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An engineered M13 phage–rGO electrochemical biosensor for rapid detection of viral protein in complex matrices

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Abstract

The demand for rapid and scalable biosensing technologies has motivated the development of antibody-free platforms capable of operating in complex sample environments. Here, we report an electrochemical biosensor based on engineered M13 bacteriophages displaying a SARS-CoV-2 spike S1–binding peptide immobilized on a reduced graphene oxide (rGO) transducer. The sensor employs a chemiresistive detection mechanism under a fixed low-voltage bias, enabling rapid electrical readout following target binding. Detection of S1 protein was achieved in buffer and in spiked complex matrices, including fetal bovine serum, pasteurized milk, and wastewater, demonstrating matrix tolerance under the tested conditions. The biosensor response is evaluated using a statistically defined binary detection criterion, with an operational limit of detection of 10^{-4} pg/mL in buffer. Compared to a previously reported antibody-functionalized rGO sensor fabricated using the same platform, the phage-based biosensor exhibits comparable sensitivity while offering advantages in genetic tunability and production scalability. While the present study focuses on proof-of-concept validation using spiked

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samples, these results highlight the potential of engineered phage–graphene interfaces as adaptable biorecognition elements for rapid electrochemical protein sensing in complex environments.

Introduction

Emerging infectious diseases underscore the urgent need for rapid, sensitive, and portable biosensing platforms capable of operating effectively in clinical and environmental settings[1-3]. While antibody-based biosensors offer strong specificity, their utility is limited by thermal instability, complex manufacturing requirements, and dependence on animal-derived production pipelines[4, 5]. These constraints are especially pronounced in resource-limited environments where cold chain logistics and batch-to-batch consistency cannot be guaranteed. Moreover, antibody production depends on hybridoma technology that is slow, animal-dependent, and poorly suited for toxic or weakly immunogenic targets[6, 7]. Aptamers, while synthetically accessible require extensive post-selection stabilization and frequently lose binding structure in protein-rich matrices[7, 8]. Their selection process, known as SELEX, involves multiple iterative steps, each of which must be optimized [9], making it time-consuming and unpredictable. To address these limitations, engineered M13 filamentous bacteriophages have emerged as a promising alternative.

M13 phages are viruses that infect *Escherichia coli*, and their rod-shaped architecture and editable surface proteins make them highly versatile for biosensor development[10, 11]. Peptides with high affinity to specific targets can be displayed on phage coat proteins, a technology known as phage display[11, 12]. This innovation enables the design of target specific biosensors for proteins, toxins, nucleic acids and small molecules[10, 13].[10, 13] Unlike antibodies and aptamers, phage display enables *in vitro* selection of high-affinity ligands, such as peptides or antibody fragments without immunization or mammalian cell culture, reducing both time and cost[6, 14]. M13 withstands harsh environmental conditions such as temperature fluctuations, pH variation, and solvent exposure, offering superior robustness beyond protein- or nucleic acid-based probes[7, 15]. Electrochemical phage-based biosensors have targeted a

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broad range of analytes, including foodborne pathogens, microbial indicators, caspase-3, interleukin-1β (IL-1β); blood coagulation proteins such as thrombin; and food allergens like ovomucoid[14, 16-23] [14, 23] [24, 25]Earlier studies revealed that graphene-based biosensors offer low noise and ultra-sensitive detection capabilities[26-28]. Graphene and its related forms exhibit outstanding electrical properties and distinctive structural features[29, 30]. Recent graphene field-effect transistor (GFET) biosensors for cytokine and exosome detection further illustrate the versatility of graphene transducers in complex physiological media[31]. Various methods are used to produce graphene, including mechanical and chemical exfoliation, epitaxial growth, and chemical or thermal reduction of graphene oxide (GO) to form reduced graphene oxide (rGO)[32-34]. rGO stands out due to its high electrical and thermal conductivity, carrier mobility, mechanical robustness, and optical characteristics, making it a promising nanomaterial for biosensor development[35, 36]. However, achieving high-performance detection in unprocessed biological or environmental samples remains challenging, especially when aiming for sub-second response times[37].

While previous work has demonstrated phage-displayed peptides in SARS-CoV-2 biosensing[24, 25], our approach introduces engineered M13 bacteriophages with a reduced graphene oxide platform for direct, antibody-free electrochemical detection of SARS-CoV-2 S1 protein. Here we differ from prior approaches by combining whole-phage immobilization on rGO with sub-second transient response readouts and direct bencmarking against antibody-based controls across three distinct complex sample types. In this work, we focus explicitly on the S1 antigen as a surrogate target for SARS-CoV-2, rather than intact virions. Clinical validation with the whole virus remains beyond the scope of this study. A comparative summary of representative electrochemical biosensors for SARS-CoV-2 detection is presented in Table 1.

Here, we report a phage-based electrochemical biosensor that leverages engineered M13 bacteriophages as the biorecognition element, enabling antibody-free detection of SARS-CoV-2 S1 protein on a reduced graphene

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oxide platform previously validated for protein sensing[27, 28]. We directly compare phage-based and antibody-based biosensors under identical conditions. While demonstrating comparable analytical performance, the phage-based system offers practical advantages, including lower production costs, reduced reliance on commercial antibody supply chains, and eliminates the need for recurrent batch validation typically required for commercial antibodies. To evaluate real-world applicability, we tested sensor performance in

municipal wastewater, blood serum, and pasteurized milk. We engineered M13 bacteriophages displaying either S-protein-binding peptides (SBP) or scrambled control peptides (SC), immobilized them on rGO and validated surface functionalization and specificity[25]. Our results demonstrate: (i) immobilization of whole M13KE phages on rGO, (ii) sub-second electrochemical readout, (iii) selective detection across three complex matrices, and (iv) direct benchmarking against antibody-based capture. To our knowledge, this is the first demonstration of whole-engineered phage functionalization of rGO for rapid, label-free antigen detection, establishing a scalable, antibody-independent biosensing strategy.

Materials and Methods

Construction of recombinant M13 bacteriophages

M13 bacteriophages were engineered to display either a SBP or a SC on the pIII coat protein, using a modified M13KE phage vector cloning strategy. Two sets of complimentary oligonucleotides were designed for the genetic modification of the pIII gene in the M13 genome. Both of these oligonucleotide sets were designed to produce KpnI (GGTAC/C) and EagI (C/GGCCG) overhangs when annealed. The first oligonucleotide set codes for a polypeptide sequence with previously described binding affinity to the SARS-CoV-2 receptor-binding domain[25]. The second oligonucleotide set contained a scrambled sequence as served as a control. PCR primers were also designed to amplify the region of

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the pIII gene spanning the insertion site to verify successful cloning. All oligonucleotide sequences are listed in Supplementary Table 1.

Equimolar amounts of the complementary oligonucleotides were mixed and annealed by heating to 90°C, before gradual cooling to room temperature. The M13KE pIII Cloning Vector (New England Biolabs; Ph.D.™ Peptide Display M13KE-based vector) was digested with EagI and KpnI, and purified using the GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Fisher Scientific), following the manufacturer's protocol. The annealed SBP and SC inserts were ligated into the digested vector using T4 DNA Ligase.

Competent *E. coli* K12 ER2738 cells (New England Biolabs) were prepared by inoculating 500 µL of overnight culture into 50 mL of LB medium supplemented with 20 µg/mL tetracycline (LB-Tet) and incubating at 37 °C with shaking until mid-log phase (OD_{600} = 0.4–0.6). Cells were pelleted by centrifugation at 3,900 × g for 15 min at 4 °C, resuspended in 20 mL of ice-cold 100 mM $CaCl_2$, and incubated on ice for 20 min. Following a second centrifugation step under the same conditions, the pellet was resuspended in 2.5 mL of 100 mM $CaCl_2$ to yield competent cells.

For transformation, 200 µL of competent cells were mixed with 10 µL of ligation reaction product (SBP or SC construct). The mixture was incubated on ice for 30 min, heat-shocked at 42 °C for 90 sec, then returned to ice for 2 min. After recovery in 1 mL LB at 37 °C for 30 min, cells were centrifuged, resuspended in 100 µL LB, and mixed with 200 µL of turbid *E. coli* K12 culture. This infection mixture was combined with 3 mL of top agar and poured onto LB/IPTG/X-Gal plates, followed by overnight incubation at 37 °C. Blue plaques from M13_SBP and M13_SC plates were selected and resuspended in 100 µL of phage buffer (50 mM Tris-HCl, pH 7.5; 80 mM $MgSO_4 \cdot 7H_2O$; 100 mM NaCl). The plaques were amplified by adding plaque suspensions to 15 mL of mid-log *E. coli* K12 ER2738 in LB-Tet and incubated overnight at 37 °C with agitation. Cultures were centrifuged at 4,400 × g for 10 min, and the supernatant containing phage was concentrated to ~500 µL using a 3 kDa centrifugal filter unit (Amicon®Ultra, Millipore). Recombinant clones were isolated and

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amplified using the standard M13KE phage-vector plaque workflow without the use of a helper phage.

To verify the constructs, Viral DNA from M13_SBP and M13_SC phage clones was extracted using the AllPrep® PowerViral® DNA/RNA Kit (Qiagen), following the manufacturer's instructions. DNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific) with the ssDNA setting. To verify successful cloning, PCR was performed on extracted viral DNA from M13_SBP, M13_SC, the unmodified M13KE gIII Cloning Vector (positive control for no insertion), and nuclease-free water (negative control) with primers depicted in Supplementary Table 1. PCR products were analyzed on a 1% agarose gel, where a band at 797 bp indicated successful cloning and insertion of the annealed oligonucleotide sequence, and 750 bp indicated an unmodified vector (no insert present).

Biosensor Construction and Functionalization

A schematic overview of the phage engineering and biosensor functionalization process is shown in (Figure 1). The spike-binding peptide used for phage display in this study was not newly engineered, but selected from a previously published phage display screen that identified peptides with high affinity for the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein. In that earlier work, the peptide's specificity was validated through ELISA and surface plasmon resonance assays, confirming its suitability for biosensing applications[25].

Phage amplification and purification: 200 µL of either M13_SBP or M13_SC recombinant phage were amplified in 1 L of 1:100 *E. coli* K12 ER2738 culture (LB-Tet medium) grown to mid-log phase. Cultures were incubated overnight at 37 °C with agitation. Following amplification, cultures were clarified by filtration through 0.2 µm PES membrane filters, then concentrated to 30 mL using Centricon® Plus-70 centrifugal filter units (Millipore). Final buffer exchange and volume reduction were performed using Amicon® Ultra-15

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centrifugal filter units, resuspending the phages in Phosphate Buffered Saline (PBS), pH 7.4. Phage titers were determined by plaque assay on LB/IPTG/X-Gal plates. Final titers were approximately 10^{11} PFU/mL for both M13_SBP and M13_SC.

Graphene oxide synthesis, deposition, and reduction: graphene oxide (GO) was synthesized via a simplified Hummers method as described previously, involving the oxidation of graphitic flakes using $KMnO_4$ in an H_2SO_4/H_3PO_4 mixture, followed by H_2O_2 treatment and purification steps[28]. Glass slides were cut into 1.5 × 1.5 cm squares and treated with Piranha solution (3:1 sulfuric acid to hydrogen peroxide) for 40 min[38]. Substrates were then rinsed thoroughly with distilled water, and dried at room temperature. A 70 µL of GO solution (2mg/mL) was drop-cast onto the glass. The temperature was gradually increased in 10 °C increments, followed by drying at 100 °C overnight on a hot plate. GO reduction was carried out in a horizontal tube furnace under a nitrogen atmosphere. The sensors were placed in the furnace, and the temperature was increased from room temperature to 450°C over 3 hours, then held at 450 °C for 1.5 hours, and cooled to room temperature.

Biosensor surface functionalization: First, the sensors were covered by a 2 mM solution of 1-pyrenebutanoic acid succinimidyl ester (PBASE) for 1 hour to enable non-covalent π–π stacking with the graphene surface. After functionalization, the sensors were rinsed with methanol and deionized (DI) water, dried, and contacts were applied using silver paste. Residual NHS esters on the PBASE-functionalized rGO were not quenched, and no blocking. Although the rinsing steps and short sample exposure times limited background activation, residual reactivity may contribute to non-specific adsorption. Future versions of the sensor will incorporate protein blocking (e.g., BSA or casein) to minimize such effects if required.

Two types of functionalization were then performed: (i) M13 Phage Functionalization, where M13 bacteriophages M13_SBP and M13_SC were immobilized by applying 10 µL of phage solution onto the PBASE-treated sensor surface. Sensors were incubated for 3 hours at room temperature, then

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rinsed with PBS and DI water, and stored at 4°C until use. (ii) Antibody Functionalization (AB_SBP); 10µL of 0.25 mg/ml anti-SARS-CoV-2 spike S1 monoclonal antibody (Thermo Fisher Cat# MA5-35940) was applied to a second set of sensors. Sensors were similarly incubated for 3 hours at room temperature, rinsed with PBS and DI water, and stored at 4°C until use. PBASE functionalizes rGO through strong π–π stacking between its pyrene ring and the sp2 carbon domains of rGO, creating a stable, non-covalent interface that preserves high conductivity of rGO. The exposed NHS ester then reacts selectively with primary amines on the M13 phage coat proteins via nucleophilic attack, forming a stable amide bond after displacement of the NHS leaving group. Because each M13 virion presents hundreds of accessible amines along the pVIII coat, the resulting immobilization is non directional, but produces a dense monolayer-like phage layer limited primarily limited by steric hinderance rather than linker availability, ensuring robust and reproducible immobilization across electrode surface. Across fabrication batches, approximately 85-90% of sensors met the predefined quality criteria (stable baseline current, linear I-V characteristics and proper ΔI peak behavior) and were therefore included in testing.

Structural and Surface Characterization of the Constructed Biosensors

The morphology of the biosensor at each fabrication step was characterized using scanning electron microscopy (SEM). Prior to imaging, all samples were gold-coated using a sputter coater to improve surface conductivity and minimize charging effects. A Nova NanoSEM (FEI, USA) was employed to image the sensor surface after each key modification stage: pristine GO, rGO functionalized with PBASE (rGO/PBASE), and biosensors functionalized with M13_SBP (rGO/PBASE/M13_SBP), M13_SC (rGO/PBASE/M13_SC), and anti-S1 antibodies (rGO/PBASE/AB_SBP). SEM imaging was conducted at an accelerating voltage of 5.00 kV, with a spot size of 3.0 µm, and a working distance of 5 mm. To validate the thermal reduction of GO to rGO, analysis of the oxygen content of the GO and rGO surfaces was qualitatively and

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quantitatively assessed using a Phenom XL Desktop SEM (Thermo Fisher Scientific, Netherlands) equipped with energy-dispersive X-ray spectroscopy (EDS). Additionally X-ray diffraction (XRD) was used to investigate the difference in crystal phase between GO and rGO. Atomic force microscopy (AFM) was also utilized to assess the surface roughness at each construction stage, providing additional topographical insights.

Electrochemical Measurements and Biosensor Testing

Electrical measurements were performed using a Keithley 2400 SourceMeter (Keithley Instruments, Cleveland, OH) controlled via KickStart software. Biosensors were connected to the instrument using crocodile clips that made contact with the silver paste electrodes. Conductivity was assessed at various fabrication stages by applying a linear voltage sweep from –1 V to +1 V and recording the resulting current. Resistance values were calculated from the slope of the I–V curves using Ohm's law. A four-wire sensing configuration was used to ensure high measurement accuracy.

For biosensor response measurements, a constant voltage was applied, and real-time current was recorded following the direct addition of analyte onto the biosensor surface. Changes in current over time were used to evaluate the sensor's response to target binding analytes.

All current values were normalized using the following equation (1):

$$\frac{\Delta I}{I_0} = \frac{I - I_0}{I_0} \quad (1)$$

where I_0 is the baseline or initial current value current and I is the real-time current. This normalization enabled comparison across different sensor platforms and minimized the effects of baseline variation. All the data collected and analyzed during our experiments are also provided in the "Supplementary Raw Data" file included separately.

Signal transduction at fixed voltage

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Time-dependent current measurements were used because this time-current approach follows the operating principle we established in our prior antibody-based rGO biosensor, where target binding produced reproducible transient peaks under low-voltage bias. The present phage-based sensor exhibits the same gating behavior, making real-time I–t measurement the most appropriate readout for this chemiresistive platform. The sensor does not rely on Faradaic electrochemical reactions but instead when the target binds to sensor surface it changes electron transfer rate and the charge distribution, inducing perturbations in the rGO conductivity that manifest as measurable change in electrical current under a fixed low-voltage bias. This measurement mode is therefore essential for capturing the fast (<300 ms) interfacial gating events that constitute the sensor's detection mechanism. All real-time measurements in this study were performed at a constant voltage of −0.8 mV across the rGO biosensor, following the optimized low-field operating regime established in our previous antibody-based biosensor for SARS-CoV-2 detection (Kadadou et al., 2023). In that work, a fixed low voltage produced rapid and reproducible transient current peaks within ~240–300 ms that corresponded to the binding of the S1 protein to surface-immobilized antibodies. Increasing the applied voltage was found to decrease sensitivity and stability, confirming that the biosensor operates most effectively under weak-field conditions that maintain the structural integrity of the functional layer.

Consistent with those findings, the present phage-based biosensor was operated under the same low-voltage conditions after validation. Upon sample addition, a transient change in current was observed due to interfacial electrochemical interactions between the phage layer and the rGO/PBASE surface, followed by signal returning to baseline. The magnitude of this transient (ΔI_{peak}) serves as the analytical metric, reflecting rapid interfacial electron transfer and polarization changes at the rGO surface upon specific target binding.

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SARS-CoV-2 S1 Protein Detection and Limit of Detection Analysis

The M13_SBP biosensor was evaluated for its ability to detect the SARS-CoV-2 S1 protein and selectivity in the presence of non-target analytes and complex matrices. The S1 protein, containing the receptor-binding domain (RBD) of the virus[39], was initially diluted to 0.1 mg/mL in PBS and further serially diluted to determine the sensor's limit of detection (LOD). In addition to S1, the M13_SBP biosensor was tested against bovine serum albumin (BSA), K12 *E. coli*, and complex biological/environmental matrices including pasteurized milk, fetal bovine serum (FBS), and wastewater (WW) — both in native form and spiked with S1 protein. For comparison, the phage scrambled control (M13_SC) biosensor, was tested against the S1 protein and K12 *E. coli*. The AB_SBP biosensor was evaluated using wastewater and wastewater spiked with S1 protein to compare its performance against the M13_SBP biosensor.

To define a binary detection threshold for the M13_SBP biosensor in complex matrices, control measurements were performed using analyte-free samples of pasteurized milk, FBS, and filtered WW. For each matrix, five replicates were tested using independently fabricated M13_SBP biosensors. The average normalized current response of the blank samples (I_blanks) and its standard deviation (σ_blanks) were calculated[40, 41]. A conservative detection threshold was defined using eq (2):

$$\text{Threshold} = I_{blanks} + 3 \times \sigma_{blanks} \quad (2)$$

This threshold corresponds to a 99.7% confidence interval under the assumption of normally distributed background noise, minimizing the likelihood of false positives.

To determine the limit of detection (LOD), the M13_SBP biosensor was exposed to a series of S1 concentrations ranging from 10^{-4} pg/mL to 10 pg/mL in PBS. Given the binary nature of the biosensor output, where detection is defined as a statistically significant deviation from the baseline, a one-sample t-test was applied to each concentration group to compare it to the noise average.

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Statistical significance was defined a priori as $p < 0.05$. The LOD was defined as the lowest concentration at which the biosensor consistently produced a statistically significant signal relative to the baseline across independent biosensor replicates. Sensor sensitivity was defined as the slope of the calibration curve obtained by plotting normalized response versus $\log_{10}$(S1 concentration), consistent with standard analytical definitions42. The fabricated biosensor exhibited consistent performance across multiple independently fabricated electrodes. Reproducibility was assessed using at least $n = 3$ independently fabricated sensors tested under identical conditions. The mean ± SD were computed for ΔI_peak across replicates. All sensors were fabricated from the same material batch and measured within 3 days of preparation. The low variation observed (< 5% relative standard deviation) confirms the high reproducibility of both fabrication and measurement processes. Long-term storage stability and inter-batch reproducibility will be evaluated in future studies.

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Results

Physical characterization of the fabricated biosensors

Scanning electron microscopy (SEM) was used to characterize the biosensor surface at various stages of functionalization (Figure 2). Initial graphene oxide (GO) layer (Figure 2a) exhibited a wrinkled morphology under SEM, which is characteristic of graphene-based materials due to their ultrathin and flexible structure. Upon reduction to form rGO (Figure 2b) the surface also appeared wrinkled but with a darker contrast, suggesting changes in surface conductivity and reduced charging, consistent with partial removal of oxygen-containing groups[43, 44]. After modification with PBASE (Figure 2c), the rGO morphology appeared similar, although a slightly rougher texture was observed, indicating surface coverage by the linker molecules [27]. Upon immobilization of the M13_SBP phage (Figure 2d and e) SEM revealed irregular nanoscale features and aggregates (Supplementary Figure S1a and b) [17], which may correspond to areas of phage adsorption or clustering on the rGO surface. A similar morphology was observed for the M13_SC control phage (Figure 2f and g), although the surface appeared less uniformly covered. Functionalization with the AB_SBP monoclonal antibody (Figures 2h and i) produced a distribution of nanoscale features across the field of view, suggesting uniform modification of the surface, (Supplementary Figure S1c). Complementary AFM topographical characterization and corresponding height profiles of the biosensor surface at four key fabrication stages: (a) rGO, (b) rGO/PBASE, (c) rGO/PBASE/M13-SBP, and (d) rGO/PBASE/M13-SC was also conducted (Supplementary Figure S2). Each image includes a 2D height map and a line-scan profile used to calculate the average surface roughness. The bare rGO surface (a) shows an average roughness of 16.46 nm, consistent with the typical wrinkled morphology of reduced graphene oxide. After functionalization with PBASE (b), the roughness increases to 17.75 nm, indicating successful attachment of the linker molecules. Immobilization of the SBP-displaying M13 phage (c) results in a pronounced rise in roughness to 28.76 nm, reflecting the presence of filamentous phage structures on the

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surface. In the final step (d), immobilization of the SC phage gives a roughness of 24.13 nm, also higher than the unmodified surface, confirming biomolecule deposition. Electrical characterization further confirmed successful stepwise functionalization, with progressive increases in resistance observed after PBASE and phage immobilization (Supplementary Figure S3). This behavior is consistent with our previous antibody-based rGO biosensor, where analogous resistance increases were reported after rGO/PBASE/antibody functionalization[28].

To confirm the structural transition from GO to rGO, a combination of X-ray diffraction (XRD) and energy-dispersive X-ray spectroscopy (EDS) was employed (Supplementary Figures S4 and S5). XRD analysis revealed a shift in the characteristic GO peak at approximately 2θ = 8.99°, corresponding to the (001) plane, to a broader and less intense peak near 2θ = 22.16° in rGO after the thermal reduction (Supplementary Figure S4), indicative of decreased interlayer spacing and partial restoration of the graphitic structure[45]. This structural change is consistent with the reduction of oxygenated groups[46]. Complementary elemental composition was analyzed using energy-dispersive X-ray spectroscopy (EDS) (Supplementary Figure S5). The oxygen atomic concentration decreased from 31.5% in GO to 26.5% in rGO, indicating partial removal of oxygen-containing functional groups. This chemical transition is consistent with the increased wrinkling observed in the SEM images of rGO (Figure 2b), which is commonly attributed to the elimination of hydroxyl, epoxy, and carboxyl groups during the reduction process[47].

Voltage optimization for the fabricated biosensor

Each sample addition on the surface of the biosensor produced a transient change in current (ΔI) that peaked within ~300 ms and gradually returned to baseline. Accordingly, the analytical metric was defined as the peak current change (ΔI_peak) relative to the pre-addition baseline. For all figures, the

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normalized current response represents the average ΔI_peak value obtained from replicate measurements.

To determine the optimal working voltage for the biosensor, it was connected to a source meter and tested in real time upon exposure to the SARS-CoV 2 S1 protein. Measurements were performed at four different applied voltages: −200 mV, −15 mV, −5 mV, and −0.8 mV. As shown in (Figure 3a), the normalized current response profiles varied with applied voltage. The signal amplitude varied strongly with applied voltage, with −0.8 mV providing the highest signal-to-noise ratio. This behavior is consistent with an interfacial charge-gating mechanism, attributed to an electrostatic gating effect, where the charge of captured analytes modulates the conductivity of the rGO layer[18]. To ensure reproducibility, the same voltage conditions were tested across three independently fabricated biosensors, and the average peak current values were quantified (Figure 3b). The results confirmed that −0.8 mV consistently yielded the highest signal response with low variance. Notably, this is the same working voltage previously identified in a monoclonal antibody-based version of the biosensor targeting the same analyte (S1 protein)[28].

To further investigate the biosensor's analytical specificity and sensitivity, we tested the biosensor at −0.9 mV, −0.8 mV, and −0.4 mV using both the target S1 protein and a non-target protein, BSA. As shown in (Figure 3c), at −0.9 mV, the current response remained low for both S1 and BSA, and the difference between them was not statistically significant, indicating low sensitivity at this potential. At −0.8 mV, however, the biosensor showed a significantly stronger response to S1 relative to BSA ($p < 0.05$), confirming high sensitivity and specificity. In contrast, at −0.4 mV, elevated current responses were observed for both S1 and BSA, indicating non-specific activation and reduced selectivity at this voltage. And, shows how specificity and sensitivity were electronically modulated through controlled adjustment of the applied voltage rather than by extensive surface blocking, allowing the rGO interface to maintain responsiveness while minimizing non-specific activation. These findings

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establish −0.8 mV as the optimal detection voltage, offering strong target recognition with minimal background from non-specific proteins. This operating voltage was used in all subsequent experiments in this study.

Specificity of M13_SBP functionalized biosensors for SARS-CoV-2 S1 protein detection

To validate that the M13_SBP phage biosensor specifically binds to the SARS-CoV-2 S1 protein through the inserted peptide, comparative tests were performed using a scrambled control phage biosensor (M13_SC) lacking the S1-targeting sequence. Both biosensors were tested against K12 *E. coli*, the natural host of M13, to assess baseline interactions, as any M13 phage would be expected to specifically bind to this bacterial strain. As expected, both M13_SBP and M13_SC biosensors produced comparable current responses when exposed to *E. coli* k12 (Figure 4), indicating that both phage variants remained functionally intact and capable of interacting with their host. However, only the M13_SBP biosensor exhibited a significant current response upon exposure to the S1 protein, while the scrambled M13_SC control showed minimal signal. This contrast confirms that the enhanced S1 binding in M13_SBP is conferred by the engineered peptide and not due to nonspecific phage-surface interactions. These results demonstrate the successful insertion of a functional S1-binding peptide into the M13 scaffold and support the platform's modularity for target-specific biosensing.

Given the binary nature of the biosensor output—where detection is defined as a statistically significant deviation from baseline— A one-sample t-test ($p < 0.05$) was used to compare each concentration's signal to the baseline. The LOD was defined as the lowest concentration with a consistently significant deviation from baseline[48]. This approach reflects the sensor's intended application for presence or absence diagnostics. A significant increase in signal relative to the baseline was first observed at a concentration of 10^{-4} pg/mL, with a corresponding p-value below 0.05 (Figure 5a). No statistically distinguishable signal was observed at lower concentrations (data not shown).

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The biosensor response at 10^{-4} pg/mL was also consistent across replicates, indicating reliable detection at this level (Figure 5b) with a response time of an average of 300 ms. To explore the potential for quantitative use, a calibration curve was generated by plotting the mean current responses against the logarithm (base 10) of S1 protein concentrations (Figure 5c). Linear regression of the buffer calibration data yielded a slope of 0.05755 per decade ($y = 0.05755x + 0.4352$, $R^2 = 0.8832$), which is reported here as the sensor sensitivity in buffer. Linear regression analysis yielded a correlation coefficient (R^2) value of 0.88, indicating a moderate correlation between signal intensity and analyte concentration. Although the primary design of the biosensor is for binary detection, this result suggests that quantitative calibration may be feasible in future iterations, particularly with further optimization of signal uniformity and surface functionalization.

Detection of analyte by fabricated phage-based biosensor in complex biological matrices

To evaluate the real-world applicability of the phage-functionalized (M13_SBP) biosensor, real-time current measurements were performed in various complex sample matrices, including municipal wastewater (WW), fetal bovine serum (FBS), and pasteurized milk, with and without spiked S1 protein (Figure 6). In wastewater, the biosensor showed minimal current change in the blank sample, but a clear and rapid signal increase when 10 pg/mL of S1 protein was introduced (Figure 6a). While the biosensor demonstrated a clear and statistically significant response at 10 pg/mL S1 protein in wastewater, the response at 5 pg/mL did not reach statistical significance, as shown in the corresponding normalized current (Figure 6b). This was not due to a complete lack of signal at that concentration, but rather variability across sensor replicates. For S1 protein of concentration 5pg/mL, only 67% of the biosensors tested showed a response above the threshold, resulting in higher standard deviation (SD) and an inconclusive p-value (Figure 6j). This inconsistency is likely due to fabrication variability inherent in a lab-scale biosensor

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preparation. It is reasonable to anticipate that under controlled, large-scale manufacturing condition, where surface chemistry, phage density, and electrode uniformity are tightly regulated, the biosensor could consistently detect S1 protein at 5 pg/mL in wastewater.

FBS and pasteurized milk matrices supported more consistent performance. In FBS (Figure 6c), the biosensor exhibited a clear increase in current upon exposure to 5 pg/mL S1 protein, with all five replicates producing normalized ΔI_{peak} values above the detection threshold (Figure 6d). A similar trend was observed in the pasteurized milk (Figure 6e and f), where the sensor again reliably detected 5 pg/mL of S1 across replicates, despite the presence of multiple endogenous proteins. All measured current peaks (ΔI_{peak}) were subsequently normalized to the baseline current for cross-biosensor comparison.

To assess concentration-dependent behavior in complex matrices, we tested S1-spiked samples in filtered wastewater, fetal bovine serum (FBS), and pasteurized milk (Supplementary Figure S6). FBS and milk exhibited systematic increases in normalized current with increasing spike concentration across the tested points. Linear regression of the three-point data sets for each of these matrices is reported in (Supplementary Figure S6) (FBS: $y = 0.335x + 0.5189$, $R^2 = 0.9949$; milk: $y = 0.434x + 0.4562$, $R^2 = 0.9754$). In wastewater the replicate responses were more variable and the higher-concentration points were not distinguishable within experimental uncertainty (Supplementary Figure S6); consequently the wastewater data do not establish a reliable concentration–response relationship at the tested spike levels.

To benchmark the phage-based biosensor against an established platform, the AB_SBP biosensor utilizing a monoclonal antibody against S1 was tested under identical conditions. As shown in (Figure 6g and h), both biosensors (AB_SBP and M13_SBP) responded to 10 pg/mL S1 in wastewater, exhibiting statistically significant increases in normalized current response (ΔI_{peak}, $p < 0.05$) compared with the unspiked controls ($p < 0.05$). While the AB_SBP biosensor

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produced a larger absolute response, both sensors consistently distinguished S1-spiked samples from blanks across all replicates, demonstrating that the engineered phage provides reliable binary detection with sensitivity comparable, in practical terms, to the antibody-based platform.

(Figure 6i) summarizes M13_SBP biosensor's sensitivity across all matrices at both 5 pg/mL and 10 pg/mL. Notably, consistent detection at 5 pg/mL was observed in FBS, pasteurized milk, and WW at 10 pg/mL. (Figure 6j) presents the overall normalized current responses for each complex matrix, including unspiked controls (blanks). All blank samples remained below the defined detection threshold of 0.2, confirming high analytical specificity in the presence of protein-rich and potentially interfering environments. Notably, the biosensor produced consistent signals above this threshold in FBS and pasteurized milk at 5 pg/mL, as well as in WW at 10 pg/mL. Any signal exceeding the threshold value of 0.2 was interpreted as a positive test result, indicating the presence of SARS-CoV-2 S1 protein. The response time, defined as the interval between the initial stable baseline and the peak current, was approximately 300 ms, enabling real-time detection. These findings underscore the biosensor’s capability for rapid, selective, and matrix-resilient detection of SARS-CoV-2 S1 protein at biologically relevant concentrations. Collectively, these results demonstrate that the M13_SBP biosensor is capable of specific, rapid real-time detection of S1 protein in diverse biological and environmental conditions. Its performance compares favorably to antibody-based platforms, highlighting the potential of engineered phage as a viable and scalable alternative for biosensing.

Discussion

The successful fabrication and characterization of a phage-functionalized biosensor for the ultra-sensitive detection of SARS-CoV-2 S1 protein underscores the potential of bioengineered M13 phages as viable and scalable alternatives to conventional antibody-based platforms. Scanning electron microscopy (SEM) confirmed distinct morphological transitions throughout the

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biosensor assembly (Figure 2), with each functionalization step yielding expected structural features. Optimizing the detection voltage revealed −0.8 mV as the condition offering the highest signal-to-noise ratio and analytical specificity (Figure 3). This trend aligns with our previous rGO-BSA biosensor by Kadadou et al, 2022, where higher voltages similarly decreased specificity due to field-enhanced nonspecific electrostatic adsorption and increased capacitive noise. Both systems operate through an electrostatic gating mechanism in which binding-induced charge perturbations are best resolved under low field conditions. Furthermore, because the biosensor functions at -0.8 mV with µA-level currents, joule heating and faradaic side reactions are negligible, and no thermal or redox artifacts were detected[28]. This finding enhances the versatility of the platform, supporting its future adaptation to a wide range of biorecognition elements without significant reengineering of the electrical parameters. Together, the 5-20% RSD in ΔI peak and the high fabrication yield (85-90%) demonstrate that the phage-rGO platform is inherently reproducible even under non-industrial conditions.

Functional testing with scrambled peptide and the specific binding peptide engineered phages provided compelling evidence that the observed binding to the SARS CoV-2 S1 protein is sequence-specific and not attributable to nonspecific phage interactions (Figure 4). This level of selectivity is crucial for the sensor's diagnostic utility and is consistent with prior reports on the modularity and binding capabilities of M13-displayed peptides. Moreover, the clear functional distinction between M13_SBP and M13_SC biosensors validates the peptide insertion strategy and supports the use of M13 as a customizable scaffold for diverse biosensing applications[36]. While the natural tropism of M13 bacteriophage for *Escherichia coli* strains positive for F-pilus is useful for phage amplification, it presents a potential limitation for biosensing applications in microbiologically active samples, such as wastewater or stool, where *E. coli* may be present. This inherent affinity could lead to off-target binding or signal interference if the sensor is deployed in samples containing *E. coli*, especially in contexts unrelated to the detection target[49, 50]. Although

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this was not a confounding factor in the current study, integration of selective sample pretreatment steps to eliminate background bacteria may be necessary for certain clinical or environmental applications. However, this modularity also opens the door to future biosensors engineered to detect specific strains of bacteria or engineered targets. For instance, the current M13-based platform could be adapted to detect *E. coli* strains expressing the F pilus, given that the wild-type M13 phage naturally binds F-piliated cells via the pIII protein[17, 51]. Leveraging this natural host-phage interaction, similar biosensors could be developed to detect specific bacterial phenotypes or virulence factors. Furthermore, this approach holds potential for use in synthetic biology or bioengineering workflows, to verify the surface expression of engineered receptors on mammalian or microbial cells[52]. In such contexts, functional binding of a phage-displayed peptide to its cognate receptor could serve as a rapid, label-free readout of successful receptor incorporation, providing a useful screening tool. In terms of analytical performance, the sensor demonstrated a limit of detection (LOD) of 10^{-4} pg/mL in buffer (Figure 5), exceeding the sensitivity of many antibody-based assays reported in the literature[53, 54] and as shown in Table 1. Although the system was designed primarily for binary detection, the moderate correlation observed in the calibration curve (R^2 = 0.88) suggests potential for quantitative analysis following optimization.

The ability of a M13_SBP biosensor to maintain high specificity in complex biological and environmental matrices is critical for its practical deployment. In this study, the M13_SBP functionalized biosensor demonstrated robust and selective detection of the SARS-CoV-2 S1 protein in three distinct sample types: WW, FBS, and pasteurized milk (Figure 6). Each of these matrices presents unique biochemical challenges due to the presence of proteins, enzymes, and other interfering substances, making them ideal testbeds for evaluating non-specific adsorption and cross-reactivity. Wastewater is a highly heterogeneous matrix, typically containing microbial debris, organic matter, surfactants, and extracellular proteins from human and environmental

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sources. Despite this complexity, the biosensor maintained a clear signal when spiked with 10 pg/mL S1 protein, and statistical analysis confirmed significant differentiation between S1-spiked and unspiked wastewater. This underscores the sensor's surface functionalization efficacy. FBS is commonly used in biomedical research and contains a high concentration of albumin, globulins, and growth factors[55]. Remarkably, the M13_SBP biosensor produced a consistent and distinguishable response in FBS with 5 pg/mL S1 spike-in, suggesting strong binding specificity of the displayed peptide toward the target and minimal cross-reactivity with abundant serum proteins. Pasteurized milk represents a nutritionally rich and protein-dense matrix, containing caseins, whey proteins, carbohydrates, immunoglobulins, and lipids[56]. The successful detection of S1 in this medium further validates the biosensor’s robustness. Importantly, signal levels remained above the detection threshold, and no significant interference from milk components was observed, indicating that the biosensor can function effectively even in non-standard or zoonotic surveillance contexts. The tested S1 protein concentrations (5–10 pg/mL) fall within a range that is physiologically relevant for early-stage SARS-CoV-2 infections or low-copy-number contamination scenarios. Based on structural estimates, each SARS-CoV-2 virion carries approximately 24 spike trimers, corresponding to roughly 72 S1 subunits per particle[57, 58]. Molecular weight of 103 kDa for the synthetic S1 protein, 5 pg/mL corresponds to approximately 2.92×10^7 S1 molecules per mL, which would translate to an estimated 4×10^5 virions per mL. These values are consistent with viral loads reported in patient respiratory fluids and wastewater surveillance studies[59, 60]. Beyond binary detection, additional concentration-response measurements in complex matrices provide further insight into matrix-dependent sensor behavior (Supplementary Figure S6). The calibration-style measurements in complex matrices demonstrate the sensor’s ability to produce concentration-dependent responses in protein-rich matrices such as fetal bovine serum and pasteurized milk, while also highlighting limitations under environmental conditions. The high R^2 values observed for the three-point FBS and milk data

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are influenced by the restricted concentration ranges and limited number of points and therefore should be interpreted cautiously rather than as evidence of robust quantitative calibration. In wastewater, replicate variability was substantially higher and responses at higher spike concentrations were not statistically distinguishable within experimental uncertainty, consistent with the heterogeneous chemical and physical composition of environmental samples. Accordingly, the matrix-specific behaviors observed here provide important context for the wastewater, FBS, and milk performance discussed below, and indicate that full quantitative validation in environmental samples will require denser concentration series, increased replicate numbers, and matrix-aware sample preparation.

Although the biosensor was tested in complex simulated matrices, its performance in actual clinical samples (e.g., nasopharyngeal swabs, saliva) remains unverified. Clinical translation will require extensive validation in patient-derived specimens, alongside assessments of diagnostic sensitivity, specificity, and cross-reactivity in real-world settings[49, 50]. When benchmarked against a monoclonal antibody-functionalized sensor under identical conditions, the M13_SBP biosensor exhibited comparable performance in terms of sensitivity and specificity. This equivalency not only validates the phage approach but also emphasizes its advantages in terms of production scalability, stability, and ease of genetic modification. Unlike monoclonal antibodies, which require complex and costly production processes, M13 phages can be rapidly engineered and amplified using standard bacterial expression systems, presenting a cost-effective alternative for decentralized and resource-limited diagnostic applications. This study demonstrates sub-second, matrix-tolerant S1 antigen detection using an engineered phage–rGO chemiresistor.

As shown in Table 1, most graphene oxide-based electrochemical biosensors for SARS-CoV-2 detection rely on monoclonal antibodies as biorecognition elements. Recent graphene-based biosensors further contextualize the present platform, including graphene oxide–modified capacitive chips[61], label-

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free capacitive biosensors for protein detection such as $A\beta_{1-42}$[62], and broader diagnostic strategies for SARS-CoV-2 [63]. In parallel, recent advances in two-dimensional graphene nano-biosensing and emerging perspectives on borophene-based biosensors highlight ongoing efforts to exploit charge-transfer modulation and ultrathin materials for next-generation sensing[64]. Phage-based biosensing strategies, while less frequently reported than antibody- or aptamer-based systems, offer unique advantages in robustness and genetic tunability, and have recently been explored in combination with graphene and other nanomaterials for label-free detection.

While these platforms exhibit high sensitivity, they often require longer assay times and are limited by factors such as thermal instability, high production costs, and dependency on commercial supply chains. A previously developed antibody-based biosensor[28] demonstrated strong performance, achieving a 0.5 fg/mL detection limit and a 240 ms response time. In the present work, the same sensor configuration was reproduced, substituting the antibody with an engineered M13 phage displaying an S1-binding peptide. The resulting biosensor achieved a lower detection limit of 0.1 fg/mL and a response time of 300 ms, demonstrating comparable or superior performance at a lower production cost.

As summarized in Table 1, recent graphene oxide-based electrochemical biosensors for SARS-CoV-2 detection have primarily employed monoclonal antibodies. Amperometric and DPV-based systems[65, 66] demonstrated nanogram- to femtogram-level sensitivity but required longer detection time. Cu_2O nanocube-enhanced sensors[67] and 3D microelectrode arrays[68] achieved high sensitivity and rapid responses but involve complex fabrication. Other immunosensors using human serum[69] also showed strong performance but rely on cold-chain–sensitive antibodies. Table 1 clearly shows that this Engineered M13 phage biosensor have similar performance of antibody-based counterparts, establishing M13 as a viable, low-cost alternative for rapid, sensitive, and scalable diagnostic platforms. The use of whole M13KE phage offers manufacturing and robustness advantages. Phage production is

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inexpensive and scalable in bacterial hosts, and the filamentous structure endures temperature and pH fluctuations that commonly denature antibodies or aptamers. Although immobilization is non-directional and pIII display is limited to five copies per virion, the phage layer provides a multivalent, high-surface-area scaffold that produced analytical performance comparable to the antibody benchmark.

Building on previously reported work using an rGO-based electrochemical biosensor platform with monoclonal antibodies, the present study investigates whether similar or enhanced performance can be achieved through biorecognition element substitution. Maintaining the original sensor configuration and detection method suggest that the rGO-based sensor platform derives its high sensitivity not solely from the antibody component, but from the synergistic interaction between the transducer and the engineered biorecognition interface. The results highlight the potential of phage-based recognition elements as scalable, robust alternatives in high-performance diagnostic applications.

Given the biosensor's demonstrated ability to detect SARS-CoV-2 S1 protein in complex, protein-rich media such as fetal bovine serum and milk, this platform shows strong potential for broader use in both clinical and food-quality applications. Its high specificity, rapid response time (~300 ms), and low detection limit support its potential for detecting analytes in human serum or blood, which could be valuable in point-of-care diagnostic testing. Additionally, the successful detection of spiked protein in milk highlights its relevance for food safety applications, particularly in the dairy industry, where reliable detection of pathogenic proteins or contaminants is essential. With further validation and integration into portable devices, this biosensing strategy could be adapted for rapid screening of dairy products to ensure product quality and safety during processing, distribution, and retail.

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Table 1 Comparison of previously reported SARS-CoV-2 biosensors with M13 phage functionalized Graphene oxide-based biosensor.

Electrochemical sensor type	Recognition element	Target	Sample Matrix	LOD	Detection Time	Reference
Electrochemical Impedance Spectroscopy (EIS) with gold electrode functionalized with dimeric aptamer	Dimeric DNA aptamer (DSA1N5)	Spike (S) protein (Wild-type, Alpha variants)	Saliva (unprocessed)	1000 cp/mL (WT), 5000 cp/mL (Alpha variant)	<10 min	70
Electrochemical impedance spectroscopy (EIS) using FTO/AuNP-modified electrode	ssDNA aptamer (CoV2-RBD-1C)	(S) protein	Recombinant S1 protein in PBS buffer	0.016 fg/mL	15 min	71
Electrochemical aptamer-based (EAB) sensor using SWV on gold electrode (reagentless)	DNA aptamer (1C variant)	(S) protein	PBS buffer; validated in 50% artificial saliva and FBS	Signal detected at picomolar concentrations (not formally reported as LOD)	<20 seconds	72
Amperometric (carbon black-modified SPE + MBs)	Antibody	Spike (S) & Nucleocapsid (N) proteins	Saliva (untreated)	19 ng/mL (S) 8 ng/mL (N)	30 min	66
Electrochemical Impedance Spectroscopy (EIS) with Cu_2O nanocube-modified SPCE	Antibody	(S) protein	Buffer/Spiked sample	0.04 fg/mL	20 min	67
Paper-based electrochemical device (ePAD) using SWV	anti-SARS-CoV-2 IgM	(S) protein	Human serum	0.11 ng/mL	30 min	69
RAPID (Laser-engraved graphene, SWV)	Antibody	(S) protein	Clinical Sample	2.8 fg/mL	4 min	65
3D aerosol jet–printed electrochemical sensor (SWV)	ACE2 protein	(S) protein	Pseudovirus solution	9.2 fM	43 s	68
Graphene oxide Kelvin Immunosensor	antibody	(S) protein	Synthetic S1; raw wastewater Nasopharyngeal swab sample	0.5 fg/mL; 2.91 copies/mL	240 ms	28

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M13 phage Graphene oxide based biosensor	Engineered M13 phage	(S) protein	Synthetic S1; PBS buffer	10^{-4} pg/mL	300 ms	This study

Conclusion

This study introduces a biosensing platform that leverages genetically engineered M13 bacteriophages displayed on a rGO substrate, offering a compelling and scalable alternative to traditional antibody-based sensors. The M13_SBP biosensor achieved high sensitivity, real-time detection of the SARS-CoV-2 S1 protein across diverse complex matrices, including wastewater, fetal bovine serum, and pasteurized milk, demonstrating high robustness and analytical selectivity under our test conditions. By integrating engineered phages with conductive nanomaterials, the platform enables low-cost, scalable manufacturing and is readily adaptable to other biotargets through peptide reprogramming. Future work will focus on clinical validation in human specimens, long-term storage stability, and inter-batch reproducibility to fully establish the platform's potential for large-scale deployment. Overall, this work demonstrates a proof-of-concept phage-enabled electrochemical biosensor, paving the way for rapid, decentralized detection of pathogen's proteins and biomarkers in healthcare and environmental monitoring applications.

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DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Figure Legends:

Figure 1: Schematic Illustration of the M13/rGo-based Biosensor Design (a) M13 bacteriophage design and construct validation. (1) Oligonucleotide sequences were designed to encode S-protein-binding peptides (SBP) or scrambled control peptides (SC). (2) Forward and reverse oligonucleotides were annealed to generate double-stranded DNA (dsDNA) inserts with overhangs complementary to the M13KE phage vector. (3) The annealed dsDNA inserts were ligated into the pIII M13KE phage vector at the KpnI and EagI restriction sites using T4 DNA ligase. (4) The ligated M13KE phage vector constructs representing SBP and SC sequences were introduced into *E. coli K12* cells via transformation. (5) Transformed cells were plated on x-gal/IPTG/LB agar plates for blue-white colony screening. (6) Colonies were selected, and plasmid DNA was extracted to confirm the presence of inserts via PCR and gel electrophoresis. (7) SBP and SC M13KE phage vector constructs were amplified in host cells, quantified via plaque assay, and suspended in PBS buffer, ready for biosensor functionalization. (b) Biosensor preparation and testing. SiO_2 glass substrates were cleaned with Piranha solution, coated with graphene oxide (GO), and thermally reduced to graphene oxide (rGO). The rGO surface was functionalized with PBASE through π–π stacking interactions between the pyrene moiety and the sp^2 carbon lattice of rGO. The PBASE-modified rGO was subsequently incubated with target-specific M13 phage, enabling covalent coupling between the NHS ester of PBASE and primary amine groups on the phage coat proteins. During testing, S1 protein was applied to the functionalized surface, where specific binding to the displayed peptide on the immobilized phage induces a change in rGO conductivity, which was recorded as a real-time current response at a fixed applied voltage. Phage immobilization on rGO/PBASE occurs primarily via amine coupling along the filament (pVIII), resulting in a non-directional orientation, and no preferential pIII presentation is assumed. Figure created using BioRender.com.

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Figure 2. Scanning electron microscopy (SEM) images of the biosensor surface at different stages of fabrication. SEM images of biosensor surfaces at successive modification stages. Irregular nanoscale aggregates are consistent with biomolecular deposition; individual phage filaments or IgG molecules cannot be resolved under gold sputter. Representative SEM micrographs are shown for each stage of biosensor construction at two magnifications: 50,000× (left) and 10,000× (right). (a) show the surface of graphene oxide (GO) at 10,000×. (b) represent reduced graphene oxide (rGO) at 10,000×. (c) correspond to rGO functionalized with PBASE at 10,000×. (d,e) show the surface of rGO/PBASE surfaces after immobilization of M13_SBP phage particles at 10,000× and 50,000x. (f, g) show rGO/PBASE with M13_SC phage at 10,000× and 50,000x. (h, i) shows rGO/PBASE functionalized with anti-S1 antibody (AB_SBP) at 10,000× and 50,000×. The bright, irregular features observed in panels (e), (g), and (i) are attributed to residual buffer components (e.g., salts and surfactants from phosphate-buffered saline, PBS) remaining after drying of the phage- or antibody-functionalized surfaces prior to SEM imaging.

Figure 3: Voltage optimization and current response of the phage-SBP-Biosensor.

(a) Real-time current responses recorded at different applied voltages (−0.8, −5, −15, and −200 mV). To evaluate sensor performance, 10 µL of synthetic S1 protein (100 pg/mL) was added directly onto the sensor surface, and current changes were monitored in real time. Each voltage was tested in triplicate using independently fabricated sensors. Each sample addition produced a transient current peak (ΔI_{peak}) that reached its maximum within ~300 ms and gradually returned to baseline. (b) Bar graph showing the average current peak (ΔI_{peak}) at each voltage, with error bars representing the standard deviation (SD) from three independent tests. The raw current peaks were normalized relative to the baseline current to allow comparison across

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devices. The strongest response was observed at −0.8 mV, while higher voltages yielded reduced signal amplitudes. (c) Comparison of average normalized current responses (ΔI_{peak}) from three repeats at low applied voltages (−0.9 mV, −0.8 mV, and −0.4 mV) following the addition of S1 protein (10^7 pg/mL) or bovine serum albumin (BSA). Only S1 additions produced statistically significant increases in normalized current ($p < 0.05$), confirming selective detection at the optimized voltage. Statistical analysis was performed using a repeated-measures one-way ANOVA (within-subject factor = voltage), followed by Tukey's multiple comparisons test.

Figure 4. Validation of biosensor specificity using scrambled-phage-functionalized control biosensor. (a) Representative real-time current response of the M13_SBP biosensor against SARS-CoV-2 S1 synthetic protein (S1) and *E. coli* K12. (b) Corresponding bar graph showing the average current peak at each voltage, with error bars representing standard deviation (SD) from three repeats. (c) Representative real-time current response of the M13_SC biosensor against S1 and *E. coli* K12. (d) Corresponding bar graph showing the average current peak at each voltage, with error bars representing standard deviation (SD) from three repeats. Statistical comparisons between the responses to S1 and *E. coli* K12 were conducted using a paired t-test. Asterisks (*) indicate statistically significant differences ($p < 0.05$), and "ns" denotes non-significant differences ($p \geq 0.05$).

Figure 5. Limit of detection (LOD) evaluation of the M13_SBP biosensor for SARS-CoV-2 S1 protein. (a) Real-time current responses of the biosensor upon sequential exposure to increasing concentrations of S1 protein suspended in PBS (10^{-4} to 1 pg/mL). Arrows indicate the time points at which each concentration was applied. Each addition produced a transient current peak (ΔI_{peak}), and the recorded currents were subsequently normalized to the baseline for comparative analysis. (b) Bar graph showing the average

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normalized current response (ΔI_{peak}) at baseline and after exposure to 10^{-4} pg/mL S1. Error bars represent the standard deviation (SD) from replicate measurements. Statistical comparisons between baseline and S1 responses were performed using a paired t-test. Asterisks (*) indicate statistically significant differences ($p < 0.05$). (c) Calibration curve of the M13_SBP biosensor showing normalized current response versus the logarithm (base 10) of SARS-CoV-2 S1 protein concentration. The linear regression equation and R^2 value are indicated. Each data point represents the mean of three independent replicates.

Figure 6. Real-time current response of M13_SBP biosensor and AB_SBP biosensors in complex sample matrices.

(a) Representative real-time current response of the M13_SBP biosensor in filtered wastewater and in wastewater spiked with 10 pg/mL S1 protein. (b) Corresponding average normalized current response (ΔI_{peak}) for wastewater (WW) alone and wastewater spiked with 5 pg/mL and 10 pg/mL S1; error bars represent the standard deviation (SD) from five independent sensors.

(c) Real-time current response of the M13_SBP biosensor in fetal bovine serum (FBS) and FBS + S1 (5 pg/mL). (d) Average normalized current response (ΔI_{peak}) in FBS and FBS + S1, with SD from five replicates. (e) Real-time current response of the M13_SBP biosensor in pasteurized milk and milk spiked with 5 pg/mL S1. (f) Corresponding average normalized current response (ΔI_{peak}) in pasteurized milk and milk + S1, with SD from five replicates. (g) Representative real-time current response of the antibody-based AB_SBP biosensor tested in wastewater spiked with 10 pg/mL S1. (h) Corresponding average normalized current response (ΔI_{peak}) for AB_SBP biosensors in wastewater ± S1. (i) Comparison of normalized current responses (ΔI_{peak}) across matrices (WW, FBS, Milk) and blanks; the red dashed line represents the decision threshold used for binary classification. (j) Calculated sensitivity (true-positive rate, %) of the biosensors across different matrices. Both phage-based (M13_SBP) and antibody-based (AB_SBP) platforms produced

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statistically higher normalized current responses for S1-spiked samples than their respective blanks ($p < 0.01$), confirming consistent binary detection across all matrices. ns = not significant ($p \geq 0.05$); all statistical comparisons used unpaired two-tailed t-tests between S1-spiked samples and corresponding blanks.

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