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Portable microfluidic impedance biosensor for SARS-CoV-2 detection

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ABSTRACT

Pandemics as the one we are currently facing, where fast-spreading viruses present a threat to humanity, call for simple and reliable methods to perform early diagnosis, enabling detection of very low pathogen loads even before symptoms start showing in the host. So far, standard polymerase chain reaction (PCR) is the most reliable method for doing so, but it is rather slow and needs specialized reagents and trained personnel to operate it. Additionally, it is expensive and not easily accessible. Therefore, developing miniaturized and portable sensors which perform early detection of pathogens with high reliability is necessary to not only prevent the spreading of the disease but also to monitor the effectiveness of the developed vaccines and the appearance of new pathogenic variants. Thus, in this work we develop a sensitive microfluidic impedance biosensor for the direct detection of SARS-CoV-2, towards a mobile point-of-care (POC) platform. The operational parameters are optimized with the aid of design-of-experiment (DoE), for an accurate detection of the viral antigens using electrochemical impedance spectroscopy (EIS). We perform the biodetection of buffer samples spiked with fM concentration levels and validate the biosensor in a clinical context of relevance by analyzing 15 real patient samples up to a Ct value (cycle threshold) of 27. Finally, we demonstrate the versatility of the developed platform using different settings, including a small portable potentiostat, using multiple channels for self-validation, as well as with single biosensors for a smartphone-based readout. This work contributes to the rapid and reliable diagnostics of COVID-19 and can be extended to other infectious diseases, allowing the monitoring of viral load in vaccinated and unvaccinated people to anticipate a potential relapse of the disease.

1. Introduction

The rapid spread of SARS-CoV-2, which causes COVID-19, a respiratory illness that can be mild to severe and even fatal (Guan et al., 2020; Ravi et al., 2020), prompted the World Health Organization (WHO) to declare a pandemic in 2020 (Santiago, 2020). Despite the existence of vaccines, the disparity in their distribution continues to cause high infection rates in many countries (Bayati et al., 2022).

The gold standard for its diagnosis is the Reverse Transcript Polymerase Chain Reaction (RT-PCR). Although it provides sensitive and specific quantification of the viral load, it is costly in terms of

consumables, needs trained personnel and equipment and has long awaiting times (6–72 h) (Morales-Narváez and Dincer, 2020). This makes it unable to combat highly infectious pathogens with high spread rates, especially in states of low-resources (Adams et al., 2020; Drain et al., 2014; Sheridan, 2020; Xu et al., 2020). On the other hand, an early modeling study by Larremore et al. showed that a 3-day continuous testing scheme could terminate the disease transmission, even if the tests were imprecise (Larremore et al., 2021).

Although a lot of effort has gone into developing Point-of-Care (POC) devices that meet WHO's ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment free, and Deliverable to end-

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users) standard, there is still a need to improve existing platforms (Vásquez and Orozco, 2022). Lateral flow immunoassays (LFIA) have the highest potential to become suitable POC platforms, however they have lower analytical performance. (Everitt et al., 2021; Peeling and Olliaro, 2021; Scohy et al., 2020). The literature still debates their sensitivity and accuracy, which can range from 30% to 95% (Cerutti et al., 2020; de Michelena et al., 2022; Escribano et al., 2022; Osterman et al., 2021). They are recommended for individuals showing symptoms. (Linares et al., 2020; Schildgen et al., 2021).

Electrochemical transduction mechanisms are more promising to our view because they are inexpensive, compact, scalable, and user-friendly (Imran et al., 2021; Kumar et al., 2022; Vásquez and Orozco, 2022; Lim et al., 2021; Z. Zhao et al., 2021). Moreover, they show high sensitivity compared to other POC devices like LFIA (Akhavan et al., 2012), and can be quantitative or semi-quantitative (Drobysh et al., 2022; Merkoçi et al., 2021). In these methods, electrical signal changes are measured at the surface of the sensing area related to analyte presence (Ensafi, 2019).

Since the pandemic began, researchers have studied electrochemical biosensors for SARS-CoV-2 detection, comparing several methodologies and materials (Alafeef et al., 2020; Amouzadeh Tabrizi et al., 2022; Antiochia, 2022; Ashur et al., 2022; Ayala-Charca et al., 2022; Cho et al., 2022; Ditte et al., 2021; Ehsan et al., 2021; Fabiani et al., 2021; Hashemi et al., 2021; Idili et al., 2021; Kiew et al., 2021; Kim et al., 2021; Lasserre et al., 2022; Rahmati et al., n.d.; Rashed et al., 2021; Salahandish et al., 2022; Seo et al., 2020; Soares et al., 2022; Soto and Orozco, 2022; Tabrizi and Acedo, 2022; Torrente-Rodríguez et al., 2020; Torres et al., 2021; H. Zhao et al., 2021). However, although other techniques can be faster, electrochemical impedance spectroscopy or EIS-based biosensors are increasingly popular because they are highly sensitive and do not require labeling or amplification for detection. (Antiochia, 2022). EIS applies a modest amplitude of AC potential or current to the electrochemical cell at a frequency range and measures the reflected signal (current or potential). Cell impedance—potential over current in frequency domain—is determined by the result signal. The data is then modeled as an Equivalent Electrical Circuit (EEC) with non-constant constituents and associated to an electrochemical reaction in the cell. (Wang et al., 2021).

Therefore, in order to design a competitive POC platform, it should have the following characteristics: (1) should be scalable, inexpensive, reliable, and reproducible; (2) must have an optimized and simple preparation protocol, including the bio-functionalization steps; (3) sample handling and data acquisition must be automated or semi-automated; and (4) the readout platform must be sensitive and accurate (Ensafi, 2019; Salahandish et al., 2022). Over the last two years, POC for infectious diseases including SARS-CoV-2 have improved dramatically, however just few works fulfill most the above-mentioned requirements. Table S1 compares SARS-CoV-2 biosensors by material, technique, sensitivity, and characteristics. As shown, there has been substantial research on materials and methods to improve sensor performance, particularly sensitivity, focused on the first and to some extent the second requirement. Comprehensive comparisons on SARS-CoV-2 diagnosis tests can be found in these reviews (Abdelhamid and Badr, 2021; Novodchuk et al., 2022; Rahman, 2022; Rasmi et al., 2021).

This work establishes an EIS-based electrochemical biosensor platform capable of satisfying all requirements with simple material-systems and functionalization procedures. A microfluidic channel has been designed for semi-automated sample handling, as well as integration on a card-edge Printed Circuit Board (PCB) for simple plug-in connection. The chip was tested with a desktop-portable electrochemical workstation and a smartphone. Fig. 1a presents the concept of the developed microfluidic biosensor. We adopted DoE to optimize the operational performance of the biosensor toward spike glycoprotein, presenting a statistical model to predict and optimize the most impactful operation parameters (AC and DC potential, incubation time and flow rate). After optimization, a Limit of Detection (LOD) and Limit of Quantification

(LOQ) of less than 20 fM and 60 fM were achieved respectively. The performance of the biosensor was also evaluated with a low concentration of cultured virus ($\sim 9.56 \times 10^2$ PFU/mL). Finally, 15 nasopharyngeal swabs from clinical were tested in order to demonstrate the feasibility of using such a platform for clinical diagnosis.

2. Materials and methods

2.1. Fabrication of microelectrode arrays

It has been already shown that the structural design of the electrodes, such as spiral, meandered, serpentine (SRE) or Interdigitated Electrode (IDE) layouts, plays an important role on the sensor's sensitivity (Rivadeneira et al., 2016). Among them, IDE electrodes show the best compromise between sensitivity, active sensing area and manufacturing yield (benefiting from separately connected digits). Thus, we used curved IDE layout, which also maximizes the ratio of perimeter over the area of electrode (Faulkner and Bard, 2002). Table S2 presents the geometrical dimensions of the electrodes, which were optimized experimentally, as another important factor.

In order to fabricate the microelectrode array, we adopted a common silicon-based technique, to pattern gold electrodes on borosilicate substrates. Fig. 1b presents a 2D top view of the fabrication steps. Briefly, a photoresist (PR) was spin-coated on the substrate and baked. Afterward, the exposure, a post exposure baking, flood exposure and developing were performed. Next, we used physical vapor deposition to deposit 10 nm Cr (as adhesion layer) and 50 nm Au. After deposition, wet etching was performed to lift off the remaining PR with dimethyl sulfoxide (DMSO). Finally, a 400 nm thick SiO₂ passivation layer was deposited, using plasma-enhanced chemical vapor deposition, defining the active area of the electrode. Fig. 1c shows a microscopy image of a fabricated electrode, and Fig. 1d shows their wafer-scale production, obtaining 32 sensors from a single 4-inch wafer.

2.2. Integration of the biosensor

The sensors were integrated into a polydimethylsiloxane (PDMS) microfluidic channel to make the sample handling semi-automated and to increase the sensitivity and reliability. Fig. S1a shows a schematic of integration steps and Fig. S1b presents the fluidic channels integrated on top of the electrodes, which are designed for three different purposes; for smartphone readout analysis, multiplexed for different samples, and parallel analysis of a single sample (Fig. S2 presents the dimensions and 2D and 3D schematic of the channels). To reduce the variation among measurements due to poor electrical connections, and to make the sensor user-friendly, we adopted a card-edge PCB (see SI Section 2.2 for details).

2.3. Biofunctionalization

A solution of 10 mM 11-mercaptoundecanoic acid (MUA) in absolute ethanol was pumped into the fluidic channel with a flow rate of 10 $\mu\text{L min}^{-1}$ during 12 h. MUA forms a self-assembled monolayer (SAM), attaching from the thiol end to the Au surface, and leaving exposed a functional carboxyl group on the other end for the covalent binding of the bioreceptor (antibody against the spike protein of SARS-CoV-2). Next, the channel was washed with ethanol.

For the antibody immobilization, 10 mM N-(3-(dimethylamino) propyl)-N'-ethylcarbodiimide hydrochloride (EDC), 5 mM of N-hydroxysuccinimide (NHS) and 50 $\mu\text{g mL}^{-1}$ of anti-SARS-CoV-2 antibody in phosphate buffered saline (PBS) 1X were mixed and pumped into the channel with a flow rate of 5 $\mu\text{L min}^{-1}$ for 2 h. The EDC-NHS chemistry improves the immobilization by facilitating covalent bonds between amino groups of antibodies and carboxylic groups of the SAM (Mrksich and Whitesides, 1996; Whitesides et al., 2005).

After washing the channel with PBS 1X, a solution of 3 μM of

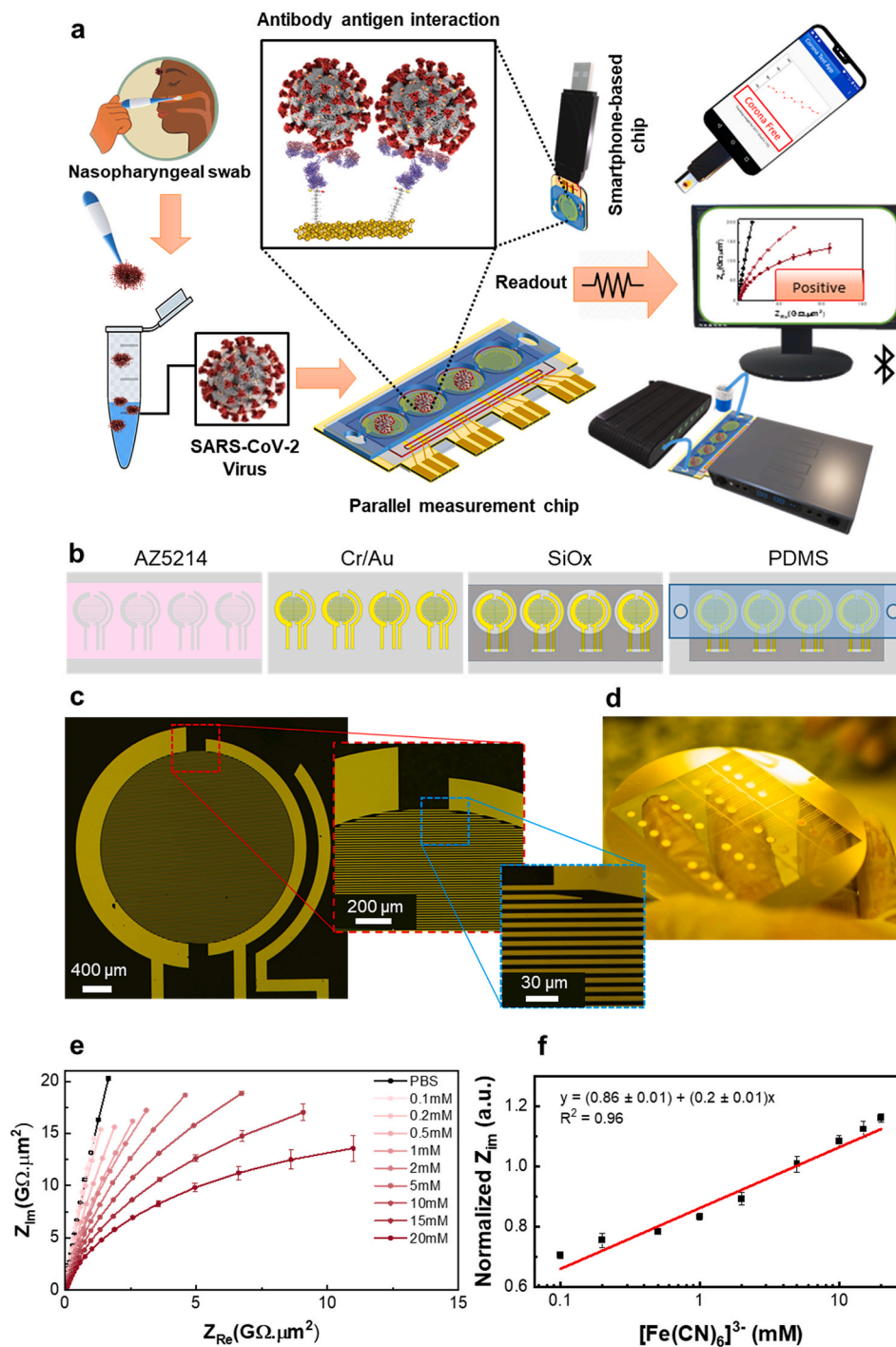


Fig. 1. Representation of the proposed biosensor platform for SARS-CoV-2 detection. a) conceptualization of the platform for rapid and accurate detection of SARS-CoV-2, b) a 2D schematic of the fabrication steps (photoresist patterning, deposition of electrodes, passivation of the surface and integration with microfluidic channel), c) microscopic image of the fabricated electrodes, d) Electrodes' array, fabricated on a 4-inch wafer, e) characterization of the fabricated electrode by EIS using different concentration of potassium ferricyanide in PBS 1X (error bars show the standard deviation of at least three consecutive measurements), and f) calibration curve for different concentration of Potassium Ferricyanide in PBS 1X (error bars in calibration curve corresponds to measurements with at least three different chips).

ethanolamine (MEA) in PBS was introduced at $2 \mu\text{L min}^{-1}$ for 2 h, blocking the remaining active groups of MUA, and washed again with PBS 1X. Finally, a $5 \mu\text{M}$ solution of 6-Mercapto-1-hexanol (MCH) in PBS 1X was introduced at a $4 \mu\text{L min}^{-1}$ for 2 h to block the free gold surface. Finally, the microfluidic device was washed with PBS 1X. A schematic of the biofunctionalization steps is depicted in Fig. 2a.

2.4. EIS data validation and modelling

Linearity, stability and causality of the data were checked by help of Kramers–Kronig Transforms (KKT) and measurement model (Agarwal et al., 1995a, 1995b; Orazem et al., 1996; Orazem and Tribollet, 2008; Pachimatla and Srinivasan, 2018; Srinivasan and Fasmin, 2021; Watson and Orazem, 2020) using a Python code by Orazem (Watson and Orazem, 2020). After validation, the Levenberg–Marquardt algorithm (LMA) was used to model the data to an EEC (see Fig. 4d). LMA is being used for non-linear least-square curve fitting generally and specifically for EIS data, because it is fast and precise. (Boukamp, 1995; Boukamp and Ross Macdonald, 1994; Orazem and Tribollet, 2008; Ossai and Egwuotuoha, 2021; Risse et al., 2016; Srinivasan and Fasmin, 2021; Watson, 1977; Žic et al., 2020, 2021). All the models had the chi squared less than 0.012 (See SI section 2.4 for details).

2.5. Clinical samples and cultured virus preparation

Nasopharyngeal swabs of anonymous patients were collected and inactivated by adding 0.1% betapropiolactone. A virus isolate was obtained by adding a nasopharyngeal swab to Vero E6 cells cultured in DMEM GlutaMAX supplemented with 10% fetal bovine serum, 1% non-essential amino acids and 1% penicillin/streptomycin.

Details on all the reagents and devices used in this study can be found in SI section 2.1.

3. Results and discussion

3.1. Characterization of the microelectrodes

For the characterization of the electrodes prior to biofunctionalization, we employed EIS measurements toward potassium ferricyanide in PBS ranging from 0.1 to 20 mM. Fig. 1e shows the resulting Nyquist plots. The Bode plot is presented in Fig. S3. The data was acquired in a frequency range of 1 kHz to 1 Hz, under a perturbation of 5 mV and with no DC voltage, in static flow mode. As expected, the impedance and charge transfer resistance (R_{ct}) decreased with increasing ferricyanide concentration, with a linear range of detection between 0.1 mM and 20 mM (Fig. 1f). In order to make the normalization for imaginary impedance, equation (1) was used, where Z_{im_0} is

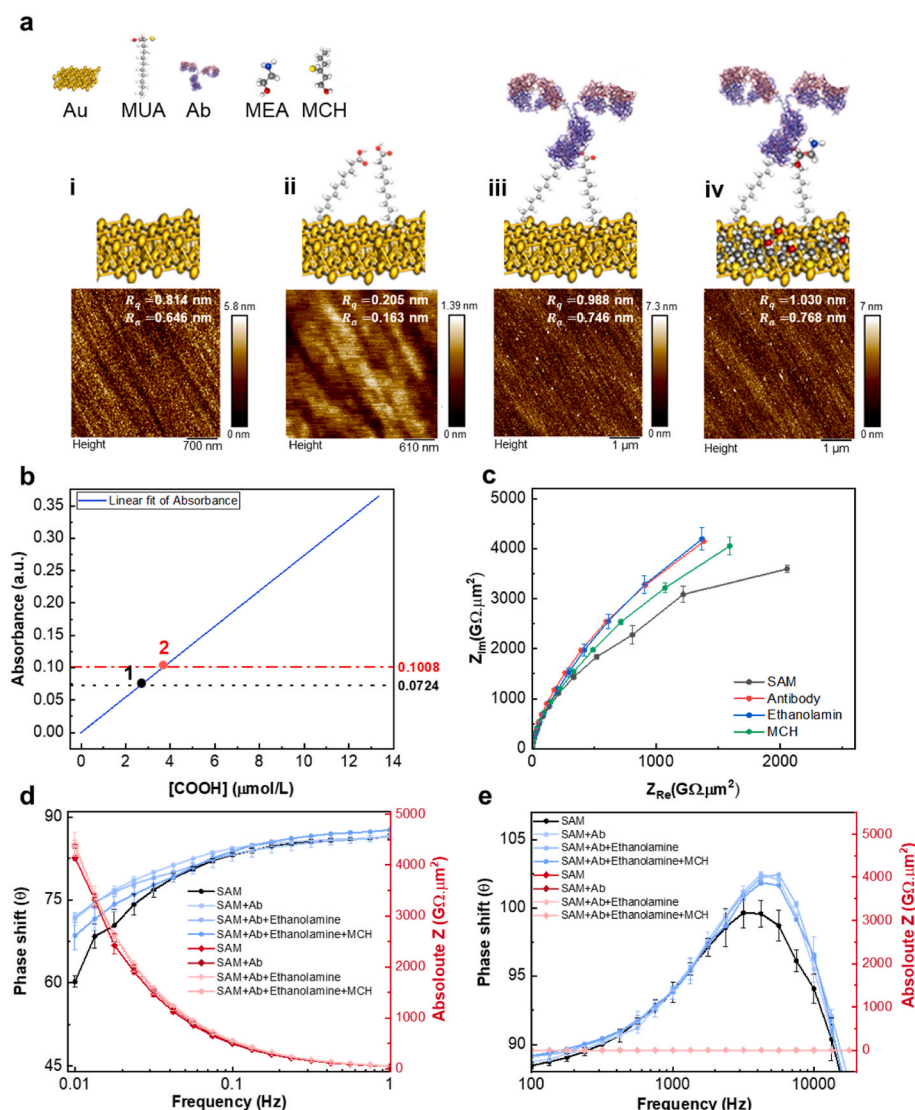


Fig. 2. Functionalization of the biosensor. a) schematics and AFM results for different functionalization steps; Unmodified surface of gold electrode (i), formation of a SAM of MUA as probe linker (ii), attachment of antibody to probe linker (iii), blocking unbonded MUA and free surface of gold (iv), b) calibration curve for the calculation of $-\text{COOH}$ density changes on the surface before (1) and after (2) SAM formation with colorimetric measurement by TBO, c) Nyquist plot showing the electrochemical characterization of the sensor at different functionalization steps, and d-e) Bode diagram showing the corresponding EIS signal at different functionalization steps at the low and high frequency ranges, respectively. Measurements were done in the frequency range from 1 MHz to 1 mHz. Error bars show three consecutive measurements.

the imaginary impedance of blank and Z_{imC} is the imaginary impedance of the concentration in use. The LOD and LOQ were also calculated based on Equations (2) and (3) (Faulkner and Bard, 2002; Orazem and Tribollet, 2008; Srinivasan and Fasmin, 2021). The obtained LOD and LOQ were 0.176 ± 0.012 mM and 0.589 ± 0.053 mM respectively.

$$Z_{im_n} = \frac{Z_{imC} - Z_{im_0}}{Z_{im_0}} \quad (\text{Equation 1})$$

$$LOD = \frac{3\sigma}{S} \quad (\text{Equation 2})$$

$$LOQ = \frac{10\sigma}{S} \quad (\text{Equation 3})$$

In these equations σ is standard deviation of the blank measurements for three sensors and S is the slope of the calibration curve.

3.2. Characterization of the functionalized electrodes

Following functionalization, the electrodes' surface was characterized first by Atomic Force Microscopy (AFM). Fig. 2a shows the effect of the functionalization on the surface topography. Fig. 2a, i and ii show that the SAM is formed on the surface of bare gold, considering that in the bare gold the surface roughness is more than four times higher. Moreover, the distribution of surface height, which decreased almost 5 times, is consistent with our expectation to see a smoother surface in the range of 1–4 nm after formation of a monolayer. The comparison of Fig. 2a, ii-iii which is measured after binding antibodies, shows a four times roughness increase and a height difference in the range of the antibody size (5–10 nm). This demonstrates that the antibody is attached to the surface. Fig. 2a, iv shows the blocking step of the remaining free surface. Here, the unmodified surface of the gold or the portion of the probe linker which are not connected to the antibody, are supposed to be blocked. As expected, there is a little increase in the roughness and a decrease in the height which is caused by a very thin layer of MCH.

As supplementary confirmation, we quantified the carboxyl groups after SAM formation with a colorimetric assay based on toluidine blue O (TBO), returning a value of $33.23 \mu\text{M cm}^{-2}$ (Fig. 2b, Fig. S4–S5, details on the SI section 3.1).

All the functionalization steps were characterized by EIS. When the SAM is formed, R_{ct} significantly increases (see Fig. S6). Fig. 2c presents a Nyquist plot showing all the functionalization steps after SAM formation. When the antibody is linked to the SAM, impedance and R_{ct} increase due to presence of a charged macromolecule on the surface. When MEA is added changes are insignificant, which means that the concentration or packing of the antibodies on the surface was good enough that almost all probe linkers were used. After adding MCH there was a slight decrease in impedance which deactivated the gold surface and guided the charge transfer. Fig. 2d and e presents the corresponding Bode diagram at high and low frequencies. Although changes on the surface for each step were more visible at lower frequencies (1 mHz), in higher frequencies (around 5 KHz) the presence of antibodies can also be detected (see Fig. 2e).

3.3. Optimization of biosensor performance with spike glycoprotein in buffer solution

After characterization, we used DoE, a statistical tool to minimize the number of required experiments especially appealing when multiple parameters need to be considered (Gilman et al., 2021; Habbema and Samuels, 1990; Dean et al., 2017). We used it to screen the most important operational parameters and to optimize them. Despite saving time and resources, DoE reveals more information about the subject matter comparing to conventional One Factor At Time (OFAT), because it considers the effect of interaction between parameters (Gilman et al.,

2021; Hinkelmann, 2012; Laleh et al., 2017; Habbema and Samuels, 1990).

Eight operational parameters were considered (see Table 1), which are reported to have an impact on the ultimate EIS response (Orazem and Tribollet, 2008; Srinivasan and Fasmin, 2021; Wang et al., 2021). Table S3 shows the parameters for screening experiments and their definitions. To evaluate their impact, we defined two responses: normalized signal of imaginary impedance (Z_{im}) which represents how the signal strength is changing, and its standard deviation (STDEV Z_{im}) for repeatability. Table S4 presents the responses with their definitions. A Minimum Screening Factorial Design (MSFD) scheme (an experimental scheme to rank the effect of many factors on the response with the lowest number of the experiments) was adopted to decide what are the independent and most affecting factors to be considered; the experimental scheme is presented in Table S5. After running the experiments and performing statistical analysis based on ANOVA table, the first ten important factors are depicted in the form of Pareto chart for each response separately in Fig. 3a and b. In these figures, the impact of different factors on X axis is related to the attributed t-value on Y axis, while orange and blue colors show a direct or reverse relation respectively. For example, factor H has a strong reverse impact on signaling (second ranked and blue). By the help of screening, non-independent or insignificant parameters were ruled out; therefore, to find the optimum of operational conditions, four parameters are considered as changing factors and the rest are kept constant as presented in Table 1 (See SI Section 4 for more details on DoE).

Next, a Central Composite Design (CCD), which is based on Response Surface Method (RSM) with a symmetric alpha value of 1 was adopted to specify the optimized operational condition (Table S6 shows the DoE scheme). Figs. S8 and S9 show the effect of these four factors on the signaling and standard deviation of imaginary impedance. Based on the obtained results and with the help of the ANOVA table (Tables S7 and S8) we statistically modeled the impact of these four parameters on normalized Z_{im} and STDEV Z_{im} as following:

$$(\text{Normalized Signal of } Z_{im})^A = +0.8556 + 0.0277 A - 0.0102 B + 0.0900 G + 0.0592 H + 0.0480 AB. (\text{Equation 4})$$

$$\text{STDEV for } Z_{im} = +1.01 + 0.3193 A + 0.1100 B - 0.0271 G + 0.0949 H - 0.0176 AG + 0.0501 AH + 0.0867 A^2 + 0.0717 B^2. (\text{Equation 5})$$

In these Equations A is dc potential, B is ac potential, G is incubation time and H is gauge pressure between inlet and outlet, which represents the flow rate.

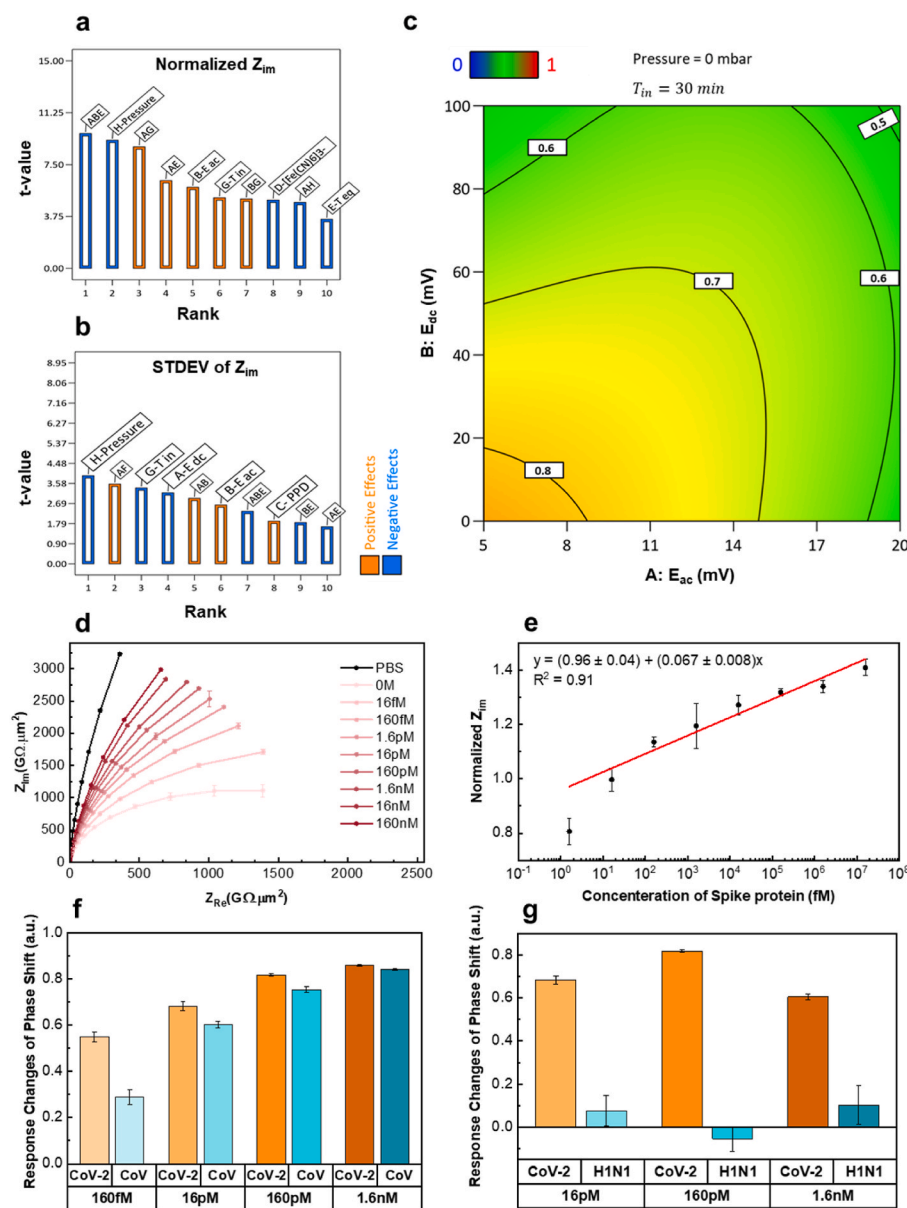
Finally, we defined a desirability point to maximize Z_{im} , minimize STDEV Z_{im} and keep the flow rate and applied potential in low values. After solving the optimization problem, a desirability contour was depicted in Fig. 3c and the optimal operational condition was reached (Table 1). All the data acquisition in the next sections were done in this optimal condition if it is not noted otherwise (see SI, section 4, Figures S7–S10 for more details on DoE).

3.4. LOD and LOQ of the biosensor

To assess the sensitivity of the sensor, its performance was evaluated

Table 1
Optimized parameters for EIS measurement.

	Parameter	value
Optimized	A: E dc	0 mV
	B: E ac	6 mV
	G: T in	30 Minutes
	H: Pressure	0 mbar
Fixed	E: T eq	0 s
	F: Frequency Range	1 kHz–100 mHz
	C: PPD (point per Decade)	8
	D: Concentration of potassium ferricyanide	1 mM



in PBS with a concentration range of 16 fM to 160 nM of recombinant SARS-CoV-2 spike glycoprotein, considering the previously optimized conditions. The Nyquist plot presented in Fig. 3d shows the response of the biosensor. First, we incubated the sensor in PBS for 30 min, and measured the response from PBS (black line). Next potassium ferricyanide (1 mM in PBS) was added, and the measurement was noted as the 0 M concentration after incubation time. Finally, we added a known concentration of spike glycoprotein and left it for incubation, and then performed the measurements. As it can be seen, when the concentration of spike glycoprotein increases, the slope of the curve increases as well, which indeed impedes the charge transfer. Antibody-antigen complexes work as a barrier for the electron transfer, therefore, R_{ct} increases with increasing analyte concentration. This trend is visible from the presented graph where the radius of the semicircle increases (each line represents a portion of a semicircle). To minimize buffer drift, the first incubation step is necessary, and we checked the PBS response to be equal with a confidence level of 95% over a time of 2 h experiments (final response for the buffer is an average of all the responses).

To determine the LOD and LOQ of the biosensor, a calibration curve corresponding to the normalized imaginary impedance (using Equation

(1)) over the concentration of SARS-CoV-2 Spike Glycoprotein is presented in Fig. 3e. Using this calibration curve, the obtained LOD is 19.09 ± 0.65 fM and the LOQ is 63.66 ± 2.1 fM, where the uncertainties are defined both sided (the calculation is based on Equations (2) and (3)). This LOD corresponds to the concentration around 4×10^5 copies/mL (details in SI section 7.1), which is less than the viral load in the day 0 of symptoms for most of the patients according to reported data from 17 studies (Challenger et al., 2022).

3.5. Selectivity of the biosensor against influenza a virus (IAV) H1N1 and SARS-CoV

The selectivity was tested with two other viruses: influenza A virus (IAV) H1N1 and SARS-CoV. The three viruses are respiratory viruses and they are widely used in SARS-CoV-2 diagnosis studies (Zhang et al., 2020). Moreover, IAV continuously poses a burden to global health and coexists with SARS-CoV-2.

Fig. 3f–g and Fig. S11 represent a comparison between the phase shift responses of the biosensor. The biosensor shows distinct selectivity for SARS-CoV-2 towards both IAV H1N1 and SARS-CoV. As expected,

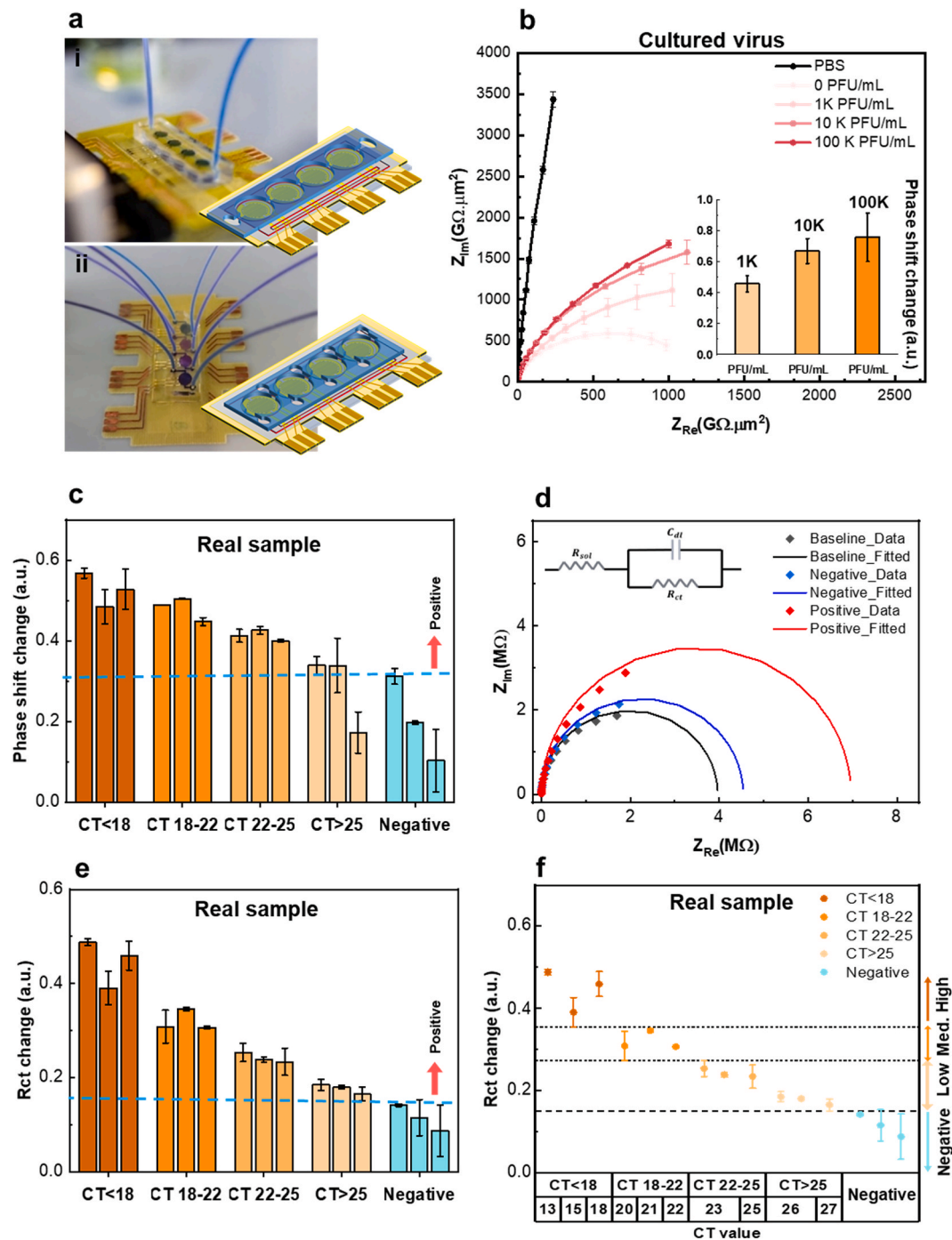


Fig. 4. Biosensor performance with cultured and real sample of SARS-CoV-2 virus. a) a real image and schematic of the biosensor being used for data acquisition (i: Parallel design, used for cultured virus sample and ii: Multiplexed design used for real samples), b) Nyquist plot for three concentration of cultured virus, diluted in PBS 1X. Inset shows the response changes of the phase shift measured for three different biosensors, c) response changes of the phase shift directly measured from data, d) an example of data fitting with an EEC (Inset shows the EEC), e) response changes of the charge transfer R_{ct} for the real samples, and f) categorization of real samples into 4 distinct group after measurement: Negative, Low, Medium and High, referring to the load of the virus and based on the response changes of the charge transfer.

SARS-CoV selectivity is less pronounced, since these two viruses share almost 70% of their spike glycoprotein, (Zhang et al., 2020), as seen in Fig. 3f, but remains significant at lower concentrations. Figs. S11a–b show the response of the biosensor to these viruses in the form of normalized Nyquist plot.

It is noteworthy to mention that the selectivity can vary when the virus mutates, because its binding affinity towards antibodies can change. However, as viruses consists of many epitopes (25–40 per virus)

(Ke et al., 2020; Laue et al., 2021; Turoňová et al., 2020) the response to other mutations could be lower or higher, but it is still expected to be more significant when compared to IAV H1N1 and SARS-CoV.

3.6. Performance of the biosensor with cultured SARS-CoV-2 virus

The biosensor performance was examined with cultured SARS-CoV-2 prior to proceeding with clinical samples, with the aim of confirming the

possibility to detect inactivated virus. A highly concentrated batch of virus ($\sim 9.56 \times 10^6$ PFU mL⁻¹) was diluted in PBS resulting in three different concentrations corresponding to $\sim 9.56 \times 10^2$ PFU mL⁻¹, $\sim 9.56 \times 10^3$ PFU mL⁻¹ and $\sim 9.56 \times 10^4$ PFU mL⁻¹. Fig. 4b shows the Nyquist graph of the acquired data and the inset depicts the response changes of phase shift for three chips (the corresponding Bode graph is presented in Fig. S12). There is a distinctive signal from a concentration as low as 9.56×10^2 PFU mL⁻¹, while the error is not significant. Moreover, there is a trend in phase shift change, increasing with the concentration. It is worthy to mention that these concentrations are still lower than the viral loads reported in patients' samples with respect to day 0 of symptoms (Challenger et al., 2022).

In order to increase the reproducibility of the acquired data, we utilized the parallel design for cultured viruses (four readout and one sample in each chip), and the multiplexed design for clinical samples (each biosensor could be used to investigate four samples). Fig. 4a shows these two different designs in schematic and in real image.

3.7. Assessment of the clinical performance of biosensor

To demonstrate the feasibility of the biosensor as a clinical diagnostic tool, its performance was evaluated against nasopharyngeal swabs from patients. RT-PCR confirmed swabs containing 12 positives and 3 negatives samples from patients attending to the clinic, which were then inactivated and kept for further experimentation. The samples were chosen in a fashion that concentration would vary significantly. In these samples, Ct values, which represent viral load, varied from Ct = 13 (high concentration) to Ct = 27 (low concentration). Table S9 shows the composition of collected samples and their properties. In order to avoid any user interference and bias, we labeled the sample vials by numbers and ran a blind test.

Fig. 4c shows the response changes of phase shift over Ct values. As shown, in high Ct values (corresponding to lowest concentration), the signal is not significantly higher than the signal from negative samples. In fact, there is higher signal from negative sample comparing to one of the high Ct values. Therefore, due to the presence of strong response change from negative samples, one of the positive samples (lowest concentration and highest Ct value) would be tagged as negative. We used Receiver Operating Characteristic curve (ROC) analysis to determine the cut point to be 0.31 and the Area Under Curve (AUC) was 0.841, which ranks the sensor as good (Catunda et al., 2023; Mandrekar, 2010; Regina de Oliveira et al., 2023). The graph is presented in Fig. S13. In this sense, the phase shift response change has to be more than 31% for a sample, to be safely considered as positive. Consequently, the reliability of the biosensor will be lower. The sensitivity, specificity and accuracy of the biosensor are 0.88, 0.77 and 0.86 respectively (See SI Section 7.2. for details).

To address this issue, we modeled the data with an EEC using LMA and calculated the R_{ct} of each measurement. Fig. 4d shows one set of data with the fitted EEC model and extrapolation of R_{ct} , and its changes, while the inset shows the corresponding EEC, which is a simple one step electrochemical reaction. This reaction corresponds to electron transfer from and to potassium ferricyanide, where $[\text{Fe}(\text{CN})_6]^{3-}$ undergoes redox reactions; in other words we assumed that there is only a redox pair in our system and no other active reaction is involved. It is worth to mention that, in our experimentation the kinetic of antibody antigen is not being detected directly, but we trace the effect of this binding by a careful examination of the charge transfer from the surface of the electrodes. Hence, the EEC consists of electrolyte resistance (R_{sol}), double layer capacitance (C_{dl}) and R_{ct} . The first two elements of the circuit stay almost constant when the changes of the surface happen due to the antibody-antigen conjugation. However, there will be a significant change in R_{ct} . This change relates to the concentration of antigen; the more antigen in a sample, the more antigen-antibody conjugation on the surface and consequently charge transfer will be impeded more

intensely. In other words, we monitored the difference in R_{ct} before and after introduction of analyte.

As shown in Fig. 4e the changes of R_{ct} with the viral load are significant. Negative samples have an average change of less than 14%, while the change for the lowest concentration of sample containing virus is higher than 16% (here we also considered the standard deviation for different sensors and different Ct values). Based on ROC analysis, we attributed a 15% of changes to be the cut off to label a sample as positive or negative; if R_{ct} changes more than 15% the sample would be positive. The AUC improved to 0.977 which ranks the diagnosis as outstanding (Catunda et al., 2023; Mandrekar, 2010; Regina de Oliveira et al., 2023) and sensitivity, specificity and accuracy of the biosensor are 0.97, 0.88 and 0.95 respectively (see SI section 7.2 for details). Moreover, we also clustered the data, based on their R_{ct} changes to four distinctive groups; Negative, Low, Medium and High viral load, as it can be seen in Fig. 4f. Although using R_{ct} adds a post-processing step including a complex nonlinear least square (CNLS) problem solving, it returns a more precise analysis, and improves reliability of the sensor significantly (compare Fig. 4c and e).

3.8. Smartphone-based detection of SARS-CoV-2

To demonstrate the capability of the designed platform to become a point-of-care test, we specifically designed a smartphone-based version of this biosensor (Fig. 5a and b). For these measurements we used a Sensit Smart (PalmSense, Netherlands) as the electrochemical workstation which has dimensions close to a USB memory stick. Furthermore, we used the swab transport medium as the buffer for all the measurements by adding potassium ferricyanide to it and limited the measurement to a frequency range from 10 to 1 Hz with only 9 data points, to reduce the measurement time.

Fig. 5c–d presents the results from two sets of negative and positive samples, in form of Nyquist and Bode graphs. As shown in Fig. 5c (Nyquist graph), a significant R_{ct} increase occurs. In the Bode graph (Fig. 5d), there is a meaningful difference between positive and negative samples, where positive samples will have a more significant change in phase shift. Therefore, it is well possible to distinguish between negative and positive samples using the smartphone-based platform. The baseline is the swab transport medium with potassium ferricyanide.

4. Conclusion

In this study, we present a low-cost, mass-producible and versatile microfluidic impedance biosensor, which is proven to be highly sensitive, label-free and reproducible. The biosensor is conceived with different designs dedicated to be used in a variety of readout platforms for laboratory analysis, for multiple sample detection or sensor self-validation purposes. Microfluidic integration helped to reduce sample and reagent volume consumption, enhance analyte diffusion toward the sensor's surface, and improve data reproducibility and reliability. We used DoE analysis to filter, optimize, and model our operational parameters. A smartphone-based readout toward a faster testing was also implemented. All these steps resulted in a robust device with a LOD of 19 fM and a LOQ of 64 fM for spike protein, which is relevant to the reported viral load on day zero of symptoms. Such early diagnostic approach is critical to stop the chain of infections. Moreover, a clinical validation of the proposed biosensor was done with 15 patient samples showing the possibility of performing semi-quantitative virus load examination.

Various limitations restrict the usage of the hereby presented platform as a true POC test, such as the need of microfluidic pumps, no discrimination of mutants and certain degree of cross-reactivity with SARS-CoV. In future, improvements may be focused on solving these issues by implementing passive microfluidics or miniature pumps (e.g. piezo pumps), and by including more receptors toward the known mutants. Additionally, modifications on the electrodes may be considered,

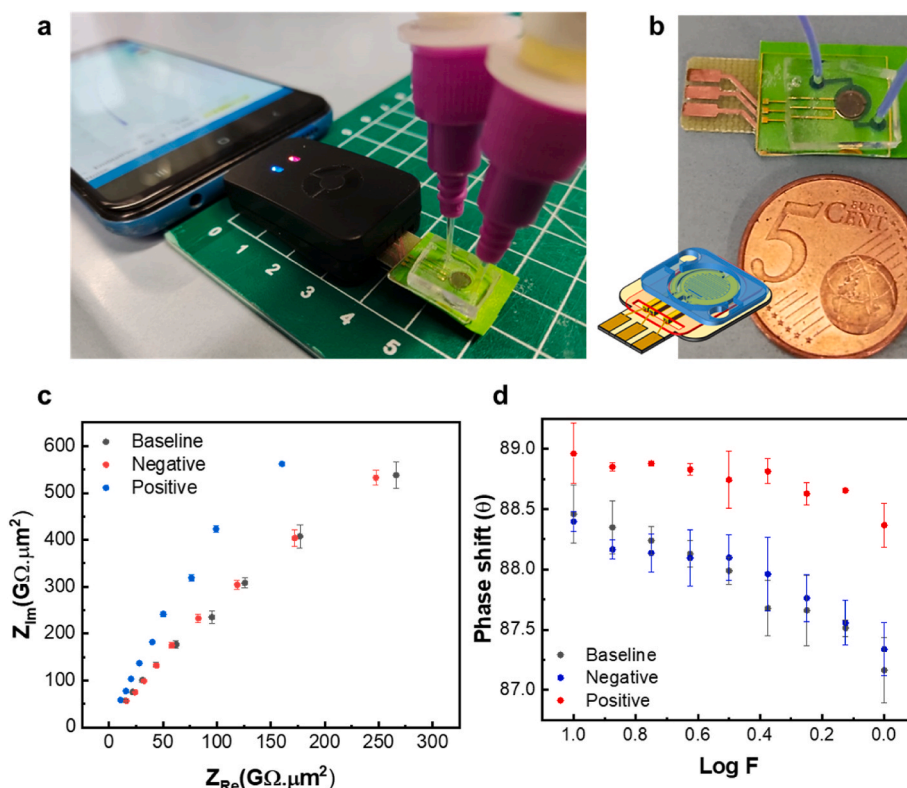


Fig. 5. Demonstration of biosensor capability to be used with a smartphone as a point of care test. a) real image of the biosensor for the smartphone-based readout platform, b) enlarged image of the smartphone-based sensor and a schematic of it, c) Nyquist plot of two samples (one negative and one positive) being detected by the biosensor using smartphone as readout, and d) Bode plot of the phase shift corresponding to the same data set.

for example employing composite materials or nanomaterials, to not only increase the sensitivity and reproducibility, but also to provide the platform with additional functions such as self-surface renewal, and pathogen in-situ elimination. Furthermore, a comprehensive study on the impact of the drift response from nasopharyngeal swab medium can help to enhance the reliability of the test. As another foreseeable improvement which surely helps to enhance the performance of the biosensor (in reliability and response time) is to improve the CNLS algorithm for fitting EEC or even to consider using Non-Linear EIS.

CRediT authorship contribution statement

Soroush Laleh: Investigation, Visualization, Data curation, Conceptualization, Formal analysis, Writing – original draft, Resources, Validation, Methodology, Writing – review & editing. **Bergoi Ibarlucea:** Conceptualization, Supervision, Validation, Methodology, Visualization, Writing – review & editing. **Marlena Stadtmüller:** Resources, Methodology, Writing – review & editing. **Gianaurelio Cuniberti:** Validation, Funding acquisition. **Mariana Medina-Sánchez:** Conceptualization, Methodology, Supervision, Validation, Funding acquisition, Data curation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declared that they have no conflicts of interest to this work.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bios.2023.115362>.

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