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An electrochemical biosensor for SARS-CoV-2 detection via its papain-like cysteine protease and the protease inhibitor screening

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\textbf{A R T I C L E   I N F O}

Keywords:
Electrochemical biosensor
SARS-CoV-2 papain-like cysteine protease
Peptide-DNA nanoprobe
Protease inhibitor

\textbf{A B S T R A C T}

The persistent coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is still infecting hundreds of thousands of people every day. Enriching the kits for SARS-CoV-2 detection and developing the drugs for patient treatments are still urgently needed for combating the spreading virus, especially after the emergence of various mutants. Herein, an electrochemical biosensor has been fabricated in this work for the detection of SARS-CoV-2 via its papain-like cysteine protease (PLpro) and the screening of protease inhibitor against SARS-CoV-2 by using our designed chimeric peptide-DNA (pDNA) nanoprobes. Utilizing this biosensor, the sensitive and specific detection of SARS-CoV-2 PLpro can be conducted in complex real environments including blood and saliva. Five positive and five negative patient throat swab samples have also been tested to verify the practical application capability of the biosensor. Moreover, we have obtained a detection limit of 27.18 fM and a linear detection range from 1 pg mL\textsuperscript{-1} to 10 \mu g mL\textsuperscript{-1} (I = 1.63 + 4.44 lnC). Meanwhile, rapid inhibitor screening against SARS-CoV-2 PLpro can be also obtained. Therefore, this electrochemical biosensor has the great potential for COVID-19 combating and drug development.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread rapidly since its discovery in December 2019, and has had a serious impact on the lives and health of people worldwide. According to the Corona Virus Disease 2019 (COVID-19) weekly epidemiology updated by the World Health Organization on January 9, 2022, as of the update time, the cumulative number of confirmed cases of COVID-19 worldwide has exceeded 304 million, and the cumulative number of deaths is approximately 5.4 million. At the same time, the global epidemic has been further deteriorated due to the emergence of mutated viruses of SARS-CoV-2, especially the Omicron (B.1.1.529) and Delta (B.1.617.2) strains with greater ability to spread [1–3]. Considering that SARS-CoV-2 is highly contagious, rapid virus detection and early diagnosis of infection are highly needed for curbing the spread of the virus [4]. In addition, drug and vaccine development are also the key to control the spread of the epidemic, thus screening of the inhibitor for the virus is crucial [5].

At present, various approaches for SARS-CoV-2 diagnosis have been proposed, which are mainly focused on the detection of SARS-CoV-2 nucleic acid or antibodies [6–10]. As the gold standard for nucleic acid detection, real-time quantitative polymerase chain reaction (RT-qPCR) has extremely high sensitivity and specificity, but it has inevitable limitations such as high equipment requirement, time-consumption, and professional technician requirement [11,12]. These limitations make it difficult to popularize in developing countries with limited resources, resulting in the huge obstacle for epidemic prevention and control in these countries. For antibodies detection in SARS-CoV-2 infected patients, they can only be detected after a long time after the human body is infected with the virus, which greatly affects the timeliness of virus detection and increases the risk of aggravation of the patient’s condition [13]. Therefore, exploring alternative biomarkers for

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https://doi.org/10.1016/jcej.2022.139646

Received 13 April 2022; Received in revised form 21 September 2022; Accepted 4 October 2022
Available online 10 October 2022
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SARS-CoV-2 diagnosis and developing matched detection methods are still highly needed.

In fact, in a very short period of time, many detection methods have been developed, including field-effect transistor method [14], electrochemical method [15-17], molecular imprinting method [18], which can reach a good detection limit [19-21]. At the same time, various potential biomarkers for SARS-CoV-2 detection have been verified by the emerging evidences, including spike proteins [22-28], nucleocapsid proteins [22,29,30], main proteases [31,32], and papain-like proteases (PLpro) [33]. Among them, SARS-CoV-2 PLpro as a cysteine protease can promote virus proliferation by processing viral polyproteins to mature proteins [34]. Moreover, SARS-CoV-2 PLpro can affect and attenuate the cellular antiviral interferon pathway by cleaving ubiquitin-interferon stimulated protein 15 (ISG15) from interferon response factor 3 (IRF3), which is one of the important means of virus immune escape [34,35]. As a relatively conserved domain, SARS-CoV-2 PLpro has only 4 mutation sites in the Omicron mutant, while there are at least 21 mutation sites in the S1 gene [36]. This suggests that SARS-CoV-2 PLpro may be suitable as a general biomarker for the detection of SARS-CoV-2 and its mutants. It is also worth mentioning that SARS-CoV-2 PLpro is a target for drug screening, and a lot of work has been conducted by targeting SARS-CoV-2 PLpro for inhibitor screening [37-39]. Therefore, detection of this protease may also contribute to the development of antiviral drugs for SARS-CoV-2.

Over the past period, some studies have also been conducted on SARS-CoV-2 detection methods in our laboratory, through nucleic acids [9,10,40] and spike proteins [26]. Based on these, we would like to avoid the complicated steps of nucleic acid extraction and improve the practical value of the detection method. So, in this work, an electrochemical biosensor has been fabricated not only for SARS-CoV-2 detection but also for antiviral inhibitor screening by using PLpro as a biomarker. Briefly, the biosensor is designed by innovatively using the specific enzymatic activity of this viral functional protein to detect SARS-CoV-2. Specifically, chimeric peptide-DNA (pDNA) nanoprobes are designed to be recognized and cleaved by the target protease. Since the released DNA strands are also designed to form double-stranded DNA (dsDNA) with the DNA strands previously immobilized on the electrode surface, electroactive molecules methylene blue (MB) can be embedded in the dsDNA, which can accelerate electron transfer and produce signal output [41-44]. This electrochemical biosensor can be further used to screen the inhibitor of the target protease, and the results show that YM155 has a significant inhibitory effect on SARS-CoV-2 PLpro activity. Therefore, this study may provide some ideas and inspirations for the detection of diseases and the development of antiviral drugs.

2. Experimental section

2.1. Materials and reagents

N-Hydroxysulfosuccinimide sodium salt (Sulfo-NHS, 98 %, Store at 4 °C) was purchased from Shanghai Yuanye Biological Technology Co., Ltd. 3-(Dimethylaminopropyl)-1-Ethylcarbodiimide hydrochloride (EDC-HCl, 97 %, crystalline, Store at 4 °C) was purchased from Nanjing Jingge Chemical Technology Co., Ltd. Dithiothreitol (DTT) was purchased from Solarbio, 6-mercaptop-1-hexanol (MCH), Potassium ferricyanide (K₃[Fe(CN)₄]) and Potassium hexacyanoferrate(II) (K₄[Fe(CN)₆]) were purchased from Sigma. 2-(N-Morpholino)ethanesulfonic acid (MES) was purchased from Aladdin. The peptide (GGLRGG-acp-C) used in this experiment were synthesized by Hefei National Peptide Biotechnology Co., Ltd. SARS-CoV-2 PLpro was purchased from Sino Biological Company, with a purity of greater than 90 % and good biological activity. Recombinant SARS-CoV-2 PLpro/papain-like protease (His Tag) consists of 324 amino acids and predicts a molecular mass of 36.79 kDa. Sequence of DNA1: 5′-HOOC-TGTGCTATTGGCTATGGCTT-3′; Sequence of DNA2: 5′-HOOC-TGTGCTATTGGCTATGGCTT-3′. The synthesis and modification of DNA was completed at Sangon Bioengineering Co., Ltd (Shanghai, China). Sepantronium Bromide (YM155) was purchased from Shanghai Beyotime Biotechnology Co., Ltd (China, Store at −20 °C). All proteins, peptides, and DNA were stored at −20 °C and avoided repeated freezing and thawing.

2.2. Synthesis of 3.5 nm, 17 nm, and 40 nm AuNPs

The synthesis of 17 nm gold nanoparticles was based on a previous study using the sodium citrate reduction method [45,46]. 40 mL of 0.5 mM HAuCl₄ aqueous solution was boiled under stirring for 10 min, and then 4 mL of 19.4 mM sodium citrate solution was quickly added to the boiling HAuCl₄ solution and stirred. The mixture was boiled and stirred for 30 min, until the solution gradually turned dark red. Then, the synthesized AuNPs were cooled to room temperature and stored at 4 °C in the dark. Before using AuNPs, Tween 20 was added at a final concentration of 0.5 %, centrifuged at 12000 rpm min⁻¹ for 6 min, washed with phosphate buffered saline (PBS) three times, reconstituted with PBS and stored at 4 °C.

The synthesis of 3.5 nm AuNPs is based on previous studies [46,47]. 0.25 mL of 25 mM HAuCl₄ was added to a mixed solution containing 37.5 mL of 2.2 mM sodium citrate (0.0243 g) and 0.025 mL of 2.5 mM tannic acid (0.0002 g), which were mixed and stirred at 70 °C for 30 min.

The synthesis of 40 nm AuNPs also refers to previous studies [48]. 1.7 mL of 17 nm AuNPs seeds (synthesized above) were diluted to 20 mL with deionized water in a 50 mL Erlenmeyer flask with magnetic stirring at room temperature. 10 mL of precursor aqueous solution containing 370 μL (1 %, w/v) of HAuCl₄ and 10 mL of reducing agent aqueous solution containing 0.6 mL (1 %, w/v, 0.006 g) of ascorbic acid (AA) aqueous solution plus 0.3 mL (1 %, w/v, 0.003 g) aqueous sodium citrate solution were prepared. Then, the two solutions were injected into the Erlenmeyer flask with a syringe pump at a rate of 12 mL h⁻¹, and boiled for 30 min after the injection was completed. The synthesized AuNPs were stored in a refrigerator at 4 °C in the dark.

2.3. Synthesis of AuNP-peptide-DNA combined probe

The DNA modified with carboxyl groups was linked to the peptide containing amino groups through a displacement reaction mediated by EDC-HCl/sulfo-NHS. In detail, DNA containing carboxyl group was dissolved in MES buffer (0.05 M MES, 0.5 M NaCl, pH 6), and then 2 mM EDC-HCl and 5 mM sulfo-NHS were added to the reaction solution. The mixture was allowed to react at room temperature for 15 min. Subsequently, amino-containing peptide dissolved in 0.1 M sodium dihydrogen phosphate (pH 7.5) was added to reaction solution, and continued to react at room temperature for 2 h (the pH of the coupling medium was higher than 7.0, which can trigger the active ester reaction) [49]. Finally, the AuNP-peptide-DNA nanoprobe was prepared by incubating the conjugation product of peptide-DNA and AuNPs (17 nm, containing 0.5 % Tween 20) for 2 h at 37 °C. And then the prepared AuNP-peptide-DNA nanoprobe was purified by centrifugation at 12000 rpm min⁻¹ for 6 min, and washed with PBS for three times to remove excess peptide-DNA.

2.4. Electrode treatment

The gold electrode was treated according to the method of our previous works [50]. First, the gold electrode was placed in piranha solution (sulfuric acid: H₂O₂ = 7:3) and soaked for 5 min, then rinsed with ultrapure water. After being polished by alumina slurry (1 µm, 0.3 µm, and 0.05 µm), the gold electrode was ultrasonically cleaned with alcohol and ultrapure water for 5 min. Then, the gold electrode was soaked in 50 % nitric acid solution for 30 min and performed electrochemical cleaning by scanning the redox and reduction potentials (−0.35 V and 0.20 V) for 6 min at 100 mV s⁻¹, and then soaked in ultrapure water for 2 min. Finally, the electrode was dried in a flow of ultrapure nitrogen.
1.5 V) in 0.5 M H$_2$SO$_4$. After rinsing with ultrapure water, the gold electrode was dried with nitrogen. To immobilize DNA on gold electrode, 10 μL of 0.5 μM DNA2 was dropped on the surface of gold electrode and incubated at 37 °C for 2 h. The modified gold electrode was rinsed with ultrapure water and dried with nitrogen. Finally, 10 μL of 1 mM MCH was added dropwise to the surface of gold electrode and incubated at room temperature for 15 min to seal the surface of gold electrode. After rinsing with ultrapure water, the gold electrode was dried with nitrogen and stored at 4 °C for later use.

2.5. Electrochemical measurements

The electrochemical measurement was performed using the three-electrode system of a CHI660D electrochemical workstation. The electrode modified with DNA was used as the working electrode, a saturated calomel electrode was used as the reference electrode and a platinum wire electrode was used as the counter electrode. Electrochemical impedance spectroscopy (EIS) was measured in 5 mM [Fe(CN)$_6$]$^{3-/4-}$ (K$_3$[Fe(CN)$_6$] and K$_4$[Fe(CN)$_6$]) including 0.1 M KCl, and the corresponding parameters were as follows: bias potential, 0.175 V; frequency range, 0.1–100000 Hz; amplitude, 0.005 V. Square wave voltammograms (SWVs) were scanned in 20 mM Tris-HCl (0.5 M NaCl, pH 7.4), and the parameters were set as follows: step potential, 4 mV; frequency, 15 Hz; amplitude, 25 mV.

2.6. SARS-CoV-2 PLpro detection using the fabricated biosensor

SARS-CoV-2 PLpro and AuNP-peptide-DNA nanoprobe were incubated at 37 °C in assay buffer [50 mM HEPES (pH 7.5), 0.1 mg mL$^{-1}$ Bovine albumin (BSA), 5 mM DTT]. In the control group, PLpro was replaced with 20 mM Tris-HCl and 500 mM NaCl (pH 7.5). The AuNP-peptide-DNA nanoprobe was added to the assay buffer containing SARS-CoV-2 PLpro at 37°C for target recognition and cleavage. After cleavage reaction, the solution was centrifuged at 12000 rpm min$^{-1}$ for 6 min. The supernatant solution was collected and added dropwise to the surface of the gold electrode to perform DNA hybridization at room temperature, and the gold electrode was washed with ultrapure water and dried with nitrogen. Finally, 10 μL of 30 μg mL$^{-1}$ MB was added dropwise to the surface of the electrode and incubated at room temperature.

2.7. Specificity and stability of this biosensor

Target specificity verification included the following proteins: 10 ng mL$^{-1}$ BSA, 10 ng mL$^{-1}$ egg albumin, 10 ng mL$^{-1}$ hemoglobin, 10 ng mL$^{-1}$ papain, and 10 ng mL$^{-1}$ SARS-CoV-2 PLpro. The prepared AuNP-peptide-DNA nanoprobe was stored in a refrigerator at 4 °C for 30 days (30 d) protected from light to verify the stability of the biosensor. At 15 d and 30 d, AuNP-peptide-DNA nanoprobes were taken out to detect 1 ng mL$^{-1}$ and 10 ng mL$^{-1}$ of SARS-CoV-2 PLpro target protease.

2.8. Target detection in complex environments and clinical samples

The performance of this biosensor in a complex environment was verified using 10 % saliva and 10 % whole blood samples, respectively. In the measurement of actual samples, 1 μL of throat swab stock solution was added to 10 μL reaction buffer containing combined nanoprobe, and the other steps were the same as above. Human whole blood samples and SARS-CoV-2 throat swab clinical specimens were provided by Nanjing Second Hospital (Nanjing, China). Testing of clinical samples was done in the hospital’s biosafety laboratory. The research was ratified by the scientific ethical committee of the Second Hospital of Nanjing and Nanjing University (the project number is 2022-LY-k003), and informed consent was procured in all cases.
2.9. Validation of the PLpro inhibitor YM155

Different concentrations (0.1 μmol/L, 1 μmol/L, 10 μmol/L, 100 μmol/L) of YM155 was added to assay buffer with 10 μg mL⁻¹ PLpro at 37 °C for 30 min. SWV signal was measured after the target protease recognition reaction. The experiment details were the same as above.

3. Results and discussion

3.1. Principle of this electrochemical biosensor

The design principle of this electrochemical biosensor is shown in Scheme 1. First, the substrate peptide (GDGLRGG-acp-C) [51] of PLpro is connected with carboxyl-modified DNA1 through EDC/sulfo-NHS cross-linking chemistry [49,52] to form the chimeric peptide-DNA (pDNA) nanoparticles. Then, the nanoprobes are conjugated on gold nanoparticles (AuNPs) via Au-S bonds to prepare the combined nanoprobes (AuNP-peptide-DNA). To improve the cleavage efficiency, the distance from the cutting site to the surface of AuNPs is increased through inserting 6-aminocaproic acid into the peptide substrate. In the presence of the target protease, the peptide substrate will be cut off, resulting in the release of DNA1 fragment from the surface of AuNPs. Subsequently, the released DNA1 can hybridize with DNA2 previously immobilized on the surface of working electrode. Consequently, signal molecules MB can be inserted into the formed double-stranded DNA (dsDNA) to produce the significant electrochemical signals for the rapid and sensitive detection of SARS-CoV-2 PLpro.

3.2. Characterization of materials

The morphology and charge distribution of the prepared nanoprobes have been characterized by transmission electron microscopy (TEM) and zeta potential. The synthesized and modified AuNPs have good dispersibility and uniform size (Fig. S1 and S2). By measuring the AuNPs in the TEM images using the software ImageJ, the average sizes of the synthesized AuNPs, AuNP-peptide, and AuNP-peptide-DNA are about 17.3 nm, 17.5 nm, and 18.8 nm, respectively (Fig. S2E). The successful modification of peptide and pDNA on the surface of AuNPs are verified by measuring Ultraviolet–visible spectroscopy (UV–vis) (Fig. S2F), dynamic light scattering (Fig. S3), and zeta potential (Fig. S4). It can be seen from Fig. S2F that when the peptide and pDNA are modified on the surface of AuNPs, the absorption peaks of AuNPs red-shifted from 521 nm to 530 nm and 528 nm. This result shows that the diameters of the modified AuNPs are significantly increased. Likewise, the dynamic light scattering results show that the sizes with the highest proportion of AuNP, AuNP-peptide, and AuNP-peptide-DNA distributions are about 17 nm, 24 nm, and 21 nm, respectively. This result has also shown that the peptide and peptide-DNA modification increases the size of the AuNPs. Additionally, the surface of AuNPs carries a large amount of citrate ions, resulting in a large number of negative charges on the surface of AuNPs. When a large number of electrically neutral peptides are modified on the surface of AuNPs, the negative charge on the surface decreases. Nevertheless, when the pDNA is conjugated to the surface of the AuNPs, more negative charges are measured compared to AuNP-peptide conjugations. The chemical cross-linking between peptide and DNA has also been verified by 12 % polycrylamide gel electrophoresis (PAGE) experiment (Fig. S5). As shown in the PAGE, a significantly larger band can be observed in pDNA lane, which can be attributed to the cross-linked products of peptide and DNA. Therefore, these results clearly suggest that the combined nanoprobes are prepared successfully.

3.3. Feasibility test of the fabricated electrochemical biosensor

The feasibility of this electrochemical biosensor has been tested by the following experiments. First, the ability of single-stranded DNA (ssDNA) and dsDNA on the surface of gold electrode to load MB molecules have been investigated by using square wave voltammetry (SWV). The electrical signal of dsDNA is much higher than that of ssDNA, indicating that the binding ability of MB molecules to dsDNA is indeed stronger than that of ssDNA (Fig. S6). Then, the feasibility of the electrochemical biosensor for target detection is verified by electrochemical impedance spectroscopy (EIS) and SWV (Fig. 1). As shown in Fig. 1A, the large impedance can be detected after the pDNA nanoprobe conjugated AuNP (AuNP-peptide-DNA) is directly attached to the electrode surface. On the contrary, when the AuNP-peptide-DNA is recognized and cut by the target, only small impedance is generated, indicating that the cleaved AuNP-peptide-DNA cannot be modified on the electrode surface. Finally, the SWV signal is measured to verify the feasibility of the electrochemical biosensor. It can be observed that a strong SWV signal can be obtained in the presence of SARS-CoV-2 PLpro, while the SWV signals of the bare electrode and the target-free electrode are lower (Fig. 1B). So, these results can effectively prove the feasibility of the electrochemical biosensor.

3.4. Analytical performance of the electrochemical biosensor

To obtain better analysis capabilities, experiment conditions of the electrochemical biosensor have been optimized. First, AuNPs with different sizes have been selected for optimization. In addition to 17 nm AuNPs, 3.5 nm and 40 nm AuNPs have also been synthesized and
characterized using TEM (Fig. S7). Next, the measurements of UV–vis absorption spectra of AuNPs (3.5 nm and 40 nm) have been measured. The results show that the UV–vis absorption peaks of 3.5 nm and 40 nm AuNPs are at 510 nm and 526 nm, respectively (Fig. S8A). Then, the average diameter (Fig. S8B) and size distribution (Fig. S8C and S8D) of 3.5 nm and 40 nm AuNPs have been obtained by measuring TEM images using ImageJ software. The actual average size of AuNPs synthesized by 3.5 nm and 40 nm synthesis methods are approximately 4 nm and 49 nm, respectively. Finally, the separation ability of AuNPs of three sizes is tested by centrifugation (12000 rpm min$^{-1}$, 6 min). It is found that only 17 nm AuNPs can be effectively centrifuged to the bottom of the tube. The 3.5 nm AuNPs cannot be centrifuged to the bottom of the tube, and

![Fig. 2.](image)

Quantitative analysis of the target protein SARS-CoV-2 PLpro. (A) The SWV curves with different concentrations of the target protein (1 pg mL$^{-1}$–10 μg mL$^{-1}$). (B) The statistical data of the peak values of the SWV curves measured with different concentrations of the target protein. The inserted graph is the linear relationship obtained by fitting the logarithm of the concentration as the abscissa. The error bars indicate means ± SD (n = 3).

![Fig. 3.](image)

Specificity verification of the electrochemical biosensor. (A) Measurement of the SWV signals with different interfering proteins, including papain, albumin, hemoglobin, and BSA. (B) The statistical data of the peak values of the SWV signals. (C) SWV signal curves of electrochemical biosensors for long-term storage stability. SARS-CoV-2 PLpro at concentrations of 1 ng mL$^{-1}$ and 10 ng mL$^{-1}$ have been tested for stability after being placed in a 4 °C refrigerator for 15 d and 30 d. (D) Histogram statistics of long-term storage stability of electrochemical biosensors. Error bars indicate means ± SD (n = 3).
40 nm AuNPs are easy to aggregate on the wall of the centrifuge tube after centrifugation (Fig. S9). Finally, AuNP-peptide-DNA nanoprobes using three sizes of AuNPs are synthesized for electrochemical measurement (Fig. S10). The results showed that after cleavage of 10 ng mL\(^{-1}\) PLpro, the 17 nm AuNPs produced the highest signal, while the 3.5 nm AuNPs produced the lowest signal due to the difficulty of centrifugation. And the signal generated by 40 nm AuNPs is significantly lower than that of 17 nm AuNPs due to the aggregation. Therefore, 17 nm AuNPs has been selected for this experiment.

In addition, the DNA hybridization time on the electrode surface (Fig. S11), the enzyme cutting time (Fig. S12), and the incubation time of the MB molecules (Fig. S13) have also been optimized. According to the experimental results, the optimal experimental conditions are as follows, 10 min for the DNA hybridization on the electrode surface, 15 min for enzyme cutting, and 2 min for MB incubation.

SWV signals of different concentrations of targets are then measured under the optimal experimental conditions. As shown in Fig. 2A, with the target concentrations ranging from 1 pg mL\(^{-1}\) to 10 μg mL\(^{-1}\), the electrochemical signal of the biosensor gradually increases. The limit of detection (LOD) of the target is 1 pg mL\(^{-1}\) (27.18 fM), and the peak current of the electrochemical biosensor has a linear relationship with the logarithm of the target concentrations (Fig. 2B, inset). The final linear regression curve is I (μA) = 1.63 + 4.44 lgC (pg mL\(^{-1}\)) (\(R^2 = 0.9157\)). The lowest detection concentration is lower than the LOD of the experimental conditions as follows, 10 min for the DNA hybridization on the electrode surface, 15 min for enzyme cutting, and 2 min for MB incubation.

SWV signals of different concentrations of targets are then measured under the optimal experimental conditions. As shown in Fig. 2A, with the target concentrations ranging from 1 pg mL\(^{-1}\) to 10 μg mL\(^{-1}\), the electrochemical signal of the biosensor gradually increases. The limit of detection (LOD) of the target is 1 pg mL\(^{-1}\) (27.18 fM), and the peak current of the electrochemical biosensor has a linear relationship with the logarithm of the target concentrations (Fig. 2B, inset). The final linear regression curve is I (μA) = 1.63 + 4.44 lgC (pg mL\(^{-1}\)) (\(R^2 = 0.9157\)). The lowest detection concentration is lower than the LOD of...
many previous studies \cite{6,53}. In addition, some recently published articles on SARS-CoV-2 protein detection methods have been compiled (Table S1). According to statistical data, it can be seen that this electrochemical biosensor has certain advantages in detection sensitivity.

3.5. Specificity and stability of this electrochemical biosensor

The specificity of the electrochemical biosensor has been tested for SARS-CoV-2 PLpro through using a variety of interfering proteins, including papain, albumin, hemoglobin, and BSA with concentration of 10 ng mL\(^{-1}\). As shown in Fig. 3A and 3B, the target protein PLpro and papain have higher SWV signals, while albumin, hemoglobin, and BSA have weaker signals. The target detected in this study is a papain-like protease with similar enzymatic activity as papain. Therefore, papain, as a proteolytic enzyme in papaya, can also hydrolyze the substrate peptide. This also reminds us that in the application of this biosensor, the contamination of plant-derived proteases must be avoided to decrease false positive results.

To further verify the long-term storage stability of the electrochemical biosensor, the prepared AuNP-peptide-DNA nanoprobes have been stored in a 4 °C refrigerator protected from light for 30 days (30 d). As shown in the Fig. 3C and 3D, the electrochemical biosensor still has good detection results and stability for the detection of SARS-CoV-2 PLpro (1 ng mL\(^{-1}\) and 10 ng mL\(^{-1}\)) after being placed in a 4 °C refrigerator protected from light for 15 d and 30 d. These results demonstrate the long-term preservation capability of this electrochemical biosensor, and further demonstrates its potential for practical application.

3.6. Performance of biosensors in real media

To verify the feasibility of the electrochemical biosensor in a complex real environment, external calibration method is used for the real sample analysis. Different concentrations of SARS-CoV-2 PLpro are added into 10 % saliva and 10 % whole blood, and then SWV signals are measured (Fig. 4A, 4B and 4C). It can be seen from the results that the SWV values measured by the electrochemical biosensor under different complex real environments have relatively small differences, which shows that the electrochemical biosensor can be used in a complex environment. At the same time, the recovery rate of the target protein is calculated in complex samples (Table S2). The data demonstrate that the high recovery rate for the detection of SARS-CoV-2 PLpro with low concentrations can be obtained. However, the low recovery rate for the detection of SARS-CoV-2 PLpro at high concentrations is calculated. We speculate that the reason for the results may be due to the inadequate sensitivity of this sensor for SARS-CoV-2 PLpro at high concentrations. In detail, due to the limited probe number of AuNPs surface, when the target concentration is high, the cleavage efficiency of the target may be reduced, and each target protease molecule cannot fully perform the cleavage function. Nevertheless, these results can still show that this electrochemical biosensor can be used in a complex real sample environment.

3.7. Clinical samples analysis

Clinical samples are used to verify the application potential of the biosensor. Throat swab solutions of 5 healthy samples (HS) and 5 SARS-CoV-2 patient samples (PS) are used for test. SARS-CoV-2 virus particles are released into body fluids by lysing cells after massive replication in cells, and SARS-CoV-2 PLpro protein is also released into body fluids (Fig. 4D). From the results, it can be known that the SWV signals generated by PS measured using this biosensor are significantly greater than that of HS (Fig. 4E and 4F). The average value of SWV peak for patient samples and healthy samples are 1.19 × 10\(^{-5}\) A and 5.95 × 10\(^{-6}\) A, respectively (Fig. 4E). By using the data analysis software GraphPad Prism 9, a threshold value line of 7.25 × 10\(^{-6}\) A has been calculated to fully distinguish patient samples from healthy samples. Among them,
the lowest electrical signal for the #4 PS sample is still obviously higher than those for all HS samples. So, these results demonstrate the feasibility and application of this biosensor in clinical samples.

4. Conclusion

In summary, an electrochemical biosensor has been fabricated in this work for the sensitive detection and inhibitor screening of SARS-CoV-2 via the recognition of the pLpro, which is expected to become a new biomarker for COVID-19. After optimizing the experimental conditions, the lowest concentration of target protein detected by the electrochemical biosensor can be 1 pg mL⁻¹ (27.18 M). The specificity of the electrochemical biosensor has also been verified in saliva, whole blood, and clinical samples. Moreover, this biosensor can be used for the screening and validation of SARS-CoV-2 inhibitors by exploring the treatment of SARS-CoV-2 pLpro with different concentrations of YM155 inhibitors. So, the electrochemical biosensor proposed in this research has great potential in practical applications in SARS-CoV-2 detection and inhibitor screening. It is hoped that this study can provide some inspiration and reference for SARS-CoV-2 detection and antiviral drug screening.

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 presented.

Data availability

The authors do not have permission to share data.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant Nos. 81772593, 31901771) and the Fundamental Research Funds for the Central Universities (Grant No. 202014380176).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/jcej.2022.139646.
