepatitis virus, HCoV-OC43 – human coronavirus OC43, and human coronavirus 229E; Table S4) and four non-coronavirus viral strains (H1N1 – A/California/2009, H3N2 – A/Nicaragua, Influenza B – B/Colorado, HSV2 – herpes simplex virus-2; Table S4). We did not detect cross-reactivity events against any of the viruses tested ($R_{CT} < 10\%$) (Table S4) thus further highlighting the translatability of our diagnostic test. Our biosensor is inexpensive and portable, enabling decentralized diagnosis at the point-of-care. The time of detection of our approach (4 minutes) is significantly lower than existing diagnostic tests^{15,16,23}, and could potentially be lowered even more by using engineered versions of human ACE2 with enhanced selective binding towards SARS-CoV-2 SP¹⁰. The use of such ACE2 variants would also help reduce the rate of false positives in complex biofluids such as saliva^{10,24,25}.

RAPID can also be multiplexed to allow detection of other emerging biological threats such as bacteria, fungi, and other viruses. Thus, our technology serves as a platform for the rapid diagnosis of COVID-19 and future endemic/pandemic outbreaks at the point-of-care. Its low cost, speed of detection, scalability, and implementation using smart devices and telemedicine platforms may facilitate much needed population-wide deployment and express use in hospitals, private companies, and public events.

STAR Methods:

- Key resource table
- Resource availability

- Lead contact
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- Data and code availability
- Experimental model and subject details
 - Human samples
- Method details
 - Fabrication of electrochemical devices
 - Modification of eChip and ePADs
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- Additional resources

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Authors contributions:

M.D.T.T., W.R.A. and C.F.N. designed the experiments. W.R.A. manufactured the electrochemical devices. M.D.T.T., L.F.L., and A.L.F. performed experiments. M.D.T.T., W.R.A. and C.F.N. wrote the manuscript.

Ethical Statement:

This study was performed under the University of Pennsylvania IRB protocol 844145.

Declaration of interests:

The authors declare no competing interests.

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Figures

Figure 1. Point-of-care detection of SARS-CoV-2 using RAPID 1.0. **a)** RAPID 1.0 enables diagnosing neat saliva and NP/OP swab samples infected with SARS-CoV-2. **b)** Schematic for the preparation of the electrodes. Briefly, the screen-printed electrodes in a three-electrode configuration cell (counter electrode – CE, working electrode – WE, and reference electrode – RE) were printed in phenolic paper circuit board or filter paper with conductive carbon and Ag/AgCl inks. The WE was functionalized with glutaraldehyde to enable anchoring of ACE2, which was stabilized by the addition of bovine serum albumin. Detection was improved by adding a Nafion permeable membrane enabling chemical preconcentration of cation species and protecting the electrode's surface against biofouling with proteins, lipids, and other macromolecules present in biological samples matrix. **c)** Cost and detection time comparison between RAPID 1.0 and existing FDA-approved antigen, serological and molecular tests^{26–28}. Note that comparisons were made for a single test of each of the different technologies.

Figure 2. Characterization and calibration of RAPID 1.0. **a)** Schematic representation of the RAPID diagnostic process. **b)** Cyclic voltammetry and **c)** Nyquist plots (inset shows the zoomed region of the curve with the semi-arc) of all functionalization steps showing progressive increased resistivity between the bare electrode (in black) and the four modification steps: addition of glutaraldehyde (in red), functionalization of ACE2 (in blue), addition of the blocking agent bovine serum albumin (in green), and addition of the Nafion permselective membrane (in purple). **d)** Nyquist plots for different SP concentrations ranging from 100 fg mL⁻¹ to 100 ng mL⁻¹ with 10-fold increments in neat saliva from a healthy donor (negative result by RT-qPCR). The inset shows the linearized correlation between normalized R_{CT} values and the concentration of SP exposed to the electrode. **e)** Nyquist plots for titrated inactivated virus solutions at concentrations ranging from 10¹ to 10⁶ PFU mL⁻¹ with 10-fold increments. The upper left inset shows the linearized correlation between the normalized R_{CT} values and concentration of

inactivated virus in solution. The lower right inset shows a zoomed region of the curve with the Nyquist plots' semi-arc (R_{CT}). The analytical curves presented in d) and e) were based on triplicate measurements. All data were recorded using the eChip version of RAPID.

Figure 3. Use of miniaturized and portable RAPID 1.0 for rapid point-of-care diagnosis of COVID-19. (a) Photo of mobile device-compatible handheld RAPID 1.0 during real-time sample analysis. **(b)** Nyquist plots were obtained using ePAD coupled to a smart-device for different concentrations of SP ranging from 1 pg mL⁻¹ to 100 ng mL⁻¹. The inset shows the calibration curve for the normalized R_{CT} values of the different concentrations of SP recorded in triplicate.

Table 1. Analytical parameters of RAPID 1.0.

Parameter	Value
Linear concentration range (SP in PBS)	10 fg mL ⁻¹ – 100 ng mL ⁻¹
Linear concentration range (SP in VTM)	10 fg mL ⁻¹ – 1 ng mL ⁻¹
Linear concentration range (SP in saliva)	100 fg mL ⁻¹ – 100 ng mL ⁻¹
Limit of detection (SP in PBS)	2.18 fg mL ⁻¹
Limit of detection (SP in VTM)	6.29 fg mL ⁻¹
Limit of detection (SP in saliva)	1.39 pg mL ⁻¹
Limit of quantification (SP in PBS)	7.26 fg mL ⁻¹
Limit of quantification (SP in VTM)	20.96 fg mL ⁻¹
Limit of quantification (SP in saliva)	4.63 pg mL ⁻¹
Working concentration range (IV in VTM)	10 ¹ – 10 ⁶ PFU mL ⁻¹
Limit of detection (IV in VTM)	1.16 PFU mL ⁻¹
Limit of quantification (IV in VTM)	3.87 PFU mL ⁻¹

Table 2. Positive and negative values obtained by RT-qPCR, and sensitivity, specificity, and accuracy of RAPID 1.0 using NP/OP and saliva samples.

RAPID (NP/OP)	RT-qPCR			Sensitivity	Specificity	Accuracy
	Positive (N=109*)	Negative (N=30)	Total (N=139)			
Positive	91	0	91	91/109 (83.5%)		121/139 (87.1%)
Negative	18	30	48		30/30 (100%)	
RAPID (Saliva)	RT-qPCR			Sensitivity	Specificity	Accuracy
	Positive (N=13)	Negative (N=37)	Total (N=50)			
Positive	13	5	18	13/13 (100.0%)		45/50 (90.0%)
Negative	0	32	32		32/37 (86.5%)	

*Clinical sample set includes a highly contagious SARS-CoV-2 UK variant B.1.1.7 from a patient.

Table 3. Comparison of methods reported to COVID-19 diagnosis.

Sensor	Technique	Biological Target	Lowest Concentration Detected	Number of Clinical Samples	Price* (US\$)	Time (min)	Reference
RAPID 1.0	EIS	SARS-CoV-2 spike protein	2.8 fg mL ⁻¹	151	4.67	4	This work
SARS-CoV-2 RapidFlex	DPV and OCP-EIS	Viral antigen nucleocapsid protein	500 pg mL ⁻¹	16	-	10	29
SARS-CoV-2 RapidFlex	DPV and OCP-EIS	IgM and IgG antibodies	250 ng mL ⁻¹	16	-	10	29
SARS-CoV-2 RapidFlex	DPV and OCP-EIS	C-reactive protein	50 ng mL ⁻¹	16	-	10	29
	SCC	SARS-CoV-2 RNA	231 RNA copies μ L ⁻¹	48	10	5	30
	DPV	SARS-CoV-2 RNA	200 RNA copies μ L ⁻¹	33	-	<5	31
	EIS	SARS-CoV-2 spike protein	0.1 mg mL ⁻¹	4	-	3	15
	SWV	IgM and IgG antibodies	1 μ g mL ⁻¹	17	-	45	32
DETECTR	CRISPR technology	<i>E</i> gene and <i>N</i> gene	10 RNA copies μ L ⁻¹	11	-	40	33
	Colorimetric assay	<i>N</i> gene	0.18 ng μ L ⁻¹	1	-	30	34
	Localized surface plasmon resonance	<i>RdRp</i>	2.26×10^4 RNA copies μ L ⁻¹	5	-	2	35
	DNA nanoscaffold-based hybrid chain reaction	Synthetic RNA conserved region	0.96 pmol L ⁻¹	0	-	10	36

RT-LAMP	<i>orf1ab</i>	20-200 RNA copies μL^{-1}	130	-	60	37
RT-LAMP	<i>N gene</i>	100 RNA copies μL^{-1}	27	-	30	38

EIS – Electrochemical impedance spectroscopy; DPV – Differential pulse voltammetry; OCP-EIS – Open-circuit potential-electrochemical impedance spectroscopy; SCC – Signal conditioning circuit; SWV – Square-wave voltammetry; RT-LAMP – Reverse transcription loop-mediated isothermal amplification.

*Prices reported correspond to a single test for each of the technologies and do not account for the equipment required in each case.

5

STAR Methods

Key resources table

Reagent or Resource	Source	Identifier
Chemicals		
Human angiotensin converting enzyme 2 (ACE2)	GenScript	Z03484-1
Sulfuric acid (H ₂ SO ₄)	Sigma	258105-1L-PC
Potassium chloride (KCl)	Sigma	P3911-1KG
Potassium ferricyanide K ₃ [Fe(CN) ₆]	Sigma	244023-5G
Potassium ferrocyanide K ₄ [Fe(CN) ₆]	Sigma	P3289-5G
Bovine serum albumine (BSA)	Sigma	A2153-10G
Glutaraldehyde (GA)	Fisher	S25341
Nafion	Sigma	527084-25ML
Phosphate saline buffer (PBS)	VWR	P32200
Software and Algorithms		
Squidstat	Admiral Instruments	
PSTrace	PalmSens	
PSTouch	PalmSens	

Resource Availability

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Cesar de la Fuente-Nunez (cfuente@upenn.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Test results used for analysis will be provided upon reasonable request.

Experimental model and subject details

Clinical samples preparation

Patient saliva, NP/OP swab samples were collected and processed according to the appropriate biosafety procedure (2020 CDC COVID-19 test protocol for details on specimen collection) at the Hospital of the University of Pennsylvania. Clinical matrices were heated at 56 °C for 1 hour for viral inactivation. Saliva used for optimization steps were collected from healthy donors (attested by negative result by RT-qPCR) and used promptly for experiments. The samples obtained are derived from patients with a wide range of symptoms (*i.e.*, from negative cases, to patients with very low viral loads, to severe COVID-19 cases) thus providing a highly representative COVID-19 population sample set. All the steps were approved and performed under conditions detailed in the University of Pennsylvania IRB 844145.

Methods details

Fabrication of electrochemical devices

The electrochemical sensors were screen-printed in a three-electrode configuration cell (dimensions: 1.8 x 1.2 cm) on two accessible substrates (i) a qualitative filter paper and (ii) phenolic paper circuit board material. First, specific patterns were wax printed on A4 size filter paper using a commercial Xerox ColorQube 8570 printer (Xerox, Brazil). The patterns consist of small white rectangles (1.1 x 1.7 cm) to delimit the electrochemical cell on paper substrates. In a

single A4 size paper, 80 patterns were printed, thus affording 80 disposable ePADs. Following, the screen-printing process was performed in the previously patterned paper using electrically conductive carbon and Ag/AgCl inks (Creative Materials, USA) to fabricate the working/auxiliary electrodes and reference electrodes, respectively. The printed filter paper sheets were then placed in a thermal oven for 30 minutes at 100 °C. The heating process induces the curing step of the conductive tracks and melts the deposited wax layer that then penetrated in the cellulosic structure, forming a 3D hydrophobic barrier around the hydrophilic patterns (electrochemical cell). Finally, the electrochemical paper-based analytical devices (ePADs) were cut with scissors and the backside of the devices was covered with a transparent tape to prevent solution leakage through the device and to add structural integrity. The phenolic paper is a material largely used as a printed circuit board substrate. The boards were washed thoroughly with deionized water and isopropyl alcohol. The screen-printing process on the paper phenolic resin was performed using the same design and dimension reported for the filter paper platform. The electrochemical circuit board-based devices (eChip) present a rigid substrate and low wettability that dispenses the use of a hydrophobic barrier. After the curing step of printed electrodes, they were cut into small pieces (2 x 2 cm) and a non-conductive layer was applied to delimit the electrode area.

Modification of the eChips and ePADs

The electrodes were washed with deionized water and cleaned/activated electrochemically by cyclic voltammetry (CV) recorded in sulfuric acid solution (0.1 mol L⁻¹) in the potential range from -1.3 to 1.5 V at the scan rate of 100 mV s⁻¹ for 5 cycles. The eChips were dried at room temperature and 4 µL of GA solution (25% in water) was added on the surface of the working electrode using the drop-casting method. After 1 hour, 4 µL of ACE2 solution (0.32 µg mL⁻¹) prepared in PBS medium was added on top of the working electrode and left to dry at room temperature for 1.5 hours. Subsequently, 4 µL of BSA solution (1 mg mL⁻¹) was added on the surface of the working electrode to stabilize the protein and block unspecific sites of the electrode. After 30 minutes, 4 µL of Nafion solution (1.0% in PBS) was added to the working electrode's surface and left for 1 hour before the final washing with deionized water. The ePADs were modified using the same protocol but applying 2.5-fold higher volume of the modifying agent solutions.

Electrochemical measurements

SquidStat Plus (Admiral Instruments) and Sensit Smart (PalmSens) potentiostats controlled by a laptop running the software SquidStat and a smartphone running the software PSTouch, respectively, were used to record all electrochemical data. The electrodes were characterized by CV technique using a mixture of 5 mmol L⁻¹ potassium ferricyanide/ferrocyanide in the medium of 0.1 mol L⁻¹ KCl solution prior and after electrode modification using a potential range of 0.7 to -0.3 V at the scan rate of 50 mV s⁻¹. Electrochemical impedance spectroscopy (EIS) was used to characterize the biosensor and for SARS-CoV-2 detection. The EIS measurements were performed using 200 µL of a mixture of 5 mmol L⁻¹ ferricyanide/ferrocyanide prepared in 0.1 mol L⁻¹ KCl solution added after the sample incubation on the electrode (10 µL of OP/NP or saliva samples) and the gentle washing process using PBS solution to remove the unbound SP/SARS-CoV-2. A sinusoidal signal was applying in the frequency range between 10⁵ and 10⁻¹ s⁻¹ using a typical open circuit potential of 0.15 V and an amplitude of 10 mV at room temperature.

Optimization tests

We evaluated the main experimental parameters and processes that affect the efficiency of the developed biosensor. For modification steps, both GA and BSA were used at high concentration levels to ensure the complete recovery of the electrode surface providing the best condition to covalently attachment of ACE2 and its stabilization. The formation of permselective membrane was evaluated by using different Nafion concentrations in the range of 0.5 to 3.0 wt%. After the biosensor preparation, we evaluate its response to different concentrations (1 pg mL⁻¹ – 10 µg mL⁻¹) of angiotensin II (AngII), the natural substrate of ACE2, to verify if the anchoring and stabilization strategies maintain the biological activity of ACE2. To assess the kinetics of interaction between SP and the architecture of the modified electrode, we carried out calibration curves ranging from 1 pg mL⁻¹ to 1 ng mL⁻¹ SP using different times of incubation (from 1-10 minutes) to obtain the best analytical response to RAPID1.0. Finally, the need for sample pretreatment of saliva samples was evaluated using 3 different approaches: (i) direct use of raw saliva, (ii) 2 minutes of centrifugation at 10,000 rpm, and (iii) simple dilution of sample 1:1 (v/v) with PBS. We performed this study with saliva samples because it presents greater matrix

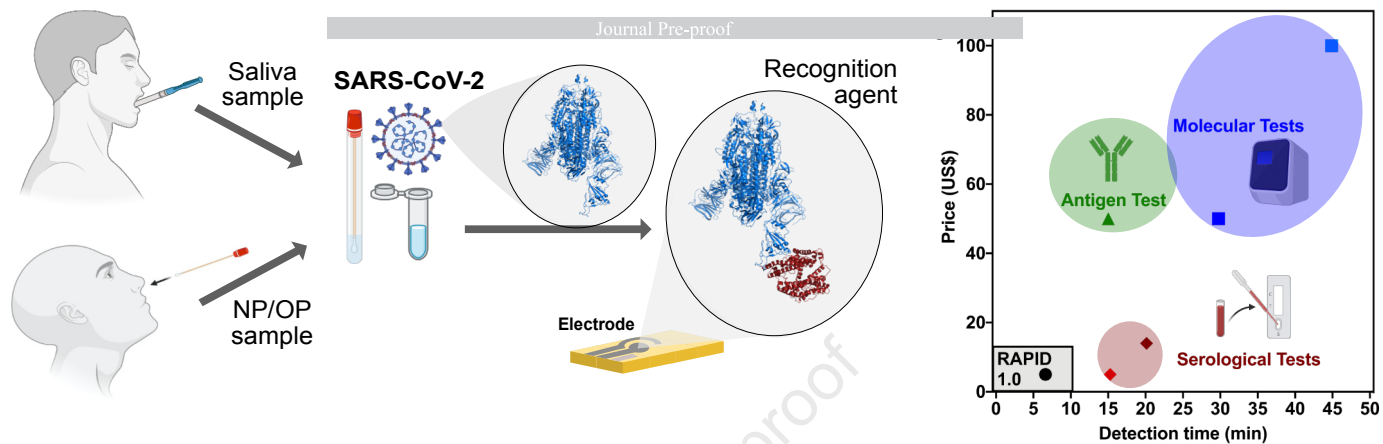
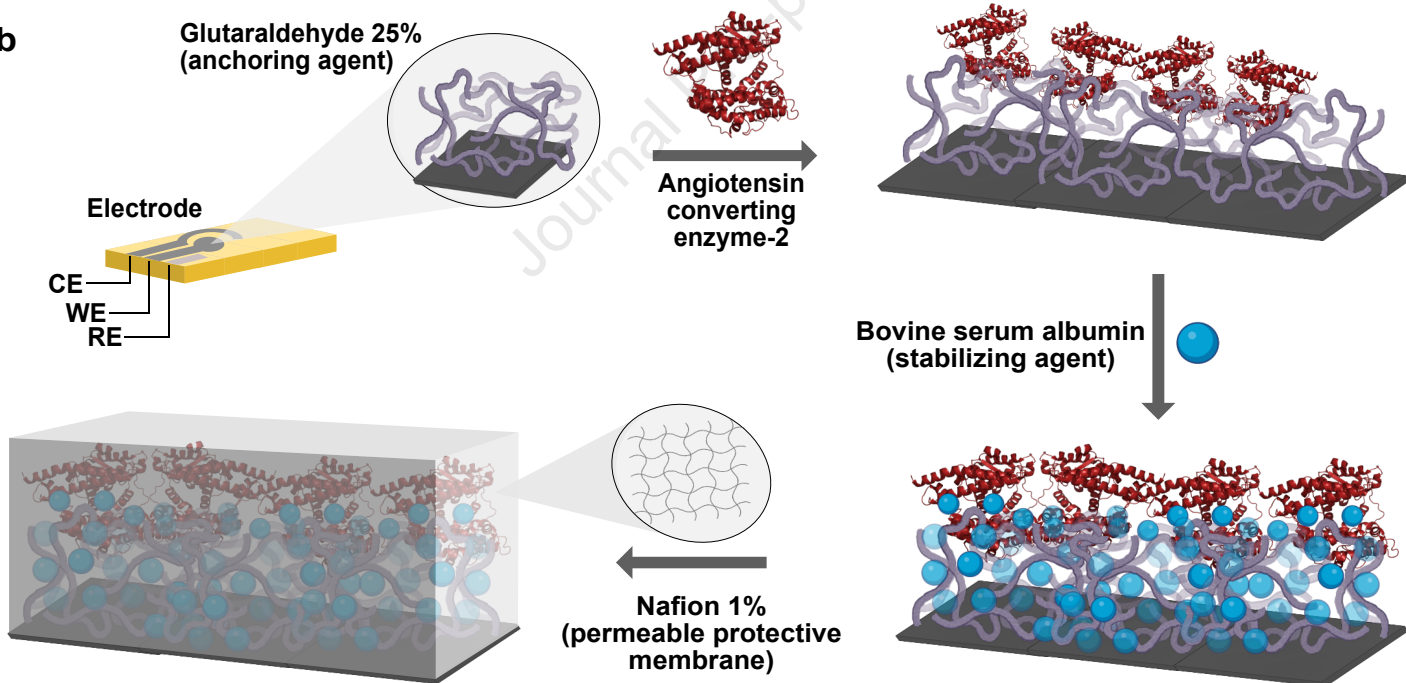
complexity (high viscosity and content of proteins, lipids, and other biomolecules that can cause biofouling of the electrodic surface) when compared to NP/OP swab samples.

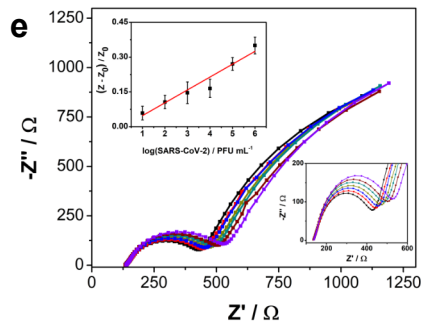
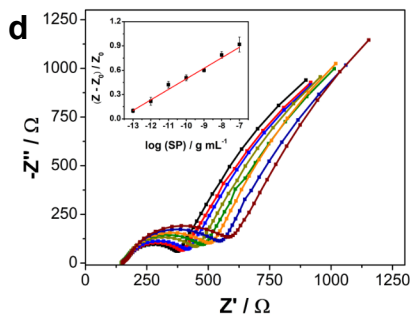
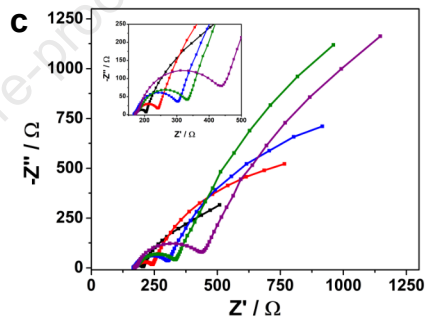
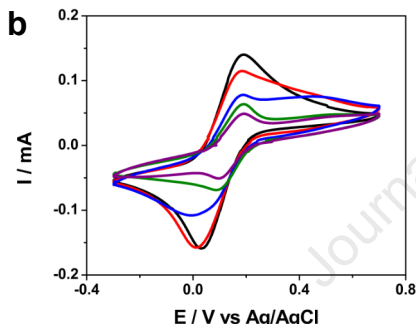
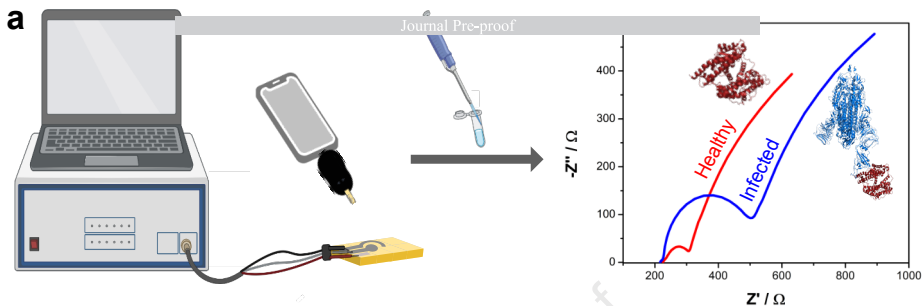
Cross-reactivity experiments

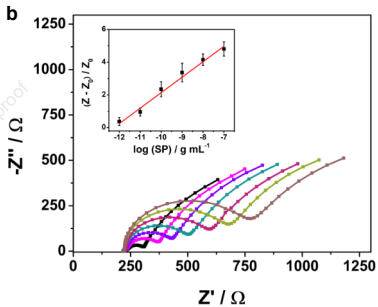
Cross-reactivity assays were carried out by exposing the sensor to three coronaviruses (MHV – murine hepatitis virus at 10^8 PFU mL⁻¹, HCoV-OC43 – human coronavirus OC43 at 10^4 PFU mL⁻¹, and human coronavirus 229E at 10^7 PFU mL⁻¹), and four non-coronavirus viral strains (H1N1 – A/California/2009, H3N2 – A/Nicaragua, Influenza B – B/Colorado, HSV2 – herpes simplex virus-2, all at 10^5 PFU mL⁻¹) were used to assess the specificity of our biosensor. The conditions used were the same as those used for all SARS-CoV-2 samples: incubation time of 5 minutes, 10 µL of virus sample, and EIS measurements as specified above (Electrochemical Measurements section).

Quantification and statistical analysis

Cyclic voltammetry and electrochemical impedimetric spectroscopy measurements are presented as an average of 3 or 7 different replicates for each condition and it is described in each figure caption. Graphs were created and statistical tests conducted in GraphPad Prism 9.

a**b**





Progress and potential statement:

To address the need for rapid, inexpensive, and scalable point-of-care diagnostics for COVID-19, we created RAPID, a biosensor that can detect SARS-CoV-2 within 4 minutes using minimal sample volume through electrochemical impedance spectroscopy. Our test detects SARS-CoV-2 and UK variant B.1.1.7 at extremely low concentrations (1.16 PFU mL^{-1}) with high specificity, sensitivity, and accuracy in both saliva and nasopharyngeal/oropharyngeal samples.

Highlights:

- RAPID can diagnose SARS-CoV-2 and a highly contagious UK variant B.1.1.7 in 4 minutes
- RAPID is inexpensive compared to existing methods for SARS-CoV-2 detection
- RAPID is highly scalable, allowing the production of millions of units per week

eTOC blurb:

RAPID is a low-cost electrochemical biosensor that diagnoses COVID-19 within 4 minutes in clinical saliva and nares swab samples.