Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
In every pandemic, it is critical to test as many people as possible and keep track of the number of new cases of infection. Therefore, there is a need for novel, fast and unambiguous testing methods. In this study, we designed a sandwich-type voltammetric immunosensor based on unlabelled- and labeled with a redox probe antibodies against virus spike protein for fast and ultrasensitive detection of SARS-CoV-2. The process of the preparation of the sensor layer included chemisorption of cysteamine layer and covalent anchoring of antibody specific for the S1 subunit of the S protein. The source of the voltametric signal was the antibody labeled with the redox probe, which was introduced onto biosensor surface only after the recognition of the virus. This easy-to-handle immunosensor was characterized by a wide analytical range (2.0 \times 10^{-10} \text{mg} \cdot \text{L}^{-1} \text{–} 0.20 \text{mg} \cdot \text{L}^{-1}) and low detection limit (8.0 \times 10^{-8} \text{mg} \cdot \text{L}^{-1} \equiv 0.08 \text{pg} \cdot \text{mL}^{-1} \equiv 4 \text{virions} \cdot \text{mL}^{-1}). The utility of the designed device was also evidenced by the detection of SARS-CoV-2 in the clinical samples. Moreover, the main advantage and a huge novelty of the developed device, compared to those already existing, is the moment of generating the analytical signal of the redox probe that appears only after the virus recognition. Thus, our diagnostic innovation may considerably contribute to controlling the COVID-19 pandemic. The as-developed immunosensor may well offer a novel alternative approach for viral detection that could complement or even replace the existing methods.
acid isolation, which adds to the complexity and duration of the test. RT-PCR has not been standardized yet. According to the American Society for Microbiology, the results obtained from the RT-PCR COVID-19 test are dependent on its limit of detection (LOD). If the LOD of a given RT-PCR kit is too high, patients infected by SARS-CoV-2 might not test positive, which leads to false-negative results. On the other hand, if the LOD is too low, contamination may occur, resulting in false-positive results [13]. Another disadvantage of RT-PCR for the diagnosis of COVID is that it cannot be used for point-of-care testing and outside of laboratory facilities. Rapid antigen detection test is an imperfect alternative as a first-line diagnostic tool for COVID-19, and its poor sensitivity in asymptomatic patients limits its usefulness [14].

In addition to molecular testing, serological assays for detection of specific antibodies are used for COVID-19 diagnosis as they allow identifying individuals with a past infection or vaccinated ones. However, these assays do not detect the acute phase of COVID-19. An alternative to the currently used COVID-19 diagnostics is voltammetric sensors which enable quick and unambiguous diagnosis based on the detection of biomarkers or other pathogen-based endpoints, and consequently help in making quick decisions regarding the appropriate treatment. In most cases, these sensors do not require tedious and time-consuming sample preparation step, thus reducing the costs of the analysis, while maintaining high sensitivity and showing a wide range of responses. In addition, unlike PCR, they do not require qualified personnel. Furthermore, the voltammetric measuring system can be miniaturized, which will allow developing portable analytical tools for electrochemical detection characterized by higher throughput and faster turnaround time. The electrochemical protocols for SARS-CoV-2 detection, described so far in the literature [15–18], have one major disadvantage that the current signal of the redox probe is present both before and after the analyte recognition process, and the detection is based on a signal changes resulting from blockage of the receptor layer surface. Unfortunately, such an approach is none Selective and may be burden to a large error due to the fact that many elements of the clinical sample, except the analyte, are able to cause such a change in the measured current signal, which is absolutely not related to the analyte recognition process.

Here we propose effective way to overcome this problem by application of unlabeled and labeled with redox probe antibodies. Unlabeled antibody is responsible for virus (antigen) recognition, whilst labeled antibody is a source of current signal. If the virus is not recognized, the current signal will not appear. The antibody labeling was done by permanent incorporation of a ferrocene (Fc) derivative into the antibody structure through an amide bond formed between the amino groups of the antibody and the carboxyl groups of the ferrocene derivative. Our proposition not only guarantees the high selectivity of the determination but also should minimizes the likelihood of occurrence the false-positive and false-negative results of the analysis. The affinity of the labeled antibody to the S protein was confirmed by surface plasmon resonance (SPR). The detection of the S protein or SARS-CoV-2 was controlled by differential pulse (DP) voltammetry based on the intensity of Fc current oxidation.

2. Materials and methods

2.1. Materials

SARS-CoV-2 spike protein recombinant antigen (S protein), SARS-CoV-2 spike protein S1 chimeric recombinant human monoclonal antibody (Ab1), SARS-CoV/SARS-CoV-2 spike protein S2 monoclonal antibody (Ab2), bovine serum albumin (BSA), cysteamine hydrochloride (CSH), triethylamine anhydrous, phosphate buffered saline (PBS), N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N-ethyl-carbodiimide hydrochloride (EDC), ferrocenecarboxylic acid, succinic acid anhydride, 4-dimethylaminopyridine (DMAP), aceton, ethylene chloride, hydrochloric acid and magnesium sulfate were purchased from Sigma-Aldrich and used without additional purification. All solutions were prepared with the ultrapure water (Hydrolab, conductivity 0.056 μS·cm⁻¹). The measurements were performed in 0.01 M PBS buffer, pH 7.4.

2.2. Synthesis of the redox probe (Fc-R-COOH; R = -CONH-C₆H₄-NHOCH₂CH₂F)

\[ \text{N-(4-Aminophenyl)ferrocenecarboxamide (1) was synthesized in two steps starting from ferrocenecarboxylic acid, following the procedure described in the literature [19]. The reaction flask containing 1 (57.6 mg, 0.18 mmol, 1 equiv.), succinic acid anhydride (2; 18.0 mg, 0.18 mmol, 1 equiv.) and 4-dimethylaminopyridine (0.04 mmol, 4.4 mg, 0.2 equiv.) was evacuated and purged with argon. Dry acetone (20 mL) was added, and the reaction mixture was stirred at 50 °C for 72 h. Next, the reaction mixture was diluted with methylene chloride (60 mL), washed with 1 M HCl (3× 15 mL), water (3× 15 mL), dried over MgSO₄, filtered off and volatiles were evaporated on a rotary evaporator. Target product (67.0 mg, 89%) in a form of orange solid was isolated by means of the column chromatography (SiO₂; 10% CH₂OH/CH₂Cl₂; Rf = 0.20). The final product 4-((4-ferroceneamido)phenyl)amino)– 4-oxobutanoic acid was confirmed by FTIR, 1³C NMR, 1³H NMR and ESI-HRMS (TOF) analysis. The obtained results are presented in Supplementary Materials.} \]

2.3. Labeling of antibody with redox probe

The preferred site for the chemical modification of the antibody is the –NH₂ group of lysine or the free –SH group of cysteine [20]. In our case, the modification of antibody with Fc-R-COOH was carried out through the formation of amide bonds between the amine groups of antibodies and the carboxyl groups of the redox probe. To label the antibody with a carboxyl derivative of ferrocene (redox probe), the carboxyl groups of the redox probe were first activated. The 100 μM Fc-R-COOH was activated in an aqueous mixture of EDC (40 mM) and NHS (10 mM) for 30 min. Then, the antibody (phosphate-buffered saline (PBS) buffer, pH 7.4) was added to the activated redox probe solution. The mixture was incubated for 2 h under gentle stirring in ThermoMixer at room temperature and then dialyzed (4 times) against PBS buffer (pH 7.4) to remove unbound ferrocene derivative. The amount of ferrocene units anchored to antibody molecules was determined by UV–vis spectroscopy. Studies on the S protein were performed using Ab2 antibody against the S2 subunit of S protein labeled with Fc derivatives, while studies on clinical samples were performed with Ab1 antibody against the S1 subunit of the S protein.

2.4. Immunosensor construction

The orientation of the antibody in relation to the surface of its immobilization is crucial for effective antigen recognition. The vertical orientation of the antibody, which can only be achieved by the involvement of carboxyl groups of its crystallizable fragment in the immobilization process, guarantees appropriate antigen–antibody recognition. Therefore, to ensure proper covalent attachment of the antibodies in the first step, the surface of the gold electrode was modified with a layer of cysteamine, a short thiol containing a terminal amino group. The chemisorption of CSH at the gold surface was performed in 1 mM ethanolic solution. To remove the noncovalently bound CSH molecules from the gold surface, the electrodes were immersed in 96% ethanol and dried with a gentle argon stream. The antibodies were covalently attached to the CSH layer through the formation of an amide bond between the carboxyl groups of the crystallizable fragment of the antibody and the amino groups of CSH. For this purpose, the –COOH groups of the antibody were activated in an aqueous mixture of 40 mM
EDC and 10 mM NHS for 30 min. Next, a 7-μL droplet of the mixture (Ab/EDS/NHS) containing 0.05 mg L⁻¹ of activated Ab1 was placed on the electrode surface and left under the cover for 2 h. To ensure the binding of the SARS-CoV-2 S protein to the receptor layer of the immunosensor by interaction with the antibody, the electrode surface was blocked with bovine serum albumin (2.0 mg immunosensor by interaction with the antibody, the electrode surface was blocked with bovine serum albumin (2.0 mg L⁻¹ in phosphate buffer (pH 4.6)) for 1 h. The isoelectric point (pI) of BSA is around pH = 4.5 – 5.5 [21–23], so at the pH 4.6 all BSA domains have positive charge [24]. At such conditions the CSH amine groups are also protonated. Therefore, the BSA molecules are not adsorbed at the thiol layer due to the electrostatic repulsion between them. However, it is known that the regularity of thiol layers depends on many factors, including the length of the carbon chain of the thiol molecule. The short alkanethiols (n ≤ 5) promote the formation of the less ordered and labile layer with low packing density [25,26]. Cysteamine is one of the shortest thiols, so to make sure that the protein components of the sensor do not have the direct contact with the gold surface, the BSA as a factor eliminating non-specific interactions of protein with gold surface was used.

Then, a 7-μL droplet of the S protein (for construction of calibration curve), in the concentration range of 2.0·10⁻⁸ to 0.20 mg L⁻¹, or the extract of nasopharyngeal swab obtained from patients was placed on the electrode surface and left under the cover for 2 h. Next, a 7-μL droplet of Ab2 (calibration curve; the S2 subunit of the S protein) or Ab1 (nasopharyngeal swab extract; S1 subunit of S protein) modified with ferrocene units (Ab2-Fc), at a concentration of 2.0 mg L⁻¹, was placed on the electrode surface and left under the cover for 2 h. The structure of the S protein consists of two subunits the S1 and S2. The S1 subunit is the outer part of the S protein, through which the virus binds with the receptor of the host cell. Whereas, the S2 subunit is a transmembrane subunit of the S protein that mediates viral cell membrane fusion. S2 is responsible for the release of virus RNA into the cell and initiation of viral replication [27,28]. To each of the S protein subunit a different antibody is dedicated. Hence, in the case of the immunosensor analytical characteristics studies, performed with using the S protein, two types of antibodies were used. The first one forming the receptor layer was dedicated to the S1 subunit of the S protein. The second one labeled with ferrocene, specific for the S2 subunit, was used as a voltammetric signal generator (Ab-Fc). Whilst, during the analysis of the clinical samples containing virus particles, both the virus recognition process and the introduction of the voltammetric signal generator were performed via the same S protein subunit (S1). Therefore, in this case only one type of antibody, specific for the S1 subunit, was used. After each step, the electrode was gently rinsed with PBS buffer. Finally, voltammetric detection was performed in degassed PBS buffer (pH 7.4). A schematic illustration of immunosensor preparation is presented in Fig. 1. Due to the fact that the detection of the SARS-CoV-2 virus was based on the recognition of the S protein, we used two immunosensors in our research. The first of them was dedicated to the detection of the S protein, using Ab1 and Ab2 antibodies characteristic for the S1 and S2 subunits, respectively, which was used to construct the calibration curve (left route in Fig. 1). The second one was used in the analysis of real samples (right route in Fig. 1). In the case of the construction of the second immunosensor, only the labeled and unlabeled Ab1 antibody characteristic for the S1 subunit of the spike protein was used.

2.5. Clinical samples

Clinical samples obtained from adult patients were tested for the presence of SARS-CoV-2 using the RT-PCR method. Nasopharyngeal swab specimens were collected using flocked swabs and deposited in Viral Transport Medium (VTM) (Biocomma, China). Two media variants were used: inactivating and classical non-inactivating. The classical buffer was composed of Hanks balanced fluid supplemented with BSA, HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid), amino acids, cryoprotectants, and other components added to strengthen the viral integrity. Inactivating VTM additionally contained guanidine salt, a surfactant for viral lysis. Immediately after collection, the swabs were placed in the refrigerator (2–8 °C), and transported within 2 h to the laboratory. After PCR analysis, the samples were stored in the freezer at –30 °C. The RT-PCR SARS-CoV-2 analysis was performed using Life-river Novel Coronavirus (2019-nCoV) Real Time Multiplex RT-PCR Kit (Shanghai ZJ Bio-Tech C, Shanghai, China), using CF96™ Real-Time PCR Detection System (Bio-Rad, USA). The test detects three target genes: SARS-CoV-2 gene E, gene N, and ORF1ab. The LOD is 1·10⁻³ copies·mL⁻¹.

2.6. Voltammetric techniques

The voltammetric measurements (differential pulse voltammetry and cyclic voltammetry) were performed using an Autolab, model PGSTAT 12 potentiostat at room temperature in the three-electrode system included: (i) working electrode (gold disc electrode; ϕ = 1.6 mm; Ageom. = 0.0201 cm², BASi, UK), (ii) reference electrode (Ag/AgCl/3 M KCl), and (iii) auxiliary electrode (Pt plate with surface area at least 1 cm²). Electrochemical measurements were performed in deoxygenated 10 mM PBS, pH 7.4. Before each measurement, the surface of the working electrode was cleaned both mechanically and electrochemically. First the gold surface was polished on a wet pad with addition of 1 μm Al₂O₃ powder. After polishing, each electrode was rinsed with a stream of ultrapure water (Hydrolab, conductivity of ~ 0.056 μS·cm⁻¹) ensuring complete removal of alumina from the electrode surface and then dried with argon. Next, gold electrode was electrochemically cleaned by recording cyclic voltammograms in 0.1 M H₂SO₄ solution in a potential range from –0.3 to 1.5 V (vs Ag/AgCl/3 M KCl) with scan rate 0.05 V·s⁻¹, until a stable voltammogram typical for a bare gold electrode was obtained.

2.7. Surface plasmon resonance

The surface plasmon resonance experiments were carried out with using Biacore X100 from Cytiva. The measurements were made on protein A sensor chip (Cytiva) with flow rate of 5 μL·min⁻¹.

3. Results and discussion

3.1. Binding kinetic studies

SPR is the best technique for studying molecular interactions. The accuracy of the developed immunosensor was verified by obtaining SPR measurements. Fig. 2 A and 2B show a typical SPR response recorded
during the analysis of the binding of Ab1 and Ab2 antibodies with S protein, as well as the labeling of antibody with the redox probe. The two different antibodies used in the detection process corresponded to the structure of the S protein, which consists of three protomers, each with S1 and S2 subunits [21, 29]. Because S protein is trimeric and can undergo structural transitions, its interaction with antibody can be best explained by a two-state reaction model. The results of the kinetic analysis are given in Fig. 2C. The $K_D$ values calculated for all the studied antibodies confirmed their high affinity to S protein. Moreover, the labeling of antibody with the redox probe did not have any influence on its interaction with S protein.
Sensors and Actuators: B. Chemical 371 (2022) 132539

5

spectra of unmodified antibody and antibody modified with Fc units

analytical signal. Selective voltammetric detection requires the appli-

troduced to transform biological recognition into a measurable
generate a voltammetric signal. Therefore, a redox probe should be

confirm the conjugation of ferrocene units with antibodies, the UV

probe (modified ferrocene) was used in immunosensor construction. To

analyte. For this purpose, a secondary antibody labeled with a redox

specific antibody. However, the antibody

to the S protein, a surface protein of SARS-CoV-2, which is recognized by a

3.2. Analytical performance

mental conditions: 0.01 M PBST (pH 7.4),

centrations of Ab2 and Ab2-Fc (2.215; 9.500; 19.00 and 38.00 nM). Experi-

C: Kinetic parameters obtained from SPR data for binding of Ab1, Ab2, Ab1-Fc and Ab2-Fc to S protein with using two state reaction model. Inset: Sensorgrams recorded for various concentration of Ab2 and Ab2-Fc (2.215; 9.500; 19.00 and 38.00 nM). Experimental conditions: 0.01 M PBST (pH 7.4), $C_{Ab} = 0.050 \text{mg}\cdot\text{L}^{-1}$.

3.2. Analytical performance

The developed sensor works based on the voltammetric detection of
the S protein, a surface protein of SARS-CoV-2, which is recognized by a
specific antibody. However, the antibody–antigen interaction does not
generate a voltammetric signal. Therefore, a redox probe should be
introduced to transform biological recognition into a measurable
analytical signal. Selective voltammetric detection requires the applica-
tion of a redox probe that clearly indicates the recognition of the
analyte. For this purpose, a secondary antibody labeled with a redox
probe (modified ferrocene) was used in immunosensor construction. To
confirm the conjugation of ferrocene units with antibodies, the UV–vis
spectra of unmodified antibody and antibody modified with Fc units

were recorded. The UV–vis spectra recorded for Ab2-Fc are presented in

Fig. 3. The concentration of Fc in the antibody-Fc conjugates was calculated using the regression equations of the calibration curves generated for the absorbance value taken at 431 nm (Ab1-Fc: $A = (0.121 \pm 0.003)C_{\text{Fc}} + (0.0094 \pm 0.0026)$; $R^2 = 0.999$; Ab2-Fc: $A = (0.118 \pm 0.0022)C_{\text{Fc}} + (0.0071 \pm 0.0058)$; $R^2 = 0.999$). The calibration curve and UV–vis spectra of Fc-COOH are presented in the insets in

Fig. 3. The final concentrations of the antibody in the antibody-Fc conjugate were 2.0 and 2.1 mg\cdot\text{L}^{-1} (for Ab1 and Ab2, respectively) and that of Fc units were 0.38 and 0.40 mM (for Ab1 and Ab2, respectively). Considering that the IgG molecules have about 84 amine groups

[30] based on the comparison of the number of moles of –NH$_2$ groups and Fc units, it can be concluded that 59 possible conjugation sites are occupied by the redox probe. The binding of such a high number of Fc units to antibody molecule guarantees high sensitivity of voltammetric detection. The method by which the redox probe was introduced eliminates its nonspecific interaction with the receptor layer, and thus allows excluding false-positive and false-negative results of the analysis.

To ensure that the antibody labeled with the redox probe (antibody-Fc) is the sole source of voltammetric signal, DP voltammograms were recorded after each step of the immunosensor construction (Fig. 4). The current signals resulting from the ferrocene redox process were observed only after the interaction of antibody-Fc conjugate with the S protein bound with receptor layer. Importantly, no interaction was observed between the antibody molecules labeled with the redox probe and the unlabeled antibody molecules forming the receptor layer (green curve in

Fig. 4B). Probably, lack of antibody and antibody labeled with redox probe interaction is due to the occupation of amino groups of antibody molecule by the redox probe. To get the information about the possible interaction between Ab1-Fc or Ab2-Fc and each component of the receptor layer the control experiments were performed. The obtained DP voltammograms are presented in Fig. S4 in Supplementary Materials. Due to the fact that the studied Ab-Fc conjugates play a role of a source of volumetric signals and were not used as a component of the receptor layer, the activation step was not needed. The recorded voltammogram curves clearly showed that Ab-Fc conjugates interact only with the S protein, not with receptor layer components.

The amount of the antibody labeled with Fc units is dependent on the
amount of the detected analyte. A higher degree of saturation of the
receptor layer with the S protein results in a greater amount of Ab2-Fc,
and subsequently a current signal with greater intensity. The DP vol-

ammograms are presented in Fig. S4 in Supplementary Materials.

Based on the changes in the current signal resulting from Fc oxida-
tion, a calibration curve was constructed (inset in Fig. 5A). A linear

Fig. 2. A: Sensorgrams recorded for Ab1 interactions with S protein in various concentrations (12.5; 25; 50 and 100 nM). B: Sensorgrams recorded for Ab2 and Ab2-Fc interactions with S protein (100 nM). C: Kinetic parameters obtained from SPR data for binding of Ab1, Ab2, Ab1-Fc and Ab2-Fc to S protein with using two state reaction model. Inset: Sensorgrams recorded for various concentration of Ab2 and Ab2-Fc (2.215; 9.500; 19.00 and 38.00 nM). Experimental conditions: 0.01 M PBST (pH 7.4), $C_{Ab} = 0.050 \text{mg}\cdot\text{L}^{-1}$.

Fig. 3. UV–vis spectra of unmodified antibody (Ab2) and modified with Fc units (Ab2-Fc). Top inset: UV–vis spectra of Fc-COOH derivative in various concentration. Bottom inset: Calibration curve. Experimental conditions: $C_{\text{Fc}} = 2.0 \text{mg}\cdot\text{L}^{-1}$, PBS buffer, pH 7.4.

Fig. 4. A: Sensorgrams recorded for Ab1 interactions with S protein in various concentration, a calibration curve was constructed (inset in Fig. 5A). A linear
response was observed for the S protein concentration range from 2.0 \times 10^{-7} to 0.20 \text{ mg L}^{-1}, with regression equation as follows: 
\log \text{I}_{\text{Fc}} = 0.43 \cdot \log C_{ \text{S protein}} + 3.21 \quad (\text{regression coefficient } R^2 = 0.9863). 

The LOD was determined from the regression equation using the formula:

\text{LOD} = \frac{3 \sigma}{a} \tag{1}

where \(\sigma\) is the standard deviation of the response observed for the lowest measurable concentration of the S protein and \(a\) is the slope of the calibration curve. The estimated LOD was 0.080 pg \text{ mL}^{-1}. A virion contains approximately 100 molecules of the S protein (trimers) on its surface [31]. Therefore, considering the value of LOD and the fact that the S protein consists of three monomers, it can be concluded that the proposed immunosensor can detect the presence of the virus at a level of about 4 virions \(\mu\text{L}^{-1}.

Cyclic voltammetry provides quantitative information on the number of redox probe (ferrocene units) molecules or antibody molecules labeled with a selected redox probe. However, it should be noted that in the case of layers characterized by the low electrical conductivity (such a layer is definitely the protein layer), the current signal recorded using cyclic voltammetry may be poorly developed. In order to determine the number of Ab-Fc molecules introduced into the electrode surface as a result of interaction with the S protein in the function of its concentration, the cyclic voltammetry measurements were performed. Fig. 5A showed the voltammetric curves for the highest and lowest concentration of the S protein from the linear range of the calibration curve (inset in Fig. 5A). For the highest concentration of the S protein, the Fc electrooxidation current signal was clearly visible and well developed, whilst it practically disappeared in the case of a low concentration of the S protein. Thus, in order to estimate the number of redox probe molecules associated with the sensor surface corresponding to the analyte concentration (0.08 pg \text{ mL}^{-1}), the linearity of the charge changes of the Fc electrooxidation peak as a function of the S protein concentration was assumed. The obtained relationship, presented in the inset in Fig. 5B, is described by the following linear regression equation: 
\log Q_{\text{Fc ox}} = 1.05 \cdot \log C_{\text{S protein}} - 7.41 \quad (\text{regression coefficient } R^2 = 0.9728). 

On its basis, the value of the charge of the ferrocene oxidation peak corresponding to the concentration of the S protein (analyte) 0.08 pg \text{ mL}^{-1} is 1.99 \times 10^{-15} \text{ C}. Therefore, the number of ferrocene units introduced into the sensor surface was calculated in accordance with the equation 
\frac{Q_{\text{Fc ox}}}{N} = 1.24 \times 10^{-4}. 

Ferrocene molecules are introduced into the receptor layer by the antibody molecules labeled with them. Based on the UV–vis measurements (Fig. 3), we showed that there are 59 Fc molecules per one antibody molecule, so the number of Ab-Fc molecules corresponding to the S protein concentration 0.08 pg \text{ mL}^{-1} is 211. Taking into account the fact that 1 virion contains ca. 100 molecules of the S protein, but only the half of them are maximally available for Ab-Fc conjugate (the other half are involved in the recognition of the analyte by the receptor layer). So the number of virions is around 5 per \(\mu\text{L}. Thus, it can be concluded that the minimum number of Fc units introduced into the electrode surface necessary for the detection of the virus SARS-CoV-2 is 211.

The proposed immunosensor was highly stable for 30 days. During
this time the observed changes in the Fc oxidation current signal were not greater than 3.6% from the initial value (obtained just after interaction of the freshly prepared immunosensor with analyte). The measurements were performed every 24 h for a period of 30 days. Between the measurements electrodes were kept in the PBS buffer, in 4 °C. In the case of longer storage times, the changes in signal intensity were much greater. The representative DP voltammograms as well as the changes in the current signal intensity obtained during long-term stability measurements are presented in Fig. 6A. The electrode-to-electrode and investigator-to-investigator reproducibility was tested by two investigators with using 11 gold disc electrodes with the same diameter. The observed relative standard deviations were low, at the level of 2.8% and 4.5%, respectively for electrode-to-electrode and investigator-to-investigator (see Fig. 6B).

Selectivity is an important parameter of each biosensor that verifies its functionality versus real samples. The selectivity studies were carried out in one-component solutions for a few selected protein components of saliva, such as: α-amylase, lysosome, lactoferrin, fibronectin and MMP-9. The concentration of potential interferents was 1000-fold higher than the S protein concentration (2.0 ng mL⁻¹). The obtained results, presented in Fig. 7, proved the high selectivity of the proposed immunosensor.

The observed values of ferrocene electrooxidation current as a result of the sensor’s contact with interferents were much lower, at the level of 0.15 – 0.23 μA. A control measurement was also performed for the mixture of the S protein with all the studied interferents, then the intensity of the current signal was only ca. 3% higher compared to the signal obtained for the sample containing only the S protein.

Biosensors based on nanomaterials are powerful tools widely applied for screening coronaviruses in clinical samples such as: saliva, nasopharyngeal swab specimens, endotracheal sputum aspirates, and blood [32, 33]. The colorimetry, fluorescence, surface plasmon resonance, and electrochemistry are the most popular detectors in nanobiosensors. The electrochemical biosensors for SARS-CoV-2 detection described in the literature, presented in Table 1, detect the RNA of SARS-CoV-2 [34], SARS-VoV-2 antibodies [15, 16, 35, 36], as well as S and N proteins [17, 37, 38]. However, most of them are based on the nonselective interaction of the redox probe (e.g. potassium hexacyanoferrate (II and III)) with a receptor layer, giving rise to a current signal with the intensity dependent on the degree of layer packing. The problem is, that the real samples collected from patients are characterized by a multielement matrix, the ingredients of which can also react with the receptor layer, consequently leading to layer sealing. In such a case, a decrease in current signal will be observed, regardless of the amount of the recognized analyte, generating false-positive results. Our approach helps to avoid these issues. The presented in Table 1 electrochemical protocols for the SARS-CoV-2 virus detection are mainly focus on the detection of the S protein. They are characterized by different ranges of linear response and LOD values. Comparing our proposal with other biosensors based on the detection of the S protein, our concept of virus detection is characterized by one of the lowest limit of detection also for clinical samples. Moreover, the high sensitivity of our device (4 virions·μL⁻¹) enables early diagnosis of COVID-19, without the necessity of sample amplification. Also, the materials used for the construction of the immunosensor showed no toxicity.

3.3. SARS-CoV-2 identification

To verify whether the developed voltammetric immunosensor may be used in the diagnosis of SARS-CoV-2, experiments with clinical samples were performed. A total of 28 clinical samples were used, which included 17 samples from COVID-19-infected patients (8 in non-inactivating buffer and 9 in inactivating buffer) and 11 samples from healthy individuals (5 in non-inactivating buffer and 6 in inactivating buffer), resulting in a test accuracy of 92.8%. The typical DP voltammograms recorded after the interaction of the recognition layer with clinical samples are presented in Fig. 8A. As can be seen from Fig. 4B and Table 2, a current response higher than 0.26 μA indicated that the
A. Kowalczyk et al.

Electrochemical sensors for SARS-CoV-2 detection and their analytical parameters.

**Table 1**

| Receptor layer | Analyte | Technique | Source of current signal | Analytical range | LOD | Ref. |
|----------------|---------|-----------|--------------------------|------------------|-----|------|
| Au/ncovNP-MIP  | ncovNP  | DPV       | Fe(CN)₆⁴⁻ in solution     | 2 – 111 fM       | 15 fM (~0.7 pg mL⁻¹) | [39] |
| ePAD/GO/SP RB/STKI | IgG/IgM | SWV       | Fe(CN)₆⁴⁻ in solution     | 1 – 1000 ng mL⁻¹ | IgG: 0.96 ng mL⁻¹ IgM: 0.14 ng mL⁻¹ 260 nM (20 pg mL⁻¹) | [15] |
| GE/PBase/Ab/BSA | S protein | SWV     | Fe(CN)₆⁴⁻ in solution     | 20 – 80 µg mL⁻¹ |          | [18] |
| Au/dDNA-Fc/Ab   | S protein / SARS-CoV-2 | ChA     | Fc in receptor layer      | 1 – 100 pg mL⁻¹ | –       | [40] |
| SPE/CNF/N protein/Ab | SARS-CoV-2 | SWV       | Fe(CN)₆⁴⁻ in solution     | 0.1 – 1000 pg mL⁻¹ | 0.8 pg mL⁻¹ | [17] |
| SPE/AuNs/MUA/Ab | AuNP    | SWV       | S protein                 | 1 – 10 000 pg mL⁻¹ | 1 pg mL⁻¹ | [41] |
| CP-MNB/CT-MNB/SiMNP-R gene or SiAO-RP-S gene | N-gene | DPV       | MB or AO in receptor layer | 1 – 1×10⁹ copies µL⁻¹ | 1 copy µL⁻¹ | [42] |
| SPE/AuNs/Hs-aptamer/MCH | S protein | EIS     | Fe(CN)₆⁴⁻ in solution     | 0.01 – 100 nM    | 1.30 pM (66 pg mL⁻¹) | [43] |
| SPE/SA/biotin-SARS-CoV-2 RBD/analyte/Ab/ALP | IgG/IgM | EIS     | pAPP in solution          | IgG: 0.0101 – 60 pg mL⁻¹ IgM: 0.0016 – 50 pg mL⁻¹ | IgG: 10.1 ng mL⁻¹ IgM: 1.64 ng mL⁻¹ | [44] |
| GCE/TGO/Ab/BSA  | S protein | Voltammetry | Fe(CN)₆⁴⁻ in solution     | 1 ag mL⁻¹ – 10 fg mL⁻¹ (1.25 – 125 copy µL⁻¹) | 1.25 copy µL⁻¹ | [45] |
| SPE/CAB/S1 protein/DAb/HRP-IgG | S protein | ChA     | TMB in solution           | 0.5 – 10 ng mL⁻¹ | 0.19 ng mL⁻¹ | [46] |
| SPE-GNP/mAb/S1 protein/poly rabbit-anti-S1/GAR/GAR | S protein | DPV       | HQDP in solution          | 0.5 – 5 pg µL⁻¹ | 12 ng µL⁻¹ | [47] |
| SPE/Au/MAA/Ab/BSA/ | S protein | EIS     | Fe(CN)₆⁴⁻ in solution     | 10⁻¹¹ – 10⁻⁷ M    | 3.16 pM (83.7 pg mL⁻¹) | [48] |
| Au/MUA/cpDNA    | ssDNA-SARS-CoV-2 | EIS | Fe(CN)₆⁴⁻ in solution | 1.0×10⁻¹⁸ – 1.0×10⁻⁶ M | 0.5 mM | [49] |
| Au/CSH/Ab1/BSA/analyte/Ab2-Fc | S protein / SARS-CoV-2 | DPV | Fe | 0.2 – 2.0×10⁵ pg mL⁻¹ | 0.08 pg mL⁻¹ (4 virions µL⁻¹) | [this paper] |

Ab: antibody; ALP: alkaline phosphatase; AO: acridine orange; AuNPs: gold nanoparticles; BSA: bovine serum albumin; CAB: capture primary antibody; ChA: chronoamperometry; CNF: carbon nanofiber; cpDNA: capture probe DNA; CP-MNB: capture probe-conjugated magnetic bead particle; CT-MNB: capture target-conjugated magnetic bead particle; DAb: seconder antibody; DPV: differential pulse voltammetry; dDNA-Fc: double stranded DNA conjugated with ferrocene; ePAD: electrochemical paper-based analytical device; EIS: electrochemical impedance spectroscopy; Fe: ferrocene; Fc: ferrocene; FP: filter paper; GAR-AP: goat anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase; GCE: glassy carbon electrode; GE: graphene electrode; GNP: gold nanoparticles; GOS: graphene oxide; HRP: horseradish peroxidase; HQDP: Hydroquinone diphosphate; IgG: antibody; mAb: monoclonal antibody; MAA: mercaptoacetic acid; MB: methylene blue; MCH: 6-mercapto-1-hexan-1-ol; MIP: molecularly imprinted polymer; MUA: 11-mercaptoundecanoid acid; ncovNP: SARS-CoV-2 nucleoprotein; N-gene: viral nucleocapsid phosphoprotein; MAA: mercaptoacetic acid; MUA: 11-mercaptoundecanoid acid; ncovNP: SARS-CoV-2 nucleoprotein; N-gene: viral nucleocapsid phosphoprotein; PAPP: p-aminophenyl phosphoric acid; PBASE: 1-pyrene butylic acid N-hydroxysuccinimide ester linker; RBD: receptor binding domain of S protein; SA: streptavidin; SiAO: silica-acridine orange; SiMB: silica-methylene blue; SKI: skin milk; SPCE: screen-printed carbon electrode; SPE: screen-printed electrode; SP RBd: receptor binding domain of S protein; ssDNA: single stranded DNA; SWV: square wave voltammetry; TMB: 3,3',5,5'-tetramethylbenzidine

Clinical sample contained SARS-CoV-2, whereas a response of 0.26 µA or lower indicated that the sample did not contain the virus. To be sure that the assumed "cut-off value" is correct and allow to discriminate between infected and non-infected patients the ROC curve was constructed (Fig. 8B). The ROC curve is a tool for assessing the correctness of the classifier, it provides a joint description of its sensitivity and specificity [50,51]. The value of the area under the curve (AUC) equalled 0.911 indicates that the applied model correctly classified the samples from the patients as 'positive' or 'negative'. The values of the recorded current signals for samples from non-infected patients ranged from 0.19 to 0.25 µA. In contrast, the intensities of the current signals recorded for samples from infected patients were at least 5 times higher and ranged from 1.15 to 3.4 µA corresponding to the values of the S protein concentration 1.62×10⁻⁵ – 2.77 µg L⁻¹ (data calculated on the basis of the regression equation of the calibration curve presented in the inset in Fig. 5A). The obtained results were in good agreement with those obtained by applying RT-PCR for clinical samples. The specificity and selectivity of the developed biosensor are equalled the 90.9% (10/11 samples for negative results) and 94.1% (16/17 samples for positive results), respectively. The use of our proposition of the SARS-CoV-2 virus detection appears to provide a sensitivity boost relative to other electrochemical detection methods described in the literature [33,38]. The typical PCR responses are presented in Fig. S5 in Supplementary Materials.

4. Conclusions

The COVID-19 pandemic is a health disaster requiring rational approaches to disease management, including an early and accurate diagnosis for rapid introduction of effective therapy in patients suffering from the disease and for isolating the infected individuals. Although various techniques like genetic methods (PCR, LAMP) and lateral flow assays are widely used to detect SARS-CoV-2 in clinical samples, there is an urgent need to overcome the limitations of the existing solutions and develop novel analytical approaches to study viral variants resulting from the mutation of the SARS-CoV-2 genome. The approaches proposed for SARS-CoV-2 detection should be rapid, sensitive, accurate, high throughput and cost-effective. A particular need to use such a tool concerns screening tests performed in medical facilities and in human communities, e.g. airports, concerts. We developed a novel voltammetric immunosensor that targets SARS-CoV-2 by recognizing the S protein. We applied monoclonal antibodies unlabeled and labeled with redox probe to achieve a sensitive voltammetric signal and thus exclude both false-positive and false-negative results. The as-developed sensor is advantageous over traditional electrochemical approaches used so far for viral detection. The main advantage and a huge novelty of the developed device, compared to those already existing, is the moment of generating the analytical signal of the redox probe; the signal appear only after the virus recognition. Moreover, the covalent conjugation of redox probe directly with antibody minimize its non-specific interaction with the receptor layer and thus allows, beyond any doubt, to exclude false positive and negative results of the analysis.
The most significant advantage of the proposed voltammetric immunoassay is its very low LOD, which is close to the PCR analysis, and simple preparation. Taking into account the numerous reports regarding the new mutations occurring in the SARS-CoV-2 genome, producing novel viral variants such as the recently discovered delta and omicron, new molecular targets, including several structural proteins, especially the S protein, are expected to be modified by the upcoming viral variants. The proposed sensor is designed in such a way that it can be easily redefined to target such modified viral structures, if any, by replacing and/or adding some novel and specific recognizing ligands. Considering these facts, we expect a significant shift in the diagnostic landscape by the introduction of the proposed biosensor. Such a tool may be developed into a potentially versatile, simple, and rapid solution for the diagnosis of COVID-19. Moreover, the proposed analytical device is more analytically sensitive and requires only a reduced volume of clinical sample. However, it should be emphasized that the proposed approach should be further analyzed and standardized, including examination of cross reactions with other Coronavirus and LOD determination, in order to facilitate its application in everyday practice. Therefore, more detailed studies are needed with the use of next-generation ligands including self-assembled nanomaterials targeting S, E, M, and N proteins.

CRediT authorship contribution statement

Agata Kowalczyk: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – Original draft preparation, Writing – review & editing, Project administration, Funding Acquisition, Resources. Artur Kasprzak: Investigation, Writing – original draft. Monika Ruzycka-Ayoush: Writing – Original draft preparation, Project administration, Funding Acquisition, Resources. Edyta Podsiady: Investigation, Formal analysis, Writing – original draft. Urszula Demkowski: Writing – original draft, Writing – review & editing. Ireneusz P. Grudzinski: Conceptualization, Writing – original draft, Writing – review & editing. Anna M. Nowicka: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The data that has been used is confidential.

Acknowledgements

This work was funding from the program of the Polish Ministry of Science and Higher Education "Excellence Initiative – Research University (2020-2026)", Action VI.1 "Strengthening and development of cooperation between the University of Warsaw and the Medical University of Warsaw in the federalization process".

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2022.132539.

References

[1] D. Almaghaslah, G. Kandasamy, M. Almanasef, R. Vasudevan, S. Chandramohan, Review on the coronavirus disease (COVID-19) pandemic: its outbreak and current status, Int. J. Clin. Pract. 74 (2020), e13637, https://doi.org/10.1111/ijcp.13637.
Agata Kowalczyk received her Ph.D. degree at Faculty of Chemistry, Warsaw University in 2012. Her research is focused on the application of nanomaterials in (bio)sensors with gravimetric and electrochemical detection. She is also focus on the influence of structural changes of biomolecules during immobilization on their activity.

Artur Kasprzak received his Ph.D. in chemistry at Faculty of Chemistry, Warsaw University of Technology in early 2020. His research interests include the synthesis and applications of molecular receptors dedicated to the recognition of ions. He also focuses on the design of new functional nanomaterials for various applications.

Monika Ruzycya-Ayoush, Ph.D., Medical Laboratory Scientist. Dr. Ruzycya-Ayoush’s scientific goals are related to cell biology, nanotoxicology and carcinogenesis. She specializes in alternative toxicology, especially 3D culture models, signal transduction and cell to extracellular matrix communication.

Edyta Podsiadly, doctor of medical sciences, laboratory diagnostician - specialist of medical microbiology, academic teacher and researcher. Head of the Department of Microbiology at University Center for Laboratory Medicine - Medical University of Warsaw and assistant professor at the Department of Pharmaceutical Microbiology at the Medical University of Warsaw. Author and co-author of numerous scientific publications in the field of bacteriology, epidemiology and infectious serology. Technical editor of "Advancements of Microbiology", deputy chairman of the Warsaw branch of the Polish Society of Microbiologists.

Urszula Demkow, MD, Ph.D, Professor of Medical and Health Sciences, head of Dept. of Laboratory Diagnostics and Clinical Immunology, Medical University of Warsaw. Principal investigator of over 20 national and international projects in the field of immunology and infectious diseases. A Currently leading a team of scientists working on molecular, genetic and immune mechanism of leukemia and other malignancies, metabolic disorders, autoimmunity, neurometabolic diseases in children, infectious diseases, neutrophil extracellular traps, biomarkers, oxidative stress. Author of over 200 peer reviewed articles published in major journals and over 50 book chapters, mainly in the field of basic immunology and diseases related to the immune system as well as translational, and clinical studies in all aspects of immunology and genetics.

Ireneusz P. Grudzinski, Ph.D, D.Sc., Professor of Medical and Health Sciences. Over 30 years of experiences in pharmacology and toxicology. Professor Grudzinski’s research goals are focused to study novel diagnostics and therapeutic approaches in medical oncology and rare diseases applying different strategies based on nanotechnology and nanosciences.

Anna M. Nowicka professor since 2021. Her research is focused on nanomaterial-based assemblies on electrodes that would effectively bind some biological materials with the electrode surface and allow the rapid transformation of chemical signals into electrical signal. She also focuses on modern nano-electrochemistry of DNA and immuno-electrosensors used in POC diagnostics and modern nanomedicine.