An Electrochemical Biosensing Platform for the SARS-CoV-2 Spike Antibody Detection Based on the Functionalised SARS-CoV-2 Spike Antigen Modified Electrode

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1. Experimental Section

Chemicals and apparatus
The SARS-CoV-2 (2019-nCoV) spike S1-his recombinant protein (verified by HPLC, Cat: 40591-V08H), SARS-CoV-2 spike antibody protein (Chimeric MAb Cat: 40150-D00), influenza A H1N1 Hemagglutinin/H0A protein (Cat: 11055-VNAB), Middle East respiratory syndrome-coronavirus (MERS-CoV) spike-S1 protein (S1 Subunit, aa 1-725, His Tag, Cat: 40069-V08B1) were supplied from Sino Biological Inc. and the native extract of *Streptococcus pneumoniae* antigen was obtained from Native Antigen Company. Gold standard solution (1000 mg/L Au in 2 M HCl traceable to SRM from NIST, Merck 170216), cysteamine hydrochloride (BioXtra, Sigma 30078), N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC, ≥98%, Sigma-Aldrich 03450), N-Hydroxysuccinimide (NHS, 98%, Sigma-Aldrich 130672), bovine serum albumin (BSA, ≥98%, Sigma-Aldrich 05470), phosphate buffered saline (PBS, tablet, Sigma-Aldrich P4417) and the other chemicals were used as analytical reagent grade.

Vitrosens RapidFor™ SARS-CoV-2 Rapid IgG/IgM test kits were used for comparative study.

All spike antigen and antibody solutions were prepared in 0.01 M (pH 7.5) of PBS solution and stored in Eppendorf protein LoBind tubes to prevent proteins from sticking to the walls of their containers. Meanwhile ultrapure water was used to prepare all other solutions and high-density polyethylene bottles or falcon tubes were used to store them. Milli-Q Direct 8 system was operated to obtain ultrapure water.

Clinical samples were analysed with commercial PCR kits (Bioeksen, Istanbul, Turkey) according to the manufacturer’s instructions using the Biorad CFX Connect RT-PCR system.

A Metrohm Autolab PGSTAT 128N potentiostat-galvanostat system consisting of chemically modified glassy carbon electrode (gold-clusters capped with CysNH₂, functionalised S-AG and BSA modified GCE– BSA/f-S-AG/CysNH₂/Au/GCE, supporting material: BASi MF-2012 GCE) as the working electrode, platinum wire (BASi MW-1032, 7.5 cm) as an auxiliary electrode and Ag/AgCl/3 M NaCl (BASi MF-2052 RE-5B) as a reference electrode for determining the SARS-CoV-2 spike antibody (S-AB) in synthetic and spiked-real samples.

A Mettler Toledo Seven Compact pH meter with InLab Expert Pro combined pH electrode and a thermostatic circulating bath (Thermo Haake DC 10 K20) was used to prepare buffer solutions. A FEI Quanta FEG 250 environmental scanning electron microscope (SEM) and a FEI Quanta 250 XFLASH 5030 energy dispersive X-ray spectroscopy (EDX) were used for the characterisation of the prepared sensors.
Preparation of the biosensing platform

To prepare BSA/f-S-AG/CysNH$_2$/Au/GCE (Figure 1), we first polished the GCE surface with 1 µm of diamond and 0.05 µm of alumina suspension on felt, rinsed it with ultrapure water and thereafter exposed it to ultrasonic waves in an ethanol–ultrapure water mixture (1:1, v/v) and in ultrapure water for 3 min each. Gold-clusters formed on the electrode’s surface as in our previous study.$^{[3,51]}$ To prepare CysNH$_2$/Au/GCE, 25 µL of 60 mM CysNH$_2$ was dropped onto the Au/GCE surface for 30 min, after which Au/GCE chemisorbed the thiol sites of CysNH$_2$ and amine terminals were placed facing the outside of the electrode. Next, 6 µL of 200 mM EDC and 4 µL of 200 mM NHS were mixed with 10 µL of 1 µg/mL S-AG to activate the carboxylic acid groups on the S-AG for 15 min in an Eppendorf protein LoBind tube. The mixture was dropped onto the surface of CysNH$_2$/Au/GCE and incubated for 15 min to obtain f-S-AG/CysNH$_2$/Au/GCE, in a reaction based on the amite formation of the carboxylic acid groups on the S-AG and the amine groups in the CysNH$_2$. Last, 2% BSA was used to block the free spaces of f-S-AG/CysNH$_2$/Au/GCE for 20 min to obtain the biosensing platform. All incubations were conducted at 21 ± 3 °C, and the biosensor was stored in refrigerator at 4 °C until next use. The biosensor preparation time of the proposed study, which is 1.5 h, comes to the fore much more than our previous studies,$^{[3,51]}$ which are 3.5 and 2.5 h.

Voltammetric measurement procedure

After the biosensor preparation, 5 µL of S-AB was incubated on the biosensor surface for 15 min, and square wave voltammetry (SWV) was performed with a potential range of 0.1 V – 1.2 V with 10 Hz of frequency, 5 mV of step potential, 0.1 s of interval time and 20 mV of pulse amplitude. Cyclic voltammetry (CV) scans were performed between -0.3 V and 1.2 V with 3 mV of step amplitude and 25 mV/s of scan rate. S-AB spiked- or non-spiked-real samples were used via adjusting a final volume of 10 mL with 0.01 M (pH 7.5) of PBS solution. The oxidation peak of the biosensing platform at 0.7 V diminished with the increasing amount of S-AB, and that signal was used for voltammetric determination of S-AB. The laboratory atmospheric conditions were adjusted to 21 ± 3 °C and 45 ± 15% RH.

LFIA measurement procedure

Measurements were performed according to the manufacturer's instructions. Briefly, 10 µL of the spiked-saliva sample was dropped onto the sample well of the test card using the plastic dropper and thereafter added 80 µL of sample diluent to the sample well. Finally, the images were interpreted for IgG.
Sample preparation procedure

Real samples originated from saliva and oropharyngeal swab were collected from six healthy individuals and treated by following a far simpler way than in our previous work.\(^3\) After half of the samples were spiked with 1 pg of S-AB for each 5 μL, the spiked- and non-spiked-samples (i.e. the other half of the samples) were analysed to determine S-AB via external calibration by depositing 5 μL of the spiked- or non-spiked-sample on the surface of biosensor without any preprocessing.

1 ng/mL, 10 ng/mL, 100 ng/mL, 1 μg/mL, 10 μg/mL and 100 μg/mL of S-AB were added to the saliva samples and analysed by LFIA.

Clinical samples consisting of gargle and mouthwash liquids were acquired using drinking water with low ion concentration. Clinical samples were chosen among these samples which were formerly examined by RT-PCR for the presence of SARS-CoV-2. 5 μL of the clinical samples was deposited on the biosensor surface and analysed via external calibration without any preprocessing. Ethical approval was obtained from Acibadem University Ethical Committee, ATADEK approval No: 2020-14/2 for the related study.

Footnote: The reference numbers given for “Experimental Section” are the same as the main text.
2. Introduction

Table S1. Electrochemical biosensing methods for determining SARS-CoV-2 and/or the related species.

| Sensing material                                                                 | Technique                        | Biosensor preparation time (h) | Measurement time (min) | LOD               | Analyte                          | Reference | [a] The reference numbers given are the same as the main text. |
|----------------------------------------------------------------------------------|----------------------------------|--------------------------------|------------------------|------------------|-----------------------------------|-----------|---------------------------------------------------------------|
| Bovine serum albumin, SARS-CoV-2 spike antigen, glutaraldehyde, cysteamine and gold-clusters modified glassy carbon electrode | Square wave voltammetry         | 3.5                            | 40                     | 0.01 ag/mL       | Spike antibody                    | 3         |                                                               |
| Graphene and gold nanoparticles conjugated with suitably designed antisense oligonucleotides | Chronopotentiometry              | >30                            | 35                     | 6900 copy/mL     | RNA                              | 41        |                                                               |
| Magnetic bead-based immunosensor combined with carbon black-modified screen-printed electrode | Differential pulse voltammetry  | 2                              | 30                     | 19 and 8 ng/mL   | Spike and nucleocapsid protein    | 42        |                                                               |
| Graphene, 1-pyrenebutyric acid N-hydroxysuccinimide ester and SARS-CoV-2 spike antibody modified field-effect transistor | Semiconductor analyzer          | >7                             | 15                     | 1 fg/mL, 242 copy/mL | Spike protein and RNA              | 43        |                                                               |
| Graphene-based telemedicine platform                                              | Amperometry                      | 5                              | 15                     | -                | Nucleocapsid protein, spike and nucleocapsid antibodies | 44        |                                                               |
| Cobalt functionalized TiO_2 nanotube-based screen-printed electrode               | Amperometry                      | >12                            | 5                      | 0.1 μg/mL        | Spike protein                     | 45        |                                                               |
| p-sulfocalix[8]arene, graphene oxide, toluidine blue functionalized gold supersandwich | Differential pulse voltammetry  | 29                             | 3                      | 200 copy/mL      | RNA                              | 46        |                                                               |
| SARS-CoV-2 spike antibody, bovine serum albumin, staphylococcal protein A, Cu_2O nanocubes modified screen-printed electrode | Electrochemical impedance spectroscopy | 12                             | 20                     | 0.04 fg/mL       | Spike protein                     | 47        |                                                               |
| Bovine serum albumin, SARS-CoV-2 spike antibody, 1-pyrenebutyric acid N-hydroxysuccinimide ester modified graphene electrode | Square wave voltammetry         | 5                              | 45                     | 20 μg/mL         | Spike protein                     | 48        |                                                               |
| Cotton-tipped, bovine serum albumin, SARS-CoV-2 nucleocapsid protein, diazonium salt and carbon nanofiber modified screen-printed electrode | Square wave voltammetry         | 25                             | 30                     | 0.8 pg/mL        | Nucleocapsid protein              | 49        |                                                               |
| Bovine serum albumin, SARS-CoV-2 spike antibody and functionalized graphene oxide modified glassy carbon or screen-printed electrode | Square wave voltammetry         | 2.5                            | 35, 5                  | 1 ag/mL          | Spike protein                     | 50        |                                                               |
3. Sensor characterisation

A  
85.2% C  
14.8% O

B  
32.1% C  
4.1% O  
63.8% Au

C  
39.4% C  
2.7% O  
53.0% Au  
4.7% N  
0.2% S
Figure S1. EDX spectra for (A) bare GCE, (B) Au/GCE, (C) CysNH$_2$/Au/GCE, (D) f-S-AG/CysNH$_2$/Au/GCE and (E) BSA/f-S-AG/CysNH$_2$/Au/GCE (EDX analysis: SLEW/30 mm$^2$ detector, Energy resolutions: manganese: $<$127 eV FWHM at Mn$k_\alpha$, fluorine: 57 eV FWHM at F$k_\alpha$ and carbon: 51 eV FWHM at C$k_\alpha$ (mass percentages were given in EDX spectra).

4. Cyclic voltammetric characteristics of the system

Figure S2. Cyclic voltammograms of (a) the BSA/f-S-AG/CysNH$_2$/Au/GCE and (b) + 100 fg/mL of the S-AB in 0.01 M (pH 7.5) of PBS solution with a scan rate of 25 mV/s.
Figure S3. Plots of the peak height ($I_p$ ($\mu$A)) – scan rate ($u$ (mV/s)) (25 – 1000 mV/s), the peak height – the square root of scan rate ($\sqrt{u}$ (\sqrt{mV/s}) (25 – 1000 mV/s) and the logarithm of peak height (log ($I_p$, $\mu$A)) – the logarithm of scan rate (log ($u$, mV/s)) (75 – 1000 mV/s) by using cyclic voltammetry.
5. Optimisation studies

![Graph A](image1.png)

**A**

Concentration of CysNH$_2$ (mM)

![Graph B](image2.png)

**B**

Concentration of S-AG (µg/mL)

![Graph C](image3.png)

**C**

The ratio of EDC to NHS

![Graph A'](image4.png)

**A’**

Normalized current (µA)

![Graph B'](image5.png)

**B’**

Normalized current (µA)

![Graph C'](image6.png)

**C’**

Normalized current (µA)
ΔIₚ (µA)

CysNH₂ binding time (min)

ΔIₚ (µA)

S-AG binding/reaction time (min)

ΔIₚ (µA)

BSA binding time (min)

ΔIₚ (µA)

S-AB binding time (min)
Figure S4. Optimisation studies for the concentration of (A, A’) cysteamine (CysNH$_2$) and (B, B’) the SARS-CoV-2 spike antigen (S-AG), (C, C’) the ratio of EDC to NHS (200 mM for each), and the binding time of (D, D’) CysNH$_2$, (E, E’) S-AG, (F, F’) bovine serum albumin (BSA) and (G, G’) the SARS-CoV-2 spike antibody (S-AB) by using SWV. Conditions: Optimal values for the sensor preparation (the concentration of CysNH$_2$, S-AG, the ratio of EDC to NHS (200 mM for each) and the binding time of CysNH$_2$, S-AG, BSA and S-AB were respectively 60 mM, 1 μg/mL, 6 μL/4 μL, 30 min, 15 min, 20 min and 15 min) were used for each optimisation study, only the examined parameter was changed in the respective measuring range. The measurements were performed with 100 fg/mL of S-AB in 0.01 M (pH 7.5) of PBS solution.

6. Method validation

Figure S5. The response of (straight black line) BSA/f-M-S-AG/CysNH$_2$/Au/GCE, (straight red line) BSA/f-InfA/CysNH$_2$/Au/GCE and (straight green line) BSA/f-Pneu/CysNH$_2$/Au/GCE to (dotted black, red and green lines, respectively) S-AB (100 fg/mL) in 0.01 M (pH 7.5) of PBS solution by using SWV. Sensor preparation: The concentration of CysNH$_2$, spike protein/antigen, the ratio of EDC to NHS (200 mM for each) and the binding time of CysNH$_2$, spike protein/antigen, BSA and S-AB were respectively 60 mM, 1 μg/mL, 6 μL/4 μL, 30 min, 15 min, 20 min and 15 min.
Figure S6. Sensor stability studies of BSA/f-S-AG/CysNH$_2$/Au/GCE at 4 °C for 30 days by using SWV. Conditions: 100 fg/mL of S-AB, 0.01 M (pH 7.5) of PBS solution. Sensor preparation: The concentration of CysNH$_2$, S-AG, the ratio of EDC to NHS (200 mM for each) and the binding time of CysNH$_2$, S-AG, BSA and S-AB were respectively 60 mM, 1 μg/mL, 6 μL/4 μL, 30 min, 15 min, 20 min and 15 min.

Figure S7. Sensor robustness studies of BSA/f-S-AG/CysNH$_2$/Au/GCE at 25 °C and 37 °C for 30 days by using SWV. Conditions: 100 fg/mL of S-AB, 0.01 M (pH 7.5) of PBS solution. Sensor preparation: The concentration of CysNH$_2$, S-AG, the ratio of EDC to NHS (200 mM for each) and the binding time of CysNH$_2$, S-AG, BSA and S-AB were respectively 60 mM, 1 μg/mL, 6 μL/4 μL, 30 min, 15 min, 20 min and 15 min.
7. Sample application

**Figure S8.** The SWV voltammograms of saliva and spiked-saliva samples obtained from six independent measurements with BSA/f-S-AG/CysNH$_2$/Au/GCE in 0.01 M (pH 7.5) of PBS solution. Sensor preparation: The concentration of CysNH$_2$, S-AG, the ratio of EDC to NHS (200 mM for each) and the binding time of CysNH$_2$, S-AG, BSA and S-AB were respectively 60 mM, 1 μg/mL, 6 μL/4 μL, 30 min, 15 min, 20 min and 15 min. Pretreatment of the sample: After half of the samples were spiked with 1 pg of S-AB for each 5 μL, the spiked- and non-spiked-samples (i.e. the other half of the samples) were analysed to determine S-AB via external calibration by depositing 5 μL of the spiked- or non-spiked-sample on the surface of biosensor without any preprocessing.

**Figure S9.** The SWV voltammograms of oropharyngeal swab and spiked-oropharyngeal swab samples obtained from six independent measurements with BSA/f-S-AG/CysNH$_2$/Au/GCE in 0.01 M (pH 7.5) of PBS solution. Sensor preparation: The concentration of CysNH$_2$, S-AG, the ratio of EDC to NHS (200 mM for each) and the binding time of CysNH$_2$, S-AG, BSA and S-AB were respectively 60 mM, 1 μg/mL, 6 μL/4 μL, 30 min, 15 min, 20 min and 15 min. Pretreatment of the sample: After half of the samples were spiked with 1 pg of S-AB for each 5 μL, the spiked- and non-spiked-samples (i.e. the other half of the samples) were analysed to determine S-AB via external calibration by depositing 5 μL of the spiked- or non-spiked-sample on the surface of biosensor without any preprocessing.