



Nanoyeast-based impedimetric biosensor with mutated single chain antigen-binding fragment anchoring for SARS-CoV-2 detection

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Abstract

Impedimetric biosensors are useful for pathogen detection as they combine electrical impedance spectroscopy with the specificity of immunological reactions. These devices can be engineered to detect minute changes in electrical impedance caused by interactions between immobilized recognition elements and target antigens in a sample. They are advantageous in allowing for label-free and real-time detection, with the ability to operate without electroactive materials. Herein, we report an impedimetric biosensor containing nanoyeast expressing SARS-CoV-2 antibody fragments as the active layer. Using nanoyeast offers key advantages such as biocompatibility and stability. The single-chain antigen-binding fragment (scFab) against receptor binding domain of SARS-CoV-2 was mutated according to in silico predictions. It was expressed in *Saccharomyces cerevisiae* fused to the agglutinin 2 (Aga2), where the binding to Aga1 on the yeast cell wall displays the scFab on the surface of nanofragmented yeast (NY). Electrical impedance monitoring confirmed the successful immobilization of NY onto an adsorbed chitosan layer. This biosensor architecture detected SARS-CoV-2 spike protein with a limit of detection (LoD) of 5×10^{-18} g/mL. It distinguished viral concentrations ranging from 0.3 to 80 plaque-forming units per milliliter (PFU/mL) and demonstrated selectivity for SARS-CoV-2 over H1N1 influenza and Dengue virus. These findings suggest that this biosensing technology could be further adapted for other biomedical and clinical analyses, being promising to improve current pathogen detection methods.

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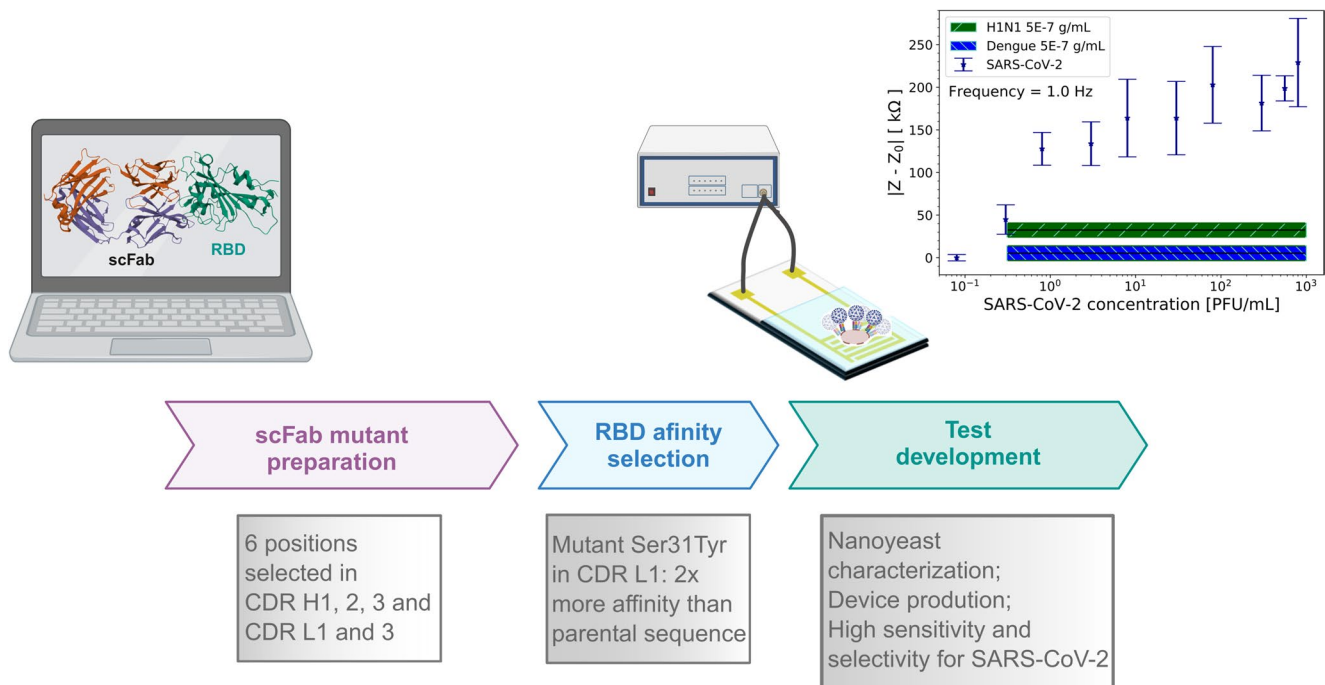
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Graphical abstract



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1 Introduction

The emergence of evolving pathogens, along with the rise of chronic and infectious diseases, highlights the need for innovative diagnostic technologies (Araujo et al. 2024). In this context, biosensors offer a significant advantage with respect to traditional methods by eliminating the need for sophisticated equipment and highly trained personnel (Song et al. 2018). Indeed, biosensors provide rapid, accurate, and minimally invasive methods for detecting and monitoring health conditions, making them ideal for point-of-care (PoC) diagnostics (Samuel and Rao 2022; Tripathi and Bonilla-Cruz 2023). By integrating principles from chemistry, biology, and electronics, biosensors convert biological responses into measurable optical, electrical, or piezoelectric signals (Chalklen et al. 2020; Singh et al. 2023; Sumitha and Xavier 2023; Wu et al. 2023). For the specific detection of a disease biomarker, a requirement is to functionalize the devices with probes, e.g., monoclonal or polyclonal antibodies, aptamers, nucleic acid, or enzymes. Although antibodies are currently the gold standard for antigen detection, their production and isolation are time-consuming, making them an expensive biorecognition element (Marx 2013a). This limitation has sparked research into high-quality affinity reagents that can be produced rapidly and cost-effectively

while preserving their target antigen specificity (Holliger and Hudson 2005; Marx 2013b).

Recombinant antibody-like molecules, such as single-chain variable fragment (scFv) and antigen-binding fragment (scFab), represent a promising alternative to monoclonal antibodies (Chao et al. 2006; Feldhaus et al. 2003; Holliger and Hudson 2005). These molecules can be displayed on the surface of phages, yeast, or ribosomes (Valldorf et al. 2022). Additionally, yeast clones that bind specifically to antigens can be developed within weeks, offering a faster and more cost-effective solution compared to monoclonal antibodies (Araujo et al. 2024). For example, Ferrara et al. (2012) used antigen 85, i.e., the most abundant secreted tuberculosis protein, in a method that combines phage and yeast display to select antibodies against any potential target. Although the methods to select yeast-displayed scFv dates to 1997 (Boder and Wittrup 1997), they performed poorly in immunoassays tests until lyophilized whole cell yeast-scFv probes were reported (Gray et al. 2012). Gray et al. (2012) used yeast-scFv probes bound specifically to putative cyst proteins of *Entamoeba histolytica*, also highlighting that lyophilization extended shelf life of this affinity reagent. However, this method was limited since the whole-cell reagents were often unsuitable for diagnostic applications due to their insolubility and large size. In this context, Grewal et al. (2013)

employed cell-free yeast-scFv reagents produced through mechanical fragmentation of whole yeast-scFv cells. The so-called nanoyeast-scFv platform was applied on label-free electrochemical detection of *Entamoeba histolytica* at concentrations lower as 100 pg/mL (Grewal et al. 2013), which is comparable to the limit of detection (LoD) achieved for whole yeast-scFv probe combined with labelled polyclonal antibody (Gray et al. 2012). Nevertheless, as the mechanical fragmentation produced nanoyeast (NY) with heterogeneous size, syringe filtering was utilized to investigate the correlation of NY fragment size on the electrochemical detection of *Entamoeba histolytica*, demonstrating that sub-100 nm NY-scFv exhibited optimal performance (Grewal et al. 2015). Subsequently, NY-scFv was designed for targeting receptor binding domain (RBD) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Li et al. 2021). This platform had a limit of detection of 17 virus/ μ L using surface-enhanced Raman scattering (Li et al. 2021), also demonstrating effective performance on fluorescence, electrochemical and colorimetric-based lateral flow systems (Li et al. 2023). Table 1 presents additional applications of recombinant antibody-like molecules.

The versatility of antibody-like constructs in single-chain format as biorecognition elements is evidenced by the diverse range of diagnostic and detection applications summarized in Table 1. While traditional platforms have successfully employed scFv for detecting various pathogens and toxins, the integration of these fragments (or other formats) into impedimetric biosensors stands out for its high sensitivity and label-free nature. Notably, the transition from conventionally purified scFv to mutated, engineered formats or displayed in yeast surfaces, as explored in this study, represents an advancement in biosensor design. This strategy not only enhances binding affinity and reagent stability but also significantly reduces fabrication costs. Beyond improving sensitivity and specificity, the integration of whole-cell or fragmented yeast platforms offers distinct logistical advantages, such as streamlined and scalable production. These attributes proved indispensable during the COVID-19 pandemic, with the demand for rapid, cost-effective, and consistent diagnostic tools.

Another essential issue to achieve low-cost, simple pathogen monitoring is the principle of detection. Label-free detection remains a primary objective in biosensor development, as labeling is often time-consuming, costly, and may interfere with the molecular binding sites. Such detection can be made with Electrochemical Impedance Spectroscopy (EIS) by probing target-analyte interactions without extrinsic markers. EIS-based devices can reach low Limit of Detection (LoD) while maintaining portability for Point-of-Care (PoC) applications (Samuel and Rao 2022).

The versatility of EIS has been demonstrated across diverse clinical scenarios. For cancer diagnostics, EIS-based platforms successfully identified circulating tumor cells (CTC) in human breast cancer lines and canine mammary cancer models (Burinaru et al. 2022; Huerta-Nuñez et al. 2019). By coupling antibodies to magnetic nanoparticles or interdigitated gold electrodes, these studies achieved high sensitivity for specific pathological stages, offering a promising tool for managing metastasis and recurrence. EIS has also proven effective for pathogen detection (viruses, bacteria, fungi, and parasites) using various recognition elements, such as DNA probes, aptamers, and antibodies, often integrated with nanomaterials like graphene oxide or chitosan (Zhang et al. 2025). For instance, oral *Streptococcus mutans* was detected at concentrations as low as 10^3 CFU (Colony Forming Unit)/mL using antibody-functionalized printed circuit boards (Dutta et al. 2019). The receptor-binding domain (RBD) of the SARS-CoV-2 spike protein was identified at 132 ng/mL using an aptamer-based impedimetric biosensor (Nemčková et al. 2024). Furthermore, wash-free technologies are highly desirable to minimize interference and reduce total assay time. For example, an EIS-based antitroponin-I biosensor achieved a detection limit of 10 pg/mL in just 10 min, six times lower than physiological levels in serum (Dutta et al. 2015). Similarly, the malaria pathogen *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) was detected within five minutes using gold electrodes, spanning a wide dynamic range from 100 ng/mL to 100 μ g/mL (Dutta and Lillehoj 2018). These advancements confirm the potential of impedimetric platforms to provide rapid, sensitive, and simplified diagnostic workflows.

In this paper, we describe the development of a label- and wash-free high-sensitivity impedimetric biosensor for SARS-CoV-2 detection, utilizing an affinity-enhanced scFab mutant derived from the CC12.1 antibody (Yuan et al. 2020). By integrating in silico structural analysis with Yeast Surface Display (YSD), we identified and selected a mutant with superior binding affinity for the S1 domain of RBD spike protein. A key innovation of our approach is the direct use of engineered yeast cells (nanoyeast, NY) as the biorecognition element. This strategy bypasses the need for laborious and costly chromatographic purification, thus enhancing the scalability and cost-effectiveness of biosensor fabrication. Our NY-based platform, anchored on chitosan-coated interdigitated electrodes, achieved a remarkable Limit of Detection (LoD), much lower than conventional monoclonal antibody-based sensors (Soares et al. 2022). Furthermore, the sensor demonstrated high selectivity against Dengue and H1N1 viruses, with a LoD of 0.3 plaque-forming unit per milliliter (PFU/mL) for inactivated SARS-CoV-2.

Table 1 Examples of Recombinant antibody-like platforms for various detection purposes

Type of antibody-like	Target	Detection platform	Achievement	Reference
scFv double mutant fused to the green fluorescent protein (GFP)	Programmed death 1 protein (PD-1) on the surface of Jurkat cells	Immunofluorescence and flow cytometry	Quantitative determination of activated T-cells in tumor biopsies (in combination with anti-CD3)	Shin et al. 2025
scFv	RE1-Silencing Transcription factor (REST)	Immunohistochemical	Enhanced detection in reduced time of cervical lesions when scFv is conjugated to biotin	Rodríguez-Nava et al. 2025
scFv fused to lactoferrin	Influenza B nucleocapsid protein (NP-B)	Dot blot, enzyme-linked immunosorbent (ELISA), and lateral flow assays	Improved performance (100x) compared to scFv non-conjugated	Nguyen et al. 2025
scFv	RBD of SARS-CoV-2 spike protein	Lateral flow immunoassay	Low-cost LFIA was developed with effective antigen recognition by scFv paired to aptamer	Çam Derin et al. 2025
scFv conjugated to biotin	OXA-23 carbapenemase of <i>Acinetobacter baumannii</i>	Paper-based immunoassay	Simple and low cost test with 100% sensitivity and specificity	Kasana et al. 2025
scFv with mutated CDR3 region in light and heavy variable chains	Eight Cry toxins of <i>Bacillus thuringiensis</i>	Indirect competitive enzyme-linked immunoassay (IC-ELISA) and indirect competitive time-resolved fluorescence immunoassay (IC-TRFIAs)	high accuracy and stability to detect the toxins in food	Xu et al. 2025
scFv conjugated with indocyanine green	Neurogenic locus notch homolog protein 2 (NOTCH2)	Near-infrared fluorescence imaging	Conjugated scFv probe accumulated and maintained the signal for long time in tumor tissue	Li et al. 2025
Bispecific antibody fragment – nanobody against antigen fused to scFv binding to a matrix	Nanobody target: RBD of SARS-CoV-2 scFv target: methoxy polyethylene glycol	Surface-enhanced Raman scattering (SERS)	RBD was detected in plasma with an LoD of 3.97 ng/mL. The platform reduced reagent, sample and time	Wang et al. 2025
Nanoyeast-scFv	Soluble programmed death 1 (sPD-1), soluble programmed death-ligand 1 (sPD-L1) and soluble epidermal growth factor receptor (sEGFR)	Surface-enhanced Raman scattering (SERS)	Proteins were detected even in multiplex with efficient recovery from human serum	Li et al. 2018
Nanoyeast-scFv	DENV2-secreted nonstructural 1 protein (NS1) in infected mosquitoes	Surface-enhanced Raman scattering (SERS)	Low cost and high sensitivity (500 fg of NS1).	Farokhinejad et al. 2022
Modified yeast two-hybrid system based on scFv or single-domain antibody V _H H	Caffeine, aflatoxin and <i>Escherichia coli</i> cells by O157 antigen	Chemiluminescence assay and flow cytometry	Versatile and low-cost system	Su et al. 2023
Nanoyeast-mutated scFab	RBD of SARS-CoV-2	Electrochemical impedance spectroscopy	High sensitivity, specificity and low-cost	This study

2 Materials and methods

2.1 Materials

All primers used in this work were synthesized by Exxtend, Brazil. Endonuclease restriction enzymes *NcoI*, *NotI* and *DpnI* (10 U/ μ L) were FastDigest Thermo Scientific, USA. The solution set of deoxyribonucleotides (dNTPs) at 100 mM were purchased from New England Biolabs, USA. Regarding the DNA polymerases, AccuPrime™ *Pfx* (2,5 U/ μ L) was purchased from Invitrogen (Thermo Scientific USA), PrimeStar HS (250 U) from Takara Bio (Japan) and Phusion High Fidelity (2000 U/mL) from New England Biolabs. GeneJET Gel Extraction Kit and GeneJET PCR Purification Kit were acquired from Thermo Scientific. For DNA sequencing reaction and purification, Big Dye Terminator v3.1 Cycle Sequencing Kit and purified with the Big Dye XTerminator Purification Kit were acquired from Applied Biosystems (Thermo Scientific, USA).

Salts, salmon single-stranded DNA (ss-DNA), bovine serum albumin (BSA), amino acids, uracil, glucose, raffinose, galactose, low molecular weight chitosan, glutaraldehyde 50% in H₂O, and 10 mM phosphate buffered saline (PBS) with MgCl₂ and CaCl₂ (liquid, sterile-filtered, and suitable for cell culture) pH 7.4 were all acquired from Sigma-Aldrich. Yeast nitrogen base without amino acids and casamino acids were purchased from Becton, Dickinson and Company and USBiological Life Sciences (USA). Flow cytometry marker streptavidin-phycoerythrin/Cyanine7 (PE/Cyanine7) was bought from Biolegend (USA) and anti-V5 tag antibody conjugated to iFluor 488, from Genscript (USA). Analytical grade solvents were acquired from Sigma-Aldrich, Exodo Científica or Synth (Brazil). SARS-CoV-2 biotinylated-RBD (amino acid sequence from residue 48 to 250 of PDB ID: 7VYR) was acquired from Biolinker (Brazil), while Spike protein was acquired from ABCAM (UK), H1N1 Recombinant Virus was obtained from ProSpec (Israel) and Dengue Envelope-3 protein from Aviva Systems Biology (USA). SARS-CoV-2 virus inactivated by UV irradiation was obtained from the Institute of Biology, University of Campinas (Brazil) (Patterson et al. 2020).

2.2 In silico analysis of potential residue site mutations

The interaction of the antigen-binding fragment (Fab) of CC12.1 and RBD was evaluated from the crystal structure downloaded from Protein Data Bank (PDB ID 6XC2; <http://www.rcsb.org/sequence/6XC2>). The structure was prepared by deleting water molecules and double chains. Then, amino acid residues in Fab were selected in the boundary

with RBD residues using Pymol. The free energy of the chosen residues in silico mutated to the other 19 amino acids was calculated using MutateX software in a range from −3.0 to 5.0 kcal/mol (Tiberti et al. 2022).

2.3 Cloning and site directed mutagenesis

CC12.1 heavy and light chain Fab sequences (PDB ID 6XC2) were obtained (SynBio Technologies, USA) codon optimized for expression in *Saccharomyces cerevisiae* (Figure S1 in Supplementary Information). They were amplified separately by polymerase chain reaction (PCR) and fused by PCR forming a flexible linker peptide (SGGSTSGSGK-PGSSEGSSGS) between both chains to build up the antigen-binding fragment single chain (scFab) (Fan et al. 2015). Amplification reactions were conducted by adding to the DNA template solution: AccuPrime™ *Pfx* DNA polymerase (1 U), 200 μ M of dNTPs, 1 μ M of each respective forward and reverse primer and 10x buffer in a thermocycler (Analytik Jena, Germany). Primers for cloning, including overlapping primers, are presented in supplementary Table S1. Fusion PCR was carried out using PrimeStar HS DNA polymerase with a first 15 cycles reaction, assembling each module in the absence of primers, followed by a second reaction with forward and reverse primers for the utmost extremities of the whole fragment (Zhou et al. 2012). According to the necessity, PCR products were directly purified or agarose-gel extracted using proper GeneJET Kit.

The fragment was inserted in frame with agglutinin 2 sequence and hemagglutinin tag (HA-tag) in N-terminus and SV5 epitope and six histidine tags in C-terminus in the yeast expression vector pYD4 [CEN/ARS *P_{GALI}-AGA2-HA-SV5-6xHIS TRP1*] kindly donated by Professor Jianlong Lou (California University, USA). The vector was linearized with *NcoI* and *NotI* restriction enzymes and transformed by lithium acetate method (Gietz and Schiestl 2007) into EBY100 simultaneously to the fused PCR product coding the scFab. Residues of CC12.1 were changed by site directed mutagenesis with single primer reaction (Edelheit et al. 2009) using the parental scFab sequence cloned in pYD4 as template. SD primers are also listed in Table S2. Briefly, the SD amplifications were conducted with Phusion High Fidelity DNA polymerase using a hot start step of 98 °C for 1 min followed by 30 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 7 min, and final polymerization at 72 °C for 10 min. Then, single-stranded amplification products were annealed by gradually decreasing the temperature 1 °C/min from 98 °C to 90 °C and then decreasing 10 °C/min up to 37 °C. DNA solutions were treated with *DpnI* and transformed into competent *Escherichia coli* DH5a cells. Mutations were confirmed by sequencing in a 3130 Genetic Analyzer (Applied Biosystems, USA) with

the Big Dye Terminator v3.1 Cycle Sequencing Kit and purified with the Big Dye XTerminator Purification Kit.

2.4 Yeast culture and scFab induction

Recombinant DNA carrying CC12.1 scFab parental and mutated sequences were introduced separately into *Saccharomyces cerevisiae* EBY100 (*MATa AGA1::P_{GALI}-AGA1::URA3 ura3-52 trp1 leu2Δ200 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*, also donated by Professor Jianlong Lou), by lithium acetate method (Gietz and Schiestl 2007) and positive transformants were selected in synthetic complete medium without tryptophan (SC-Trp, 0.67% yeast nitrogen base without amino acids, 0.2% mix of amino acids and uracil without tryptophan, 2% glucose, 2% agar). To induce scFab production, yeast cells were first cultivated in synthetic dextrose medium with casamino acids (SDCAA, 0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, 2% glucose, 0.54% Na₂HPO₄ and 0.856 g NaH₂PO₄·H₂O). After incubation, cells were reinoculated at 0.5 of optical density at 600 nm (O.D.600, measured in a spectrophotometer DU800, Beckman Coulter, USA) in SR/GCAA (0.67% yeast nitrogen base, 0.5% casamino acids, 2% raffinose, 2% galactose, 0.1% glucose, 0.54% Na₂HPO₄ and 0.856 g NaH₂PO₄·H₂O) and incubated at 22 °C for about 48 h at 200 rpm (C25KC incubator shaker, New Brunswick Scientific, USA) (Chao et al. 2006).

2.5 Yeast surface display analysis

A total of 5×10^7 yeast cells were labelled for yeast display analysis by flow cytometry. The method is fully described in the literature (Chao et al. 2006) and main adjustments are reported here. After blocking non-specific sites with bovine serum albumin at 1% in phosphate-saline buffer (1% PBSA, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 1% BSA), cells were incubated with 420 nM of biotinylated RBD in 0.5% PBSA for 1 h at room temperature and gentle rotation. Further steps were conducted at 4 °C. Unbounded antigen was washed away and labelling of RBD and scFab was performed using PE/Cyanine7 and anti-V5 tag antibody conjugated to iFluor 488. Cells were washed and resuspended in 0.5% PBSA to be analyzed by Flow Cytometer FACS Aria Fusion (BD Biosciences). The mean fluorescence intensity (MFI) was captured only for singlets of 70,000 events. Yeast cell autofluorescence was set in flow cytometry of induced cells not bound to RBD neither marked with fluorophores. The presence of scFab was confirmed by evaluating MFI of cells marked with i488. Controls of specific interaction of scFab to RBD were evaluated by MFI of cells only incubated with PE/Cy7 and incubated with both fluorophores. One control of RBD

non-specific binding to the cell was evaluated using non-induced cells, 420 nm RBD and both fluorophores labelling.

Screening of the mutant scFab with higher affinity was assayed against different concentrations of RBD: from 2500 to 25.6 nM (serially diluted 1:2.5) for CC12.1 parental sequence while 1500 to 15.36 nM was used for mutated sequences, in triplicate, using flow cytometry. Induction of scFab expression and production on yeast surface was performed as described in subsection “2.4. Yeast culture and scFab induction”. The amount of 2.5×10^6 cells were collected, incubated with RBD and labelled as described in this same subsection. The solver tool of Excel was used to estimate the dissociation constant (Kd) since it enables a sufficient nonlinear optimization of the method according to the literature (Chao et al. 2006), considering Eq. (1):

$$AB = AB_{max} \cdot \left(\frac{[B]}{[B] + Kd} \right) \quad (1)$$

where AB is the quantification of the complex measured in the experiment, i.e., the fraction of MFI from PE/Cy7 to i488 (interaction to RBD related to the expression of scFab on cell surface); AB_{max} is the MFI of the fraction PE/Cy7 at maximum signal of AB complex at saturating [B]; and [B] is the RBD concentration (Glaser 2007).

2.6 Nanoyeast generation

Nanoyeast was generated by disrupting cells after scFab expression induction, as defined in subsection 2.4. About 5×10^7 cells/mL were resuspended in 10 mL ultrapure water and submitted to sonication at 40% amplitude, 9.9 s on and 9.9 s off for 15 min (sonicator Sonics Vibra Cell VC-505, USA) in ice bath. Debris were removed after two consecutive centrifugations at 500 rpm for 10 min and 4000 rpm for 30 min, both at 4 °C. To prevent clogging and minimize backpressure, particle size was restricted by sequential filtration through 0.22 µm pore-sized membrane (Bionaky, China) and 0.1 µm low protein binding Durapore® PVDF membrane (Millex-VV, Merck, Ireland) (Farokhinejad et al. 2021).

2.7 Nanoyeast characterization

Nanoyeast particle size and shape were characterized, respectively, by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). NTA was performed using the NanoSight NS300 (Malvern Instruments) equipped with a green laser (532 nm), a sCMOS camera and a syringe pump. Samples were diluted in ultrapure water to obtain a concentration between $1 \times 10^2 - 1 \times 10^8$ particles/mL and injected with sterile syringes (1000 µL) into the

sample chamber. Five videos lasting 60 s were recorded per sample and analyzed using the NTA 3.2 software (Malvern) using a syringe pump speed of 50 (Ribeiro et al. 2022). For TEM analysis, samples were applied onto a formvar/carbon thin bar square 400 mesh grid (Electron Microscopy Sciences, USA) and visualized after drying in a JEM-2100 microscope (Jeol, USA).

2.8 Nanoyeast-based impedimetric biosensor

The fabrication of gold interdigitated electrodes (IDEs) was conducted at the National Center for Research in Energy and Materials (CNPEM, Brazil) using standard photolithography. IDEs were deposited on glass, featuring 30 pairs of digits that were 3 mm long, 200 nm high, and 10 μ m wide, spaced 10 μ m apart from each other. To immobilize bio-recognition elements, the IDEs were cleaned in a UV ozone chamber and coated by a chitosan layer. Since the glass surface exhibits negative charges, the positively charged chitosan was adsorbed spontaneously. The IDEs were immersed in the chitosan solution (1.25% of chitosan in water + 1.65% of acetic acid) for 3 h, then thoroughly rinsed with ultrapure Milli-Q water (Merck, Germany) and let to dry at 70 °C overnight. Nanoyeast (NY) particles were immobilized employing glutaraldehyde crosslinking with a (1:1:2) solution of 2% glutaraldehyde, PBS 10 mM, and NY dispersion, respectively. A droplet (10–20 μ L) of this solution was placed onto the IDEs for two hours at 22 °C. Subsequently, the devices were washed with BSA 5% in PBS, remaining in the BSA solution for 40 min. The electrical response of the devices was investigated in Milli-Q water and 10 mM PBS using an impedance analyzer (1260 A, Solartron Analytical, UK), coupled to a dielectric interface model 1296 A (Solartron Analytical). The input voltage amplitude was fixed at 50 mV, and impedance measurements were conducted across the frequency range from 1 to 10⁶ Hz. All electrical characterizations were carried out in a climate-controlled facility at a stable temperature of 22 °C.

3 Results and discussion

3.1 In silico maturation of scFab CC12.1

The analysis of scFab residues in the interface with RBD residues led to the hypothesis of 48-point mutations in heavy variable (VH) chain and 29 in light variable (VL) chain as candidates for increasing affinity. The interaction between RBD and each chosen position substituted by the other 19 amino acids was estimated using the free energy ($\Delta\Delta G$) (Tiberti et al. 2022). Six residues were predicted whose specific mutations improved the interaction with RBD, as indicated in Table 2, which also displays $\Delta\Delta G$ values and the position in Fab structure (represented in Figure S1). Three of such residues in VH were positioned in one of each of the chain's complementarity-determining regions (CDRs). In VL chain, one residue was positioned in CDR L1 and two in CDR L3. As expected, the favorable $\Delta\Delta G$ of the positions and respective mutations are in or close to specific points of dominant interaction with RBD residues and extensive hydrogen bonds calculated with 200 ns simulations (Wang et al. 2021). The predicted mutation of the serine 31 (Ser31) residue in VH could alter the neighboring asparagine-tyrosine (NY) motif, while the Ser53 mutation in VH would change the serine-glycine-glycine-serine (SGGS) motif. Together, these motifs are determinant for epitope-antibody binding. CDR L1 and L3 have a small contribution to the hydrogen bonds with RBD (Wang et al. 2021; Yuan et al. 2020). The predictions indicated one mutation in L1 and two in L3 close to the main representative residues. One of the predicted mutations was in the lysine 97 (Lys97), which seems to form an unstable salt bridge with an aspartic acid of RBD (Wang et al. 2021). Therefore, its change to the non-polar glycine could represent a possible gain of stability in the interaction. Hence, to select the mutation with the potential for higher interaction with the RBD, the Yeast Surface Display (YSD) technique was employed to estimate the affinity of the parental and mutated scFab CC12.1.

Table 2 Favorable free energy of residues sites mutated and calculated K_d to RBD

Mutation	Position	$\Delta\Delta G$ (kcal/mol)	Fold-change of bound fraction (mutant/parental)	Calculated K _d
Parental sequence	n.a.	n.a.	1	808.2 ± 34.6 nM
Ser31Asn	CDR H1	−1.25763	0.81	n.t.
Ser53Glu	CDR H2	−1.99893	1.55	478.1 ± 140.3 nM
Ser53Asp	CDR H2	−1.60229	0.91	n.t.
Asp97Leu	CDR H3	−1.99844	0.79	n.t.
Asp97Pro	CDR H3	−2.33524	1.72	441.1 ± 66.6 nM
Ser31Tyr	CDR L1	−0.09107	1.49	418.2 ± 72.2 nM
Pro95Ala	CDR L3	0.05177	0.66	n.t.
Lys97Gly	CDR L3	−0.86291	1.34	477.6 ± 130.1 nM

n.a.; not applicable, n.t.; not tested because the mutant's antigen-bound fraction showed a fold-change of less than 1.0 relative to the parental sequence

3.2 Yeast surface display (YSD) directed the selection of a mutant with higher affinity

In silico prediction of antibody-antigen interactions and energetically favorable mutations reduces the need for extensive and costly assays, which would otherwise require generating and testing a large number of mutants in the lab (Huang et al. 2022). According to the MutateX calculated $\Delta\Delta G$, the residues with higher free energy (CDR H2 Ser53Glu, H3 Asp97Pro, L1 Ser31Tyr and L3 Lys97Gly) were chosen to construct mutated scFab, separately, and were expressed in *S. cerevisiae* to measure their interaction to RBD by flow cytometry. In YSD technology, the scFab of CC12.1 parental sequence was cloned in fusion to the C-terminal of the cell wall protein agglutinin 2 (Aga2). The SV5-epitope tag was localized at the C-terminal of VL chain and both chains were joined by a flexible peptide linker (Fan et al. 2015). The PCR mutations were conducted in the final plasmidial construction pYD4-scFab_CC12.1 [CEN/ARS P_{GALI} -AGA2-HA-scFab_CC12.1-SV5-6xHIS *TRP1*].

In *S. cerevisiae*, Aga2 binds to the matting α -agglutinin Aga1 protein, thus exposing the scFab to the external side of the cell. The Aga1 sequence is chromosomally engineered to be overexpressed under the galactose 1 promoter (P_{GALI}). The same applies to Aga2, which is expressed from the centromeric plasmid. That results in approximately 5×10^4 scFab per cell. Detection of one of the SV5-tag in the construction by a binding antibody conjugated to fluorophore (i488) enabled the normalization of scFab expression in flow cytometry analysis. The interaction with the antigen RBD was determined by the detection of biotin, conjugated to the RBD, using streptavidin also bound to a fluorophore (Cy7) (Angelini et al. 2015; Kang et al. 2022). Flow cytometry was used to confirm the maintenance of interaction and functionality of mutants compared to the parental sequence of CC12.1. For the evaluation, 10^6 cells were incubated with 10x excess of RBD molecules number in comparison to the average number of scFab. To quantify the antigen-antibody interaction, the MFI ratio of PE/Cy7 to i488 was calculated within the double-positive population. This ratio represents the antigen-bound fraction, effectively normalizing the amount of bound RBD to the scFab expression level. By accounting for cell-to-cell variability in scFab expression, this approach decouples protein density from binding affinity, ensuring that mutants with high expression but low intrinsic affinity were not misidentified as hits. Mutant performance was then compared against the parental CC12.1 clone (Table 2); only those exhibiting a binding fraction higher than 1.0 (normalized to the parental scFab) were prioritized for dose-response titration curves. Then, the same technique was applied for measuring MFI of the double positive events of a titration curve of different [RBD]. This

enabled the estimation of the antibody affinity by calculating Kd, since flow cytometry could be used to screen antibody mutants with improved interaction (Chao et al. 2006; Cherf and Cochran 2015; VanAntwerp and Witttrup 2000).

According to the calculated Kd in Table 2, parental CC12.1 scFab showed Kd of 808 ± 35 nM to RBD. On the other hand, mutants generally showed a twofold improvement in affinity, with statistical significance ($p \leq 0.05$) for Asp97Pro in CDR H3 and Ser31Tyr in L1 compared to the parental CC12.1. The screening, affinity and virus neutralizing potential of different mutants of CC12.1 were obtained with YSD with flow cytometry to select Fab mutants with improved affinity to the RBD (Zhao et al. 2022). The affinity-improved mutants had at least two CDR mutated in both heavy and light chains. Dissociation and other constants were measured using the purified mutated antibody and surface plasmon resonance method. Since their Fabs had more than one mutation site, the antibody was tested in the complete and purified form. The method to evaluate protein-protein interaction was different, and the affinity values reported there were lower than the ones calculated in this work. Zhao et al. (2022) found that the more potent CC12.1 mutant antibody was capable of neutralizing both the original SARS-CoV-2 and its variants of concern, which had a mutation at Ser31 in CDR L1, along with one mutation in each of the other five CDRs. The residue was changed to a tryptophan that is an apolar but large amino acid, such as tyrosine (polar), the amino acid predicted and with better results in our mutant. Therefore, even with one single mutation, our results afforded a promising interacting mutant of CC12.1, which was employed in a biosensor to detect SARS-CoV-2.

3.3 Nanoyeast characterization

Our aim is to overcome the costly production and purification of antibodies by producing only the detection component — i.e., the antigen-binding fragment — displayed on the surface of fragmented yeast cells, as in previous works (Farokhinejad et al. 2021; Li et al. 2023). Therefore, after expression induction of scFab CC12.1 mutant Ser31Tyr the NY was generated. It was characterized by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) whose results are shown in Fig. 1. NTA provided the average particle size, concentration, and Span index based on hydrodynamic properties (Ribeiro et al. 2022). The mean particle size was 161 ± 2 nm, with the mode (indicating the most frequent particle size) of 146 ± 8 nm (Fig. 1a). The high-resolution NTA data also revealed a particle concentration of $1.64 \pm 0.08 \times 10^{12}$ particles/mL, reflecting a high yield of consistent particles. Furthermore, the Span index was 0.45, indicating a narrow and

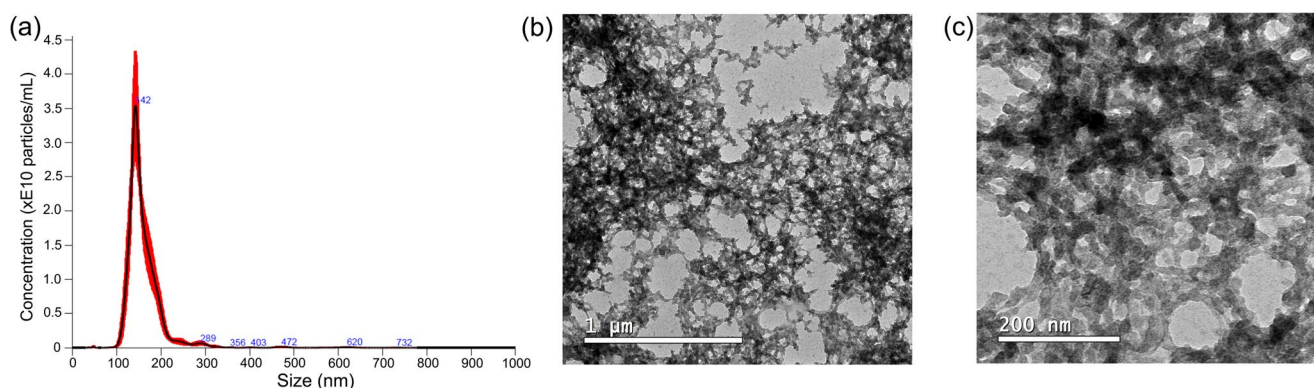


Fig. 1 Size characterization of the nanoyeast. **a** Representative plot of NY size distribution by NTA. **b** TEM micrograph of NY at 40,000x magnification with scale bar of 1 μm and **(c)** 150,000x magnification, scale bar of 200 nm

well-regulated size distribution. These findings demonstrate the efficiency of the nanofragmentation process. Nanoyeast particles tend to aggregate (Grewal et al. 2015) as observed in Fig. 1a with populations of different sizes (289, 472 and 732 nm) and confirmed by microscopy (Fig. 1b and c). Particles smaller than 200 nm with a uniform round shape were observed on the surface of the microscopy grid. Thereafter, NY with scFab CC12.1 mutant was employed in the production and evaluation of the biosensor for SARS-CoV-2 spike protein and SARS-CoV-2 detection.

3.4 Detection of SARS-CoV-2 by impedance spectroscopy

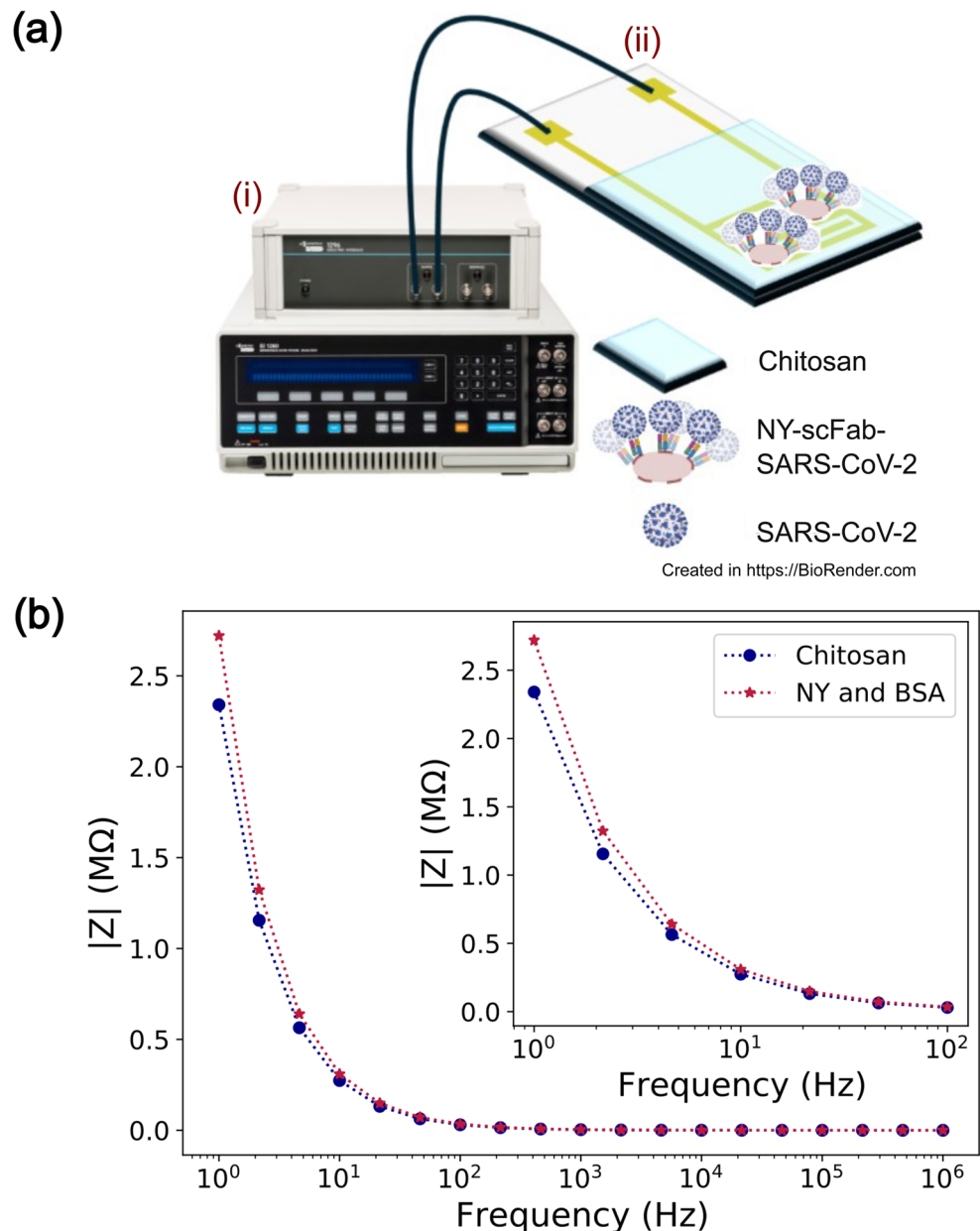
Electrical impedance spectroscopy was exploited to investigate the immobilization of NY onto the chitosan layer, in addition to passivation of non-specific binding sites using BSA. A scheme of the experimental setup is shown in Fig. 2a. The Bode plot in Fig. 2b shows that changes in the impedance spectra appear for frequencies lower than 10 Hz (inset in Fig. 2b). Since the double layer effect acts at low frequencies (Taylor and MacDonald 1987), it serves as the key factor in identifying the immobilized materials.

The performance of the immunosensor was investigated using two sets of devices prepared with NY produced in two synthesis procedures nominally identical (viz., NY M1 and NY M2). The negative control comprises a set of devices in which NY was not immobilized onto the chitosan layer. Figure 3a shows Bode plots for various concentrations of SARS-CoV-2 spike protein using devices modified with NY M1. To facilitate comparison, the impedance was subtracted from the one obtained for the 10 mM PBS solution (0 g/mL), i.e., $|Z - Z_0|$ was used in the plots. There is a clear increase in $|Z - Z_0|$ at frequencies lower than 10 Hz with increasing SARS-CoV-2 spike protein concentrations. The highest change occurs at 1 Hz, which was the frequency chosen to produce the calibration curve in Fig. 3b. The two

sets of devices modified with NY M1 and NY M2 present the same trend for $|Z - Z_0|$ as the concentration of SARS-CoV-2 spike protein increases. The logarithmic relationship between biosensor response and analyte concentrations is a characteristic trait of biosensors. This trend has consistently emerged across various studies, including those focusing on detecting specific biomarkers such as prostate cancer-specific DNA sequences (Raymundo-Pereira et al. 2021; Rodrigues et al. 2021; Soares et al. 2019), DNA methylation of the MGMT gene in head and neck cancer cell lines (Carr et al. 2020), sequences of single-stranded DNA from SARS-CoV-2 (Qiu et al. 2020; Soares et al. 2021), the spike protein and RBD from SARS-CoV-2 (Brazaca et al. 2022; Hensel et al. 2025; Soares et al. 2022). Moreover, there is a clear distinction of $|Z - Z_0|$ between the control devices, comprising both the NY unmodified and the NY lacking sc-Fab expression-modified device, and the ones modified by NY. The linear segment of the semi-log calibration curve was fitted with a straight-line equation, yielding a slope of 43.6 $\text{k}\Omega\cdot\text{mL/g}$ and a linear coefficient of 868.1 $\text{k}\Omega$. The Limit of Detection (LoD) of 5×10^{-18} g/mL was determined following the IUPAC method, where $\text{LoD} = S_{\text{BLANK}} + (3 \times \text{SD})$. S_{BLANK} represents the average signal of the control samples, and SD denotes the standard deviation. This LoD is two orders of magnitude lower than the one reported in the literature for a similar impedimetric immunosensor for SARS-CoV-2 based on monoclonal antibody (Soares et al. 2022).

The performance of the NY-based immunosensor to detect SARS-CoV-2 spike protein was evaluated by exploiting principal component analysis (PCA). Here, the selectivity of the devices was also analyzed with respect to the control devices that do not contain NY. The scatter plot in Fig. 4 feature two first principal components, PC1 and PC2, with a silhouette coefficient (SC) of 0.765. This high SC value suggests a clear separation between the clusters. Notably, PC2 distinguishes control samples (PBS 0 g/mL) from control devices without NY. Moreover, both PC1 and PC2

Fig. 2 Electrical impedance spectroscopy model built with NY containing mutated scFab. **a** Scheme of the experimental setup (i) Solartron 1260 A, coupled to a dielectric interface model 1296 A; (ii) IDE modified with chitosan, NY scFab to detect SARS-CoV-2. **b** Bode plot from 1 MHz to 1 Hz comparing the impedance spectra in PBS before (blue circles) and after the NY immobilization and BSA blocking of non-specific sites (red stars). Zoom-in of the Bode plot for the frequency range between 1 and 100 Hz



allow for discrimination between the various SARS-CoV-2 spike protein concentrations as indicated by the red arrow, and with respect to the control devices. It is worth noting that the highest concentrations (10^{-13} g/mL and 10^{-12} g/mL) are clustered together, suggesting saturation of available binding sites.

The performance of the NY-based immunosensor was investigated regarding the detection of SARS-CoV-2 inactivated virus in PBS. The detection of SARS-CoV-2 inactivated virus is shown in the Bode plots for concentrations ranging from 0.3 PFU/mL to 800 PFU/mL in Fig. 5a. $|Z-Z_0|$ increases with the concentration for frequencies lower than 100 Hz. As the higher variation of $|Z-Z_0|$ occurs at 1 Hz, we chose this frequency to obtain the calibration curve in

Fig. 5b. To be readable in semi-log scale the blank samples (0 PFU/mL) are labeled as 8×10^{-2} PFU/mL, which is the lowest value shown. The calibration curve in Fig. 5b shows an increasing trend from 0.3 PFU/mL to 80 PFU/mL, albeit with an outlier for 30 PFU/mL samples. At high concentrations, there is a noticeable saturation effect. It is also shown in Fig. 5b the $|Z-Z_0|$ at 1 Hz for H1N1 recombinant influenza virus and Dengue envelope-3 protein at a concentration of 0.5 μ g/mL. Note that NY-based immunosensors presented a clear selectivity against Dengue and H1N1; however, for the latter this occurs only for SARS-CoV-2 inactivated virus concentrations higher than 0.3 PFU/mL. The linear segment from 0.3 PFU/mL to 80 PFU/mL of the semi-log calibration curve was analyzed according to a straight-line equation,

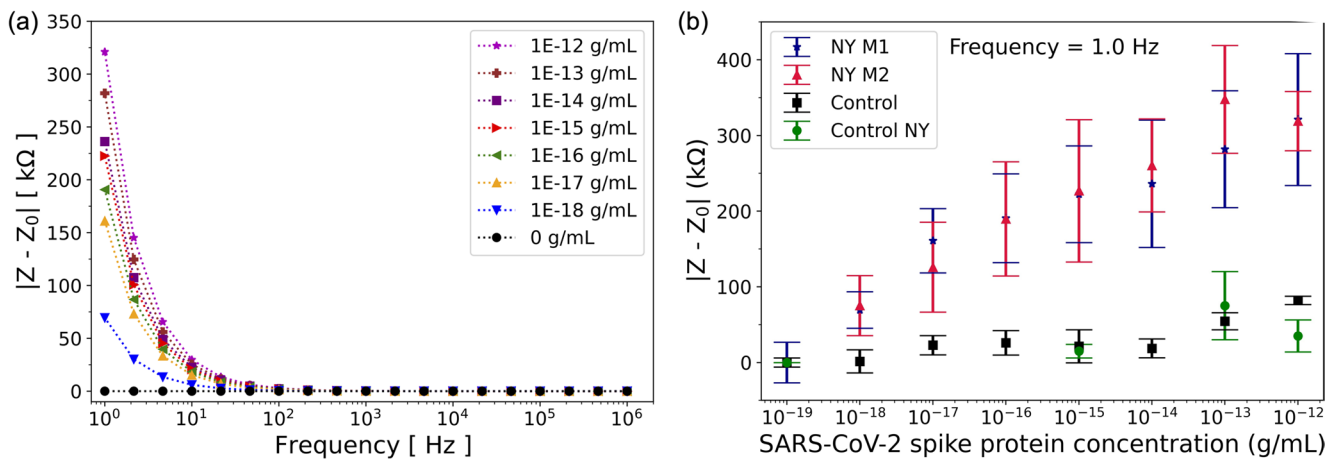
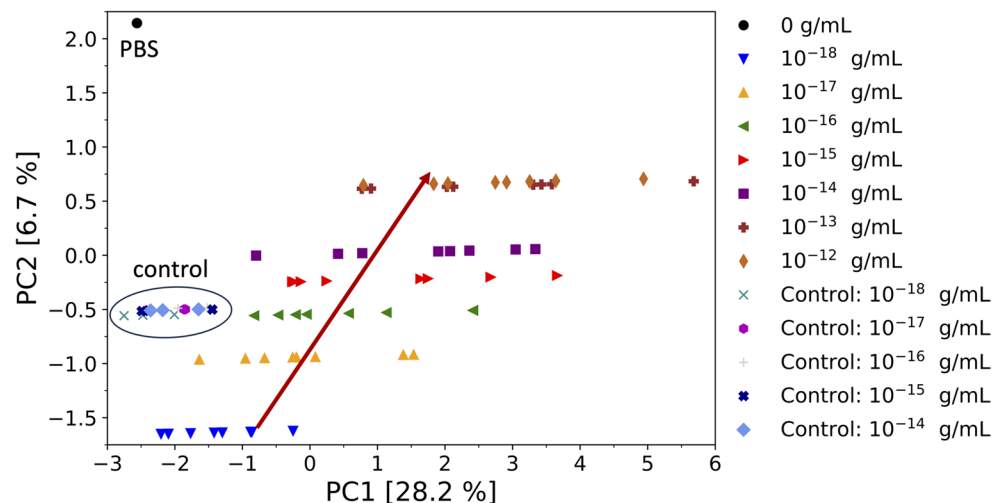


Fig. 3 Detection of SARS-CoV-2 spike protein at different concentrations with the devices modified with NY displaying the mutant scFab CC12.1. **a** Bode plots from 1 MHz to 1 Hz for each SARS-CoV-2 spike protein concentration investigated for the devices modified with NY replicate 1 (NY M1). **b** The calibration curve was obtained for two distinct syntheses of the nanoyeast (NY M1 - blue stars and NY M2 - red triangles), compared with both control cases, viz. the NY unmodi-

fied (black squares) and the NY lacking sc-Fab expression-modified (green circles) devices. The error bars correspond to the measurements in triplicate considering distinct devices. M1 and M2 are the abbreviation of two different replicates produced of the mutant CC12.1 CDR L1 Ser31Tyr. The concentration of 10^{-19} g/mL corresponds to 0 g/mL; this value was added only to be seen in the semi-log graph

Fig. 4 PCA scatter plot of the impedance spectra between 1 Hz and 10 Hz for different SARS-CoV-2 spike protein concentrations. Also shown are the data points for the control devices that were not modified with the NY. PC2 clearly distinguishes the two control samples (PBS 0 g/mL and control devices), also distinguishing the different concentrations of SARS-CoV-2 spike protein as indicated by the red arrow. Moreover, the clusters assigned to the SARS-CoV-2 spike protein are separated from the control with respect to both PC1 and PC2



yielding a slope of 41.5 kΩ.mL/g and a linear coefficient of 106.3 kΩ. The LoD calculated following the IUPAC method is 0.3 PFU/mL.

The performance of NY-based immunosensor to identify SARS-CoV-2 inactivated virus was also assessed using PCA. To investigate selectivity, we employed H1N1 influenza and Dengue viruses at 1 µg/mL, 0.5 µg/mL, and 50 ng/mL. Figure 6 presents the scatter plot with the first two principal components, which presented SC=0.802. PC2 notably distinguishes between the control samples (PBS 0 g/mL) and those containing the biomarkers or inactivated virus. Furthermore, the clusters corresponding to the SARS-CoV-2 inactivated virus are distinctly separated from those of the H1N1 influenza and Dengue viruses' samples along both PC1 and PC2 axes. There is an exception for the

SARS-CoV-2 spike protein at 0.3 PFU/mL samples, which is close to the LoD. Note that the different concentrations of the SARS-CoV-2 spike protein are effectively separated according to PC2, with an increasing trend indicated by the red arrow, with an outlier for 80 PFU/mL samples.

In summary, the biosensor demonstrated excellent selectivity for SARS-CoV-2, effectively distinguishing it from other relevant biomarkers such as H1N1 influenza and Dengue, even at low concentrations. It also demonstrated good sensitivity due to its very low LoD and clear separation of different SARS-CoV-2 inactivated virus concentrations. The robustness of these results was confirmed with the PCA analysis, where the samples with different virus concentrations could be distinguished. The high selectivity and sensitivity, and robust performance of the biosensor built with

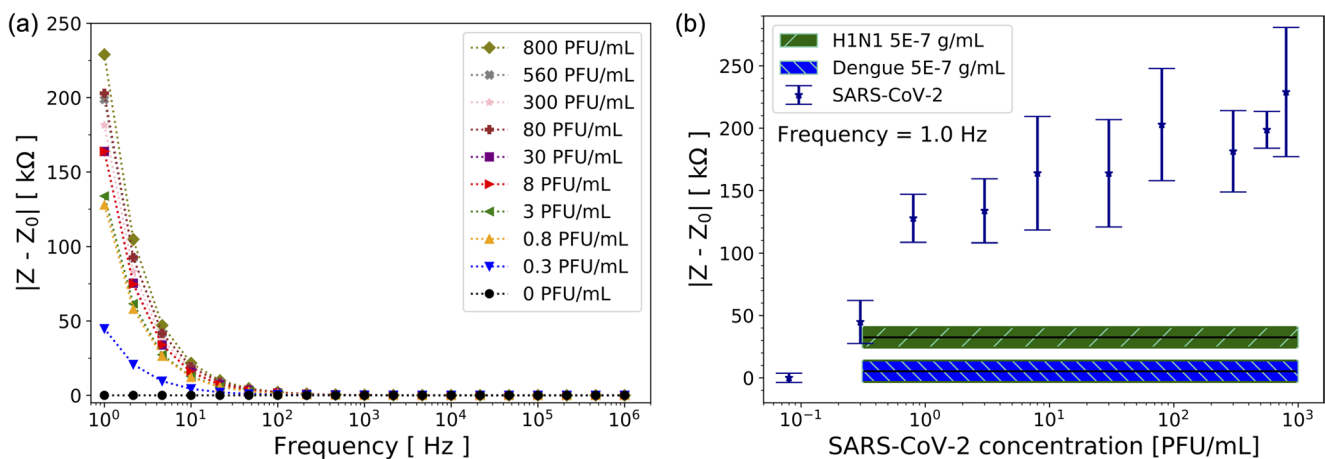
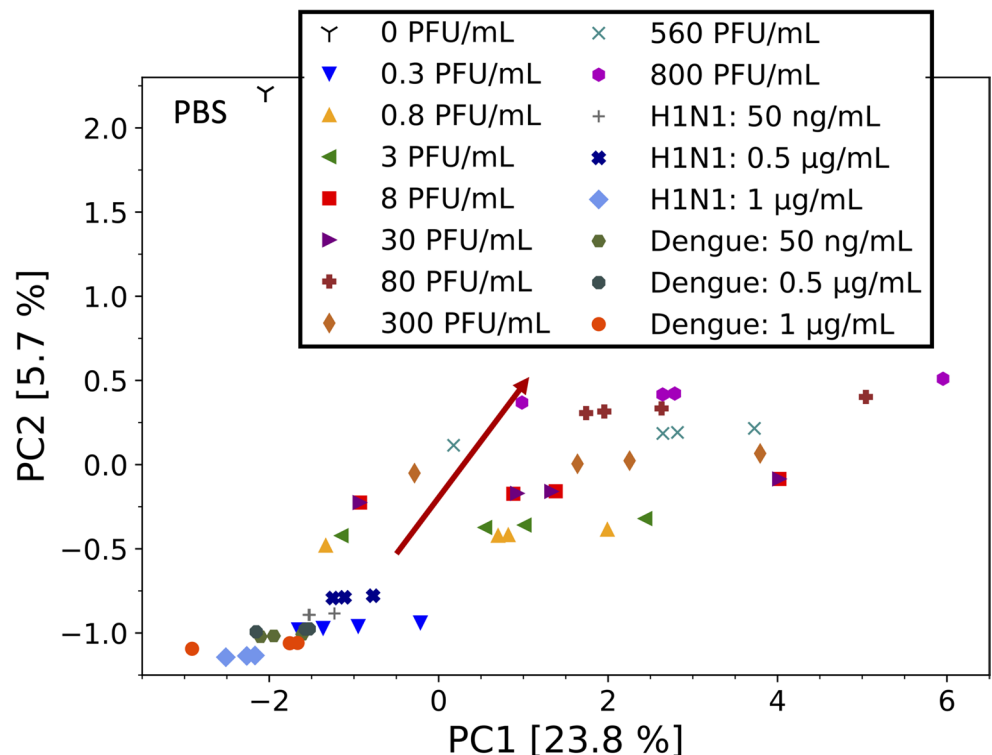


Fig. 5 Comparison of impedance spectra for SARS-CoV-2 virus detection with the generated device covered with NY. **a** Bode plot from 1 MHz to 1 Hz comparing the impedance spectra for each SARS-CoV-2 inactivated virus concentration. **b** The calibration curve obtained from the impedance spectra at 1 Hz compared with the value obtained for

H1N1 recombinant influenza virus and dengue envelope-3 protein at 0.5 μ g/mL. The error bars correspond to the measurements in triplicate considering distinct devices. Obs.: The concentration 0.08 PFU/mL corresponds to 0 PFU/mL; this value was added only to be seen in the semi-log graph

Fig. 6 PCA scatter plot of the impedance spectra between 1 Hz and 10 Hz for different SARS-CoV-2 inactivated virus concentrations. Also shown are the data points for H1N1 recombinant influenza virus and Dengue envelope-3 protein at a few concentrations. The red arrow indicates the clear analytical trend as the concentration of SARS-CoV-2 increases (from 0.3 PFU/mL to 800 PFU/mL)



NY displaying the mutated scFab of CC12.1 make it useful for further validations and clinical applications.

4 Conclusions

We demonstrated the development of a novel NY-scFab variant of the CC12.1 antibody, integrating in silico rational design with the scFab construct anchored to the surface of

nanofragment yeast. This innovative approach allowed for the precise identification of mutations that enhanced binding affinity toward the SARS-CoV-2 RBD. By utilizing a scFab fragment instead of full-length monoclonal antibodies (mAbs), we introduced a more stable, faster-to-produce, and cost-effective biorecognition element that maintained high functional integrity and could be used in diagnostics. Indeed, a high-performance impedimetric biosensor was developed, which may set a new benchmark for sensitivity

in viral detection. The mutant scFab enabled detection of the SARS-CoV-2 spike protein with a LoD of 5×10^{-18} g/mL. When compared to existing literature and similar impedimetric immunosensors that utilize traditional monoclonal antibodies, our platform demonstrated a LoD two orders of magnitude lower. This significant improvement highlights the superior orientation and density of binding sites provided by the scFab fragment on the sensor surface. Furthermore, the biosensor could distinguish inactivated SARS-CoV-2 at concentrations as low as 0.3 PFU/mL, while maintaining high selectivity as impedance values for H1N1 recombinant influenza virus and Dengue envelope-3 protein were comparable to those obtained with pure PBS solution. These results confirmed its reliability in complex biological matrices. The integration of computational engineering and impedimetric transduction presented here offered a robust solution to the limitations of current diagnostic tools. By minimizing the risk of false-positive results and lowering the detection threshold, this technology may represent a significant advancement in clinical diagnostics. Significantly, this platform can be readily refined and adapted to target a wide range of pathogens, serving as a foundational model for rapid, ultra-sensitive biomedical monitoring.

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Data availability Data will be made available on request.

Declarations

Competing interests The authors declare no competing interests.

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