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Ultrasensitive and amplification-free detection of SARS-CoV-2 RNA using an electrochemical biosensor powered by CRISPR/Cas13a

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ABSTRACT

This study proposed a CRISPR/Cas13a-powered electrochemical multiplexed biosensor for detecting SARS-CoV-2 RNA strands. Current SARS-CoV-2 diagnostic methods, such as reverse transcription PCR (RT-PCR), are primarily based on nucleic acid amplification (NAA) and reverse transcription (RT) processes, which have been linked to significant issues such as cross-contamination and long turnaround times. Using a CRISPR/Cas13a system integrated onto an electrochemical biosensor, we present a multiplexed and NAA-free strategy for detecting SARS-CoV-2 RNA fragments. SARS-CoV-2 S and Orf1ab genes were detected in both synthetic and clinical samples. The CRISPR/Cas13a-powered biosensor achieved low detection limits of 2.5 and 4.5 ag/μL for the S and Orf1ab genes, respectively, successfully meeting the sensitivity requirement. Furthermore, the biosensor's specificity, simplicity, and universality may position it as a potential rival to RT-PCR.

1. Introduction

The Coronavirus disease 2019 (COVID-19) outbreak has swiftly expanded over the world and has become a worldwide health emergency due to the high rate of transmission. Early diagnosis and prompt medical intervention for those who are at higher risk can mitigate serious complications from COVID-19. An ongoing theme of the COVID-19 pandemic necessitates the availability of accurate, rapid, and cost-effective detection methods in infected individuals. However, particular biomarkers, mostly nucleic acids and proteins, are required for reliable detection of the COVID-19 infection [1]. As a result, current diagnostics generally depend on polymerase chain reaction (PCR) and antibody-based technologies [2,3]. Although these approaches are precise, sensitive, and specific, they require costly equipment and reagents, centralized diagnostic services, and highly skilled staff, all of which pose obstacles, particularly in underserved areas [4]. Therefore, there is a high demand for innovative approaches to tackle the challenges associated with conventional detection strategies and develop straightforward, time-efficient, and cost-effective procedures for detecting COVID-19 biomarkers.

To date, several techniques for detecting Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA sequences have been

developed [5–9]. The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas effector is a new technology that has expanded the possibilities for RNA detection [10]. CRISPR/Cas-based systems employ a variety of CRISPR-associated effectors to recognize specific nucleic acid sequences [11]. CRISPR/Cas9, for example, was utilized for targeting double-stranded DNA [12–14]. CRISPR/Cas12a and CRISPR/Cas13a target single-stranded DNA (ssDNA) [15–17] and single-stranded RNA (ssRNA) [18–21], respectively. The potent collateral activity of Cas12a and Cas13a empowered CRISPR/Cas systems as an ideal tool for next-generation biosensing platforms [15,17,20,21]. CRISPR/Cas13a is the only CRISPR/Cas effector, which targets ssRNA sequences and possesses a unique RNase activity [22]. Concisely, crRNA-guided Cas13a can specifically bind to and degrade the target RNA based on complementarity between crRNA and target RNA. Following that, target recognition activates Cas13a for general nonspecific cleavage of RNA with high efficiency, thereby enabling signal amplified detection.

Zhang et al. described a Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) approach for detecting SARS-CoV-2 RNA sequences based on CRISPR-Cas13a [23,24]. Using this method, SARS-CoV-2 RNA sequences were detected at concentrations ranging from 10 to 100 copies per microliter. Another study developed a CRISPR-Cas12-based lateral flow assay to detect SARS-CoV-2 from respiratory

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swab RNA extracts [8]. This assay employs loop-mediated amplification (RT-LAMP) to perform simultaneous reverse transcription and isothermal amplification of RNA collected from clinical samples, followed by Cas12 detection of specific SARS-CoV-2 sequences, and finally, cleavage of a reporter molecule confirms detection of the virus.

The development of a mobile phone-based CRISPR-Cas13a assay was reported for direct detection of SARS-CoV-2 from nasal swab RNA by Fozouni et. al. The assay achieved sensitivity of 100 copies per milliliter as well as detected pre-extracted RNA from a set of positive clinical samples in a short period of time [25].

Although these methods are sensitive and capable of detecting COVID-19 quickly, most of them require several tedious preparation steps such as nucleic acid amplification (NAA) and/or reverse transcription (RT) processes, as well as primer design. As a result, developing universal detection methods for SARS-CoV-2 RNA that not only achieve PCR-like sensitivity but also avoid NAA-related issues is crucial.

Electrochemical biosensing platforms have piqued the interest of scientists in diagnostics [9,26–34], due to their rapid response, high sensitivity and specificity, cost-effectiveness, simplicity, and miniaturization capability. However, accuracy has always been one of the critical challenges in such biosensing platforms. The advent of CRISPR/Cas technology and its marriage with electrochemical biosensing devices can successfully tackle this obstacle, propelling electrochemical biosensing toward highly accurate as well as sensitive detection as low as attomolar levels [9,18,35,36].

In this study, a NAA-free CRISPR/Cas13a-based multiplexed electrochemical biosensor (E-CRISPR) was reported for detecting SARS-CoV-2 RNA sequences (i.e., S and Orf1ab genes) collected from clinical samples. The high sensitivity of the biosensor rises from the enzyme's collateral activity as well as the intrinsic sensitivity of the electrochemical technique, whereas the specificity results from a target-specific CRISPR RNA (crRNA) in the CRISPR/Cas13a complex, which directs Cas13a to the targeted RNA sequence [22].

To construct the E-CRISPR, a nonspecific thiolated reporter RNA (reRNA) whose other terminus was labelled with methylene blue (MB), or ferrocene (Fc) was immobilized on a gold-nanostructured electrode via sulfur–gold chemistry, then backfilled with 6-mercapto-1-hexanol (MCH) to prevent nonspecific adsorption. Following that, the sensing surface was exposed to the Cas13a-crRNA-target RNA assembly. The Cas13a cleavage capability is triggered in the presence of the target RNA, resulting in the cleavage of the redox probe labeled-reRNA and a decrease in the electrochemical signal. The robust turnover nature of Cas13a allows thousands of reporter cleavage per single target RNA binding, leading to signal amplification, and eventually quantitative readout of SARS-CoV-2 RNA.[19,22] In the absence of the target sequence, the Cas13a cleavage activity is inhibited, and the redox probe labeled-reRNA remains intact, retaining the electrochemical signal. Even across highly related RNA sequences with a single nucleotide substitution, the E-CRISPR can detect SARS-CoV-2 genes with excellent specificity and sensitivity 2.5 and 4.5 ag/ μ L (26.2 and 53.5 copies/ μ L for the S and Orf1ab genes, respectively) within an hour. Furthermore, the assay was tested on clinical samples and found to be in good agreement with qRT-PCR results. We believe that this multiplexed, NAA-free biosensing system will be widely used in diagnosis of viral infections, and a variety of genetic diseases. The present developed method offers the following benefits: (i) multiplexed detection to avoid producing false-negative results; (ii) NAA-free detection to evade NAA-related issues while maintaining PCR-like sensitivity (iii) high specificity and the ability to distinguish between closely related RNA target sequences by a single nucleotide substitution; (iv) a low LOD that meets the sensitivity requirement and could potentially be used to detect SARS-CoV-2 RNA targets in the early stages of the disease when viral gene load is low.

2. Materials and methods

2.1. Reagents and materials

Trizma hydrochloride solution (Tris-HCl), ethylenediaminetetraacetic acid (EDTA), tris (2-carboxyethyl) phosphine hydrochloride (TCEP), 3-mercaptopropionic acid (3-MPA), 6-mercapto-1-hexanol (6-MCH), 11-mercaptopundecanoic acid (11-MUA), sodium chloride (NaCl), sodium hydroxide (NaOH), potassium chloride (KCl), sulfuric acid (H_2SO_4), acetone, isopropyl alcohol (IPA), ethanol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer; pH 7.4), N,N-dimethylformamide (DMF), glycerol, 1,4-dithiothreitol (DTT), potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$), potassium ferrocyanide ($\text{K}_4[\text{Fe}(\text{CN})_6]$), hexaammineruthenium (III) chloride (RuHex), and magnesium chloride (MgCl_2) were purchased from Sigma-Aldrich (USA). Thermo Fisher Scientific (USA) provided RNase-free phosphate-buffered saline (PBS) solution (10X) pH 7.4, UltraPure™ DNase/RNase-Free Distilled Water (DW), and RNase AWAY solution. Ferrocene carboxylic N-hydroxysuccinimide ester (Fc-NHS) was supplied from Fivephoton Biochemicals (USA). AmpliScribe™ T7 High Yield Transcription Kit was purchased from Lucigen (USA). TRIzol LS reagent was purchased from Life technology Inc. (USA). Screen printed gold electrodes (SPGEs) (C220BT and X2224BT) were supplied by Metrohm DropSens Inc. (Spain). Except for the S and Orf1ab RNA sequences, all oligonucleotides used in this study were custom-made by Integrated DNA Technologies (IDT Inc., USA). The oligonucleotide sequences utilized are shown in Table S1 (Supporting Information). The stock solution of all oligonucleotides was prepared by dissolving its lyophilized powder in Ultra-Pure™ DNase/RNase-Free Distilled Water, and then diluted to the desired concentrations using buffer, prior to use. The LwCas13a protein storage buffer was prepared using 5×10^{-2} M Tris-HCl, 6×10^{-1} M NaCl, 5% glycerol, and 2×10^{-3} M DTT. The Tris-EDTA (TE) buffer was prepared using 1×10^{-2} M Tris-HCl, and 1×10^{-2} M EDTA and adjusted to pH of 8.0. The assay buffer was prepared using 4×10^{-2} M Tris-HCl, 6×10^{-2} M NaCl, and 6×10^{-3} M MgCl_2 and adjusted to pH 7.3. A buffer for square wave voltammetry (SWV) measurements was prepared using 1×10^{-2} M Tris-HCl and 1×10^{-1} M NaCl. A 1×10^{-2} M Tris buffer pH 7.5 was used as a washing buffer (WB).

2.2. Instrumentation

Field emission-scanning electron microscopy (FE-SEM) imaging was performed with the Carl Zeiss-Sigma instrument (Carl Zeiss, Germany) at an accelerating voltage of 20 kV. X-ray photoelectron spectroscopy (XPS) scanning was investigated with ThermoFisher Scientific (K-alpha +) using Al K α (mono) anode at 150 W energy in 10^7 Pa vacuum. Electrochemical experiments were carried out using CHI 1030C and CHI-660E (CH Instruments, USA) electrochemical analyzers. For the analysis of clinical samples, QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, Massachusetts, USA) was used.

2.3. Electrochemical measurements

All electrochemical measurements were performed in a three-electrode system using dual screen-printed electrodes (SPGEs) on the CHI instruments. Cyclic voltammetry (CV) was carried out in a $\text{K}_3[\text{Fe}(\text{CN})_6]$ solution (5×10^{-3} M) containing 1×10^{-1} M KCl at a scan rate of 0.1 V.s^{-1} . Electrochemical impedance spectroscopy (EIS) experiments were performed in an equimolar $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$ solution (ratio 1:1, 5×10^{-3} M) containing 1×10^{-1} M KCl. The Nyquist plots were recorded under a constant potential of 0.21 V and AC potential of 0.005 V over the frequency range (100 kHz to 0.1 Hz). The surface density of the UDH probe on the electrode surface was determined using chronocoulometry in the presence of 2×10^{-4} M RuHex solution. Chronocoulometry experiments were performed at a potential step of 0.5 V (+0.1 to −0.4 V) with a pulse width of 0.5 s and a pulse interval of

0.0025 s. SWV measurements were recorded in SWV measuring buffer at a potential range from -0.6 to $+0.5$ V, frequency of 15 Hz, amplitude of 2.5×10^{-2} V, and step potential of 5×10^{-3} V. All the measurements were performed at least in triplicate at room temperature (RT).

2.4. RNA in vitro transcription.

T7 transcription from synthesized DNA oligos was used to create the S and Orf1ab RNA sequences. Prior to in vitro transcription, a T7 promoter was added to each DNA template sequence using a PCR primer set (Table S2). The S and Orf1ab gene DNA sequences were then transcribed using the AmpliScribe™ High Yield Transcription kit as directed by the manufacturer. To summarise, all reaction components, except the AmpliScribe T7 RNA Polymerase, were first brought to RT. The reaction component, save the AmpliScribe T7 RNA Polymerase, was combined and mixed in the following order: 1 µg template DNA with appropriate promoter, 6.5 µL sterilized Nuclease-Free Water, 2 µL AmpliScribe T7 10X Reaction Buffer, 1.5 µL 1×10^{-1} M of each nucleotide (ATP, CTP, GTP, UTP), 2 µL 1×10^{-1} M DTT, and 0.5 µL RiboGuard RNase Inhibitor. Following that, 2 µL of AmpliScribe T7 RNA Polymerase was added and mixed. The resulting mixture was then incubated for 3 h at 42 °C with interval inversion of the mixture. After the reaction completed, 5×10^{-2} M EDTA was added to the mixtures to remove magnesium pyrophosphate formed. After that, the mixture was treated with RNase-Free DNase I to remove DNA template. Eventually, the RNA products were purified using TRIzol LS reagent. The purified RNA transcripts were kept at -80 °C for the future use.

2.5. Fabrication of biosensing surface

Dual SPGEs with two working zones were employed for fabricating the biosensing surface. The SPGEs were cleaned and activated using the previously described procedures prior to construction of the biosensor [9]. These electrodes were then used as substrate for nanostructured gold electrodeposition and reRNA capture. The electroplating conditions were optimized for 120 s at a concentration of 3×10^{-2} M and a plating potential of 0.2 V. Following electrodeposition, a thiolated reRNA (2×10^{-6} M) was immobilized onto both gold nanostructured working surfaces overnight at 4 °C in a humid environment. It is worth noting that thiolated reRNA was activated using TCEP solution (1×10^{-2} M) for 1 h at RT in darkness. The reRNA-modified gold nanostructured electrodes were then thoroughly washed with WB and dried with nitrogen gas flow and passivated using MCH solution (1×10^{-5} M in 1X PBS pH 7.4) for 10 min. Following the MCH treatment, the biosensor was washed using WB for 5 min. The biosensing surface was then dried with nitrogen gas before being treated by the CRISPR system and can be stored in 1×10^{-2} M Tris buffer containing 1×10^{-1} M NaCl at 4 °C for a short period.

2.6. Cas13a-crRNA assembly

LwCas13a was expressed from the pC013-Twinstrep-SUMO-hLwCas13a vector (Addgene, Massachusetts, USA) and purified by Ni-NTA affinity chromatography (Fig. S7). Cas13a-crRNA duplex assembly was performed in a tube containing 1.25×10^{-7} M purified Cas13a, 6.25×10^{-8} M crRNA in nuclease free assay buffer. The tube was then placed in an incubator at 37 °C to allow the assembly reaction to proceed for 10 min. Subsequently, for RNA target detection, variable amounts of S or Orf1ab gene target RNA was added into the Cas13a-crRNA duplex and incubated for 10 min at 37 °C. Next, the reaction mixture was introduced to the reRNA-modified sensor to perform collateral activity at 37 °C for 3 h. After the on-chip CRISPR reaction, the sensor was thoroughly washed using WB.

2.7. Biosensor performance assessment

Calibration curves were obtained in the presence of both COVID-19 RNA oligos (S, and Orf1ab genes) over a range (1×10^{-17} to 1×10^{-11} M). Experiments with one or more missing components were also carried out as controls. The control samples lacked one or more of the following components: (1) LwCas13a, (2) crRNA, (3) RNA target, and (4) reRNA. The specificity of the biosensor was evaluated using synthesized mismatched SARS-CoV-2 RNA sequences (5×10^{-13} M), as well as synthesized Influenza-A RNA sequences (5×10^{-13} M). Finally, the performance of the biosensor was evaluated for the detection of the COVID-19 RNA fragments in clinical samples. For electrochemical detection, a 1×10^{-2} M Tris buffer containing 1×10^{-1} M NaCl was applied as the electrolyte. SWV was used to recorder the signal changes before/after introduction of Cas13a-crRNA-target. A potential range from -0.6 to $+0.5$ V, a frequency of 15 Hz, an amplitude of 2.5×10^{-2} V, and a step potential of 5×10^{-3} V were used. The signal retention J/J_0 was obtained by the comparison of the resulting current density before (J_0) and after (J) the introduction of the Cas13a-crRNA-target complex to the biosensor.

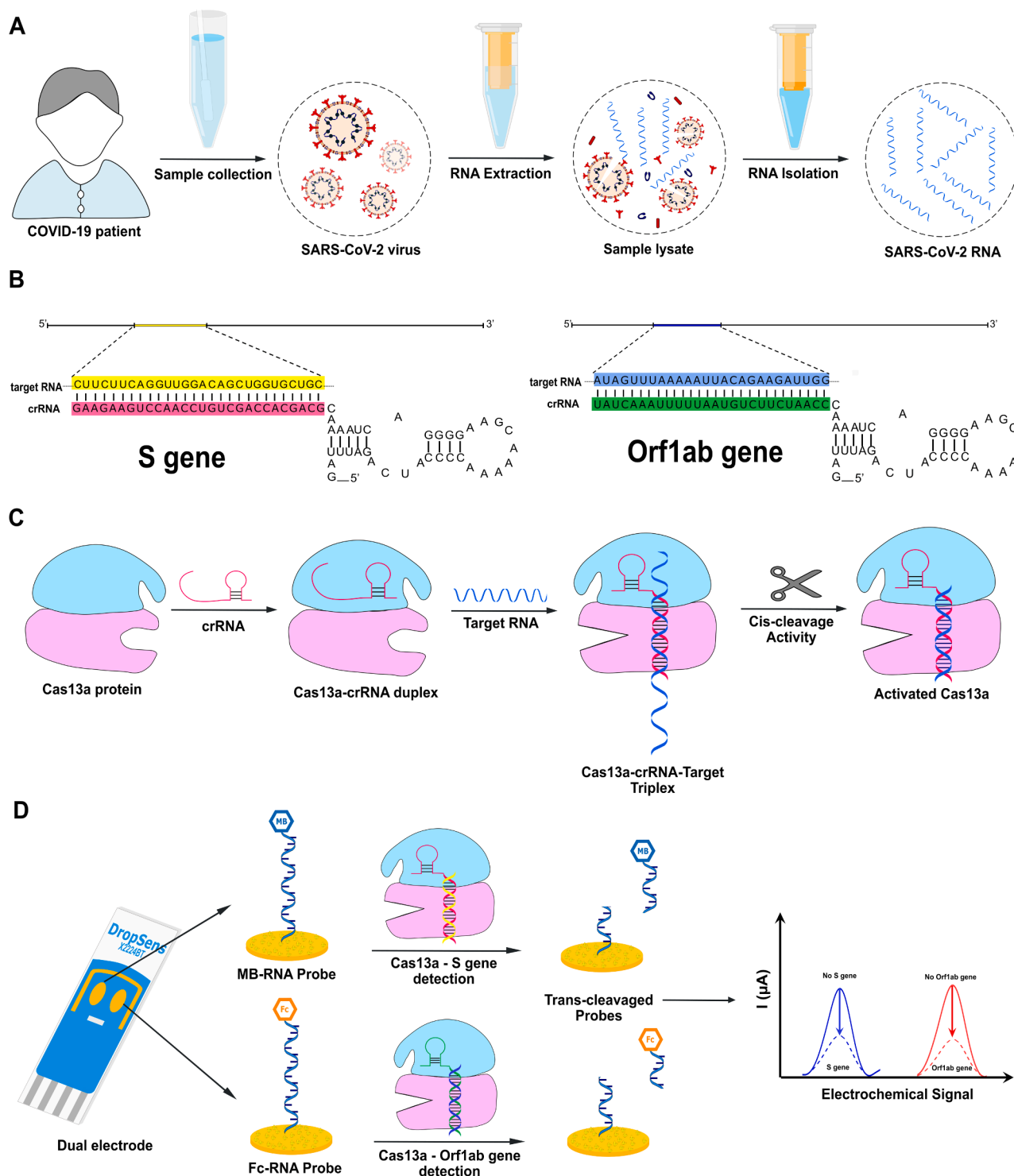
2.8. Standard PCR method

RNA samples extracted from 39 anonymized respiratory clinical patients were used as the RNA target using the electrochemical biosensor employing CRISPR platform. Samkwang Medical Laboratories (Seoul, South Korea) provided the SARS-CoV-2 positive samples in the form of purified RNA under IRB code number S-IRB-2020-029-09-17. They were ready-to-use samples that did not require pre-treatment before use. The isolated RNA samples were subjected to RT-qPCR analysis to be compared with the electrochemical results. Two different primer-probe sets were used in clinical SARS-CoV-2 RNA detection assays. The primer sets of the S and Orf1ab genes were designed by Park et al. [37]. All primer sets were synthesized by Integrated DNA Technologies. RT-qPCR assays were performed using Taq-Path™ 1-Step RT-qPCR Master Mix (ThermoFisher Scientific, Applied Biosystems™, USA) using a QuantStudio 5 Real-Time PCR System. Each 20 µL reaction mixture contained 5 µL of RT-qPCR master mix, 1 µL of each 10 µM forward and reverse primer, 2 µL of 100 nM probe, 1.2 µL of RNase-free water, and 2 µL of the template. The thermal cycling condition was 15 min at 50 °C for cDNA synthesis, 2 min at 95 °C for reverse transcription inactivation and pre-denaturation, and 45 cycles of 5 s at 95 °C and 30 s at 55 ~ 60 °C.

3. Results and discussion

3.1. Concept and construction of E-CRISPR for the detection of SARS-CoV-2

Scheme 1 represents an overall workflow of E-CRISPR for the detection of synthetic, in-vitro-transcribed (IVT) SARS-CoV-2 RNA sequences as well as SARS-CoV-2 RNA sequences extracted from patients' samples. This platform takes advantage of collateral cleavage activity of CRISPR-Cas13a assay and electrochemical biosensing simultaneously for detecting SARS-CoV-2 RNA sequences. Given that LwCas13a's collateral activity is more favorable for uracil (U) and adenine (A) cleavage [18,23], a reRNA sequence containing U/A ribonucleotides was designed and used for the biosensing. The collateral cleavage activity was investigated based on the Cas13a-crRNA duplex targeting S and Orf1ab target RNAs. First, a triplex of Cas13a-crRNA-target RNA was created by combining S and Orf1ab target RNAs with their corresponding Cas13a-crRNA duplexes. Following that, Cas13a-crRNA-target RNA was incubated on the biosensing surface. Only in the presence of target RNA that can complement with the corresponding guide region positioned in the crRNA, the Cas13a collateral activity is triggered and the ssRNA cleavage process is initiated. The cleavage process removes



Scheme 1. Schematic of the detection platform (A) The extraction of SARS-CoV-2 RNA sequences from COVID-19 patients' samples. (B) The S and Orf1ab single-stranded target RNA (highlighted in yellow and blue, respectively) as well as the S and Orf1ab crRNA (highlighted in pink and green, respectively). (C) A conformational change in Cas13a in response to crRNA and target RNA binding results in non-target collateral cleavage. (D) The E-CRISPR working principle and its main components. The redox probe conjugated reRNA-modified biosensor is exposed to the enzymatically activated Cas13a-crRNA-target RNA triplex. Activated Cas 13a cleaves the reRNA, resulting in the release of the redox probe from the reRNA and eventually, a decrease in electrochemical signal.

redox labels (of MB and Fc) from the biosensing surface, causing the electrochemical signal to decrease.

3.2. Evaluation of the optimized condition for E-CRISPR biosensing platform

Due to the low quantity of SARS-CoV-2 RNA sequences in clinical samples, the detection sensitivity is vital, and it can be improved through optimizing various parameters affecting the biosensing

platform. To begin, we optimized the parameters affecting biosensing surface performance (e.g., nanostructuring the biosensing surface, concentration of reRNA, biosensing surface coverage, passivation agents, etc.).

To create a gold-nanostructured surface, the dual SPGEs were electrodeposited with a gold solution. Nanostructure formation increases the surface-to-volume ratio, allowing for more reRNA capture. Cyclic voltammetry was used to investigate the active surface area of smooth and gold-nanostructured electrodes (GN/SPGE) (Fig. S1a). Despite the fact that both electrodes exhibit a similar cathodic peak due to electrochemical reduction of gold oxide formed during the anodic scan, because of the larger surface area, the reduction peak current of the nanostructured electrode is significantly higher than that seen on the smooth gold surface. FE-SEM was used to examine the surface roughness of both the smooth gold electrode and the GN/SPGE. The GN/SPGE surface micrograph shows a nano-flaked structure, whereas the gold substrate shows a smooth structure, which is consistent with the electrochemical results (Fig. S1b, c). Additionally, the chemical composition and its elemental state of the GN/SPGE surface was investigated by XPS. Based on the XPS survey scan on Fig. S1d, the gold element significantly dominates, with a percentage of Au atoms of 93.06%. Fig. S1e showed that the GN/SPGE surface were partially oxidized. A high-resolution spectrum of the Au4f core could be characterized by 3 pairs of Au4f_{7/2} and Au4f_{5/2} spin-orbit coupling. The position of the most significant pair with binding energies (BEs) of 84.9 and 88.9 eV were related to gold elemental (Au⁰) which respective atomic percentage was calculated

based on relative peak area as 85.3%, whereas those of the other pairs were related to the two stable gold oxide states, Au⁺ (BEs of 86 and 89.8 eV) and Au³⁺ (BEs of 87.8 and 91.3 eV), accounting for atomic percentage as 9.2% and 5.5%, respectively.

To achieve optimal immobilized reRNA onto the biosensing surface, various concentrations of reRNA-labeled with MB/Fc were immobilized on the gold-nanostructured electrode and the electrochemical signal of redox probes was monitored. The MB/Fc oxidation currents increased continuously as the concentration of reRNA increased up to 2×10^{-6} M and then plateaued (Fig. S2). As a result, for future experiments, a concentration of 2×10^{-6} M reRNA was chosen.

In addition, because the Cas13a enzyme's accessibility to the reRNA immobilized on the sensing surface is critical, reRNA surface coverage at various reRNA concentrations was tested using chronocoulometry [38]. Surface coverage was measured to be 6.4×10^{13} molecules.cm⁻² at optimal reRNA concentration (2×10^{-6} M) (Fig. S3).

Furthermore, the length of the immobilized reRNA was evaluated (Fig. 1). Because of the exposed length difference, we assumed that different lengths of reRNA would result in different cleavage efficiency. As a result, different lengths of reRNA were tested at the same concentration using the same CRISPR-Cas13a reaction condition. As Fig. 1 shows, in the presence of a short reRNA, the electrochemical oxidation current is larger than that of a long reRNA due to the short electron transfer distance between the electrode surface and the redox probe. Subsequently, short reRNA produces a high background current, whereas long strands produce a low background current. As a result,

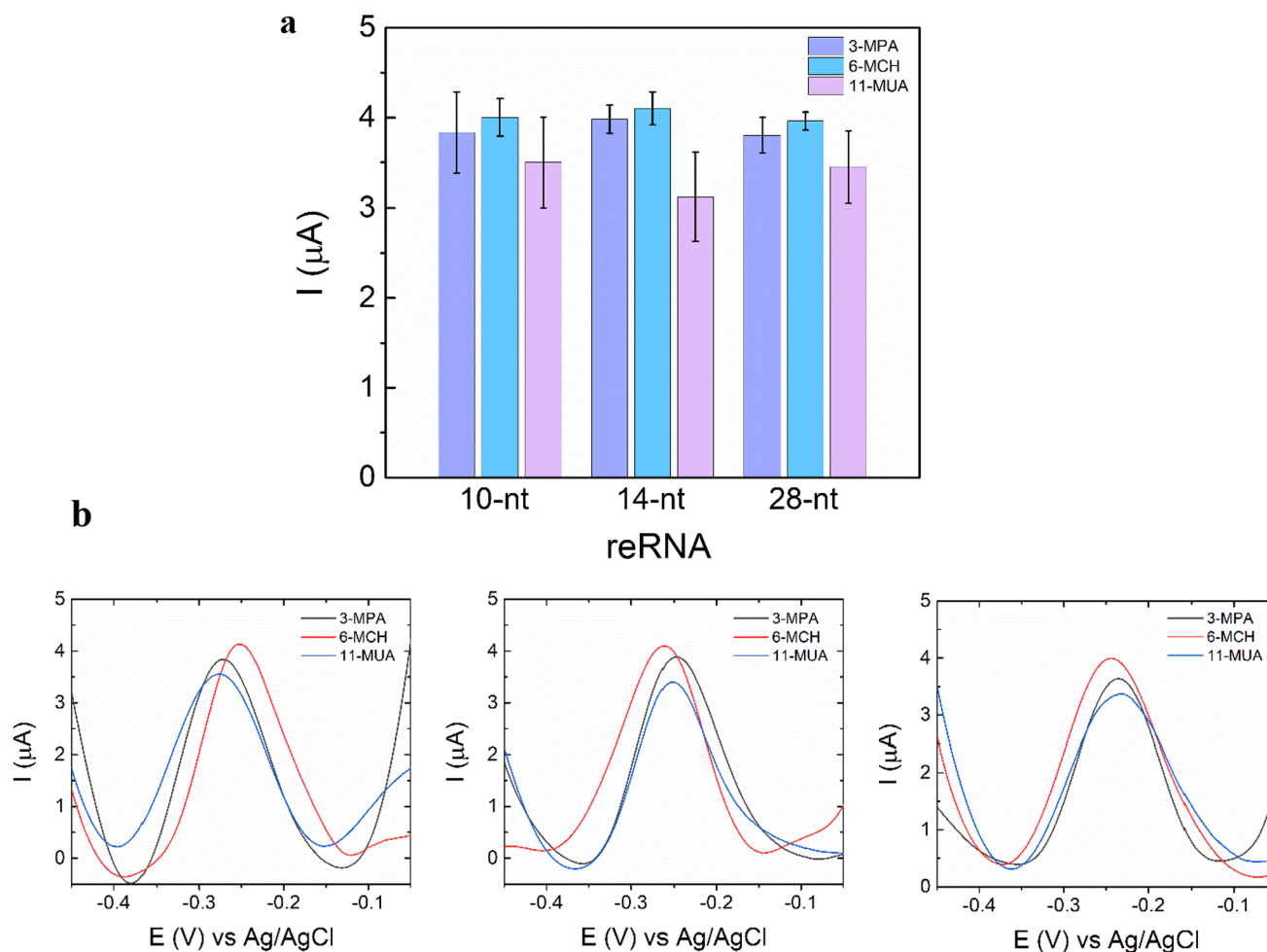


Fig. 1. (a) Current measurements of various reRNA lengths (10 nt, 14 nt, 28 nt) and passivation agent combinations (3-MPA, 6-MCH, 11-MUA). All the measurements were carried out in triplicate ($n = 3$), and the error bars represent the standard deviation for independent measurements. (b) SWV curves of three different passivation agents (3-MPA, 6-MCH, and 11-MUA) in combination with 10 nt reRNA on the left, 14 nt reRNA on the middle, and 28 nt reRNA on the right.

signal changes for long reporters are comparable with those of short strands, and reRNA length has insignificant effect on signal variations. Finally, because of its greater reproducibility, the 14 nt reRNA was chosen for additional experiments.

In the following phase, the effect of various passivation agents of varying lengths was investigated in order to achieve optimal cleavage activity followed by improved signal changes. As shown in Fig. 1, the greatest signal changes were obtained in the presence of MCH, so MCH was chosen as the favorable passivation agent. A passivation agent with a long carbon chain reduces electrostatic repulsion between reRNA strands and confines their steric movement, which can lower the redox tag's electron transfer kinetics, resulting in decreased signal changes. A short-length passivation agent, on the other hand, would be unable to lift the reRNA to the upright position and would be unable to confine the steric movement of the reRNA sufficiently, resulting in loosely oriented reRNA strands and, as a result, a high background signal.

In the final step of optimizing parameters affecting biosensing surface performance, the biosensor's shelf life was evaluated under humidified conditions at 4 °C (Fig. S4). The electrochemical signal was stable for nearly three days, which is satisfying for clinical applications [15].

The Cas13a enzyme's collateral activity is crucial for the E-CRISPR biosensing platform to transduce the electrochemical signal and directly impact on the detection sensitivity. Therefore, for electrochemical detection of SARS-CoV-2 RNA, the potential factors, influencing the collateral activity were investigated (e.g., LwCas13a concentration for cleavage process and catalytic activity time). To begin, different concentrations of Cas13a-crRNA (at a 2:1 ratio) were tested in response to the same concentration of S or Orf1ab genes (1×10^{-8} M). As shown in Fig. S5a, signal retention decreased as the Cas13a-crRNA concentration increased from 2.5×10^{-8} to 1.25×10^{-7} M. At high concentrations above 1.25×10^{-7} M, signal retention was increased because Cas13a's activity towards a nonspecific reRNA was reduced due to Cas13a's large size, which hindered its accessibility to reRNA. As a result, the optimal concentration for Cas13a-crRNA duplex collateral activity was determined to be 1.25×10^{-7} M. Furthermore, collateral cleavage activity time was evaluated since reRNA cleavage is a time-dependent process (Fig. S5b). Extending the incubation time by 3 h results in more cleaved reRNA and thus less signal retention. In contrast, a longer incubation period had no effect on signal retention. This could be because the solution contains a low concentration of target RNA sequences, resulting in an insufficient number of active Cas13a enzymes. As a result, Cas13a's total catalytic activity will be inadequate for further improvement in signal changes at very low concentrations of target genes. The effect of incubation temperature on collateral cleavage activity was investigated. The signal retention of the cleavage process was highest at 25 °C, then decreased at 37 and 42 °C, demonstrating that the higher the temperature, the more efficient the cleavage activity. However, signal retention

at 37 and 42 °C was comparable, indicating that increasing temperature above 37 °C did not further increase cleavage activity. As a result, 37 °C was chosen as the optimal temperature for the CRISPR cleavage process, in accordance with the published literature [24].

3.3. E-CRISPR for detecting SARS-CoV-2 RNA sequences

Based on optimized conditions for biosensing surface and collateral activity, the E-CRISPR platform performance was evaluated for simultaneous, multiplexed, highly sensitive, and specific detection of S and Orf1ab genes. A wide dynamic range of 7 orders of magnitude from 1×10^{-17} to 1×10^{-11} M was achieved with an LOD of 2.5 and 4.5 ag/ μ L (26.2 and 53.5 copies/ μ L) for S and Orf1ab genes, respectively. To obtain calibration curves, Cas13a-crRNA-target RNA solutions containing various concentrations (1×10^{-17} to 1×10^{-11} M) of the S and Orf1ab genes were prepared, incubated at 37 °C for 3 h, and then applied to the reRNA modified gold-nanostructured electrode (Fig. 2). The measured faradic current changes from SWV for both S and Orf1ab genes were then fitted to a four-parametric sigmoidal curve using the following regression equations:

$$\text{For S gene : } y = 0.072 + \frac{(0.840 - 0.072)}{1 + \left(\frac{x}{61.772}\right)^{0.518}}; R^2 = 0.986$$

$$\text{For Orf1ab gene : } y = 0.066 + \frac{(0.847 - 0.066)}{1 + \left(\frac{x}{69.270}\right)^{0.655}}; R^2 = 0.991$$

The LOD of 2.5 and 4.5 ag/ μ L (26.2 and 53.5 copies/ μ L) were achieved for the S and Orf1ab genes, respectively. It is worth noting that the LODs obtained by the E-CRISPR platform are comparable to, and even outperform, previously published LODs for SARS-CoV-2 RNA strands (Table 1). The overall intra- and inter-assay variability of less than 10% was also achieved for E-CRISPR (Fig. S6).

3.4. Clinical measurements and standard method

RNA samples extracted from nasopharyngeal swab samples of 23 COVID-19 positive patients and 16 healthy people were analyzed to test the feasibility of the E-CRISPR platform for SARS-CoV-2 detection in real samples (Fig. 3). For electrochemical detection, RNA sequences isolated from clinical samples were used as the target RNAs in the E-CRISPR platform. Each sample was tested using three different multiplexed biosensors. The signals obtained from COVID-19 positive patients for the S and Orf1ab genes were normalized to their blank counterparts. As illustrated in Fig. 2, the CRISPR-powered biosensor clearly discriminated COVID-19 positive patients from negative ones. All negative samples exhibited an electrochemical signal that was similar to the blank sample ($J/J_0 > 0.85$), whereas positive samples had a lower

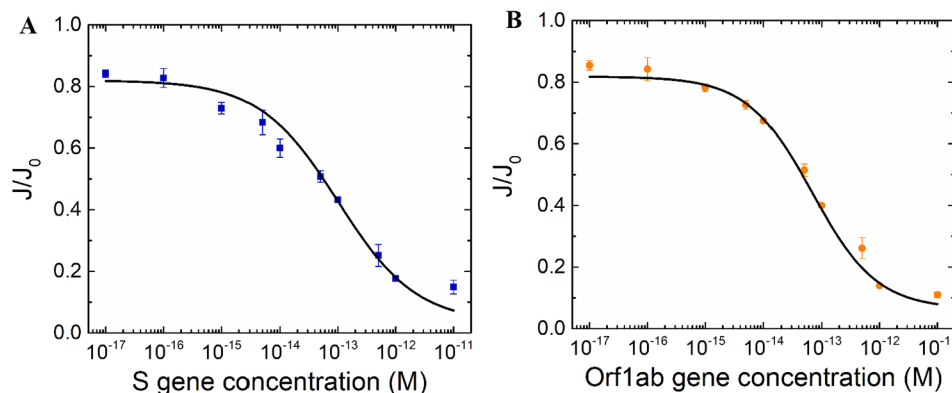


Fig. 2. E-CRISPR analysis of S and Orf1ab genes. Calibration curves for the (A) S gene and (B) Orf1ab gene. A four-parametric logistic fit was used to fit the data, yielding a LOD of 2.5 and 4.5 ag/ μ L for S and Orf1ab genes, respectively. The standard deviation (SD) for $n = 3$ replicates is represented by the error bars.

Table 1

The specifications of existing methods for detecting SARS-CoV-2 RNA fragments.

Detection Strategy	Technique	Target and LOD	Dynamic Range	Assay Time	Benefits	Challenges	Reference
Electrochemical biosensor	DPV	SARS-CoV-2 gene fragment: 26 fM	0.1 – 1000 pM	3 h	Sensitive, specific, highly stable in biological matrices	Single detection, sample preparation.	[39]
Electrochemical biosensor	DPV	ORF1ab gene: 200 copies/ml	1×10^{-17} – 1×10^{-12} M	–	Sensitive, NA amplification-free, and convenient assay	Single detection, multi-step long procedure.	[40]
Electrochemical biosensor	DPV	N gene: 3.5 fM	1×10^{-14} – 1×10^{-9} M	1 h	Rapid, stable, and sensitive	Single detection, complex structure.	[41]
Electrochemical biosensor	DPV	RdRP gene: 0.972 fg/ μ l N gene: 3.925 fg/ μ l	1×10^3 – 1×10^9 copies/ μ l	< 20 min	Rapid, sensitive, and multiplexed platform	Sample amplification is required.	[42]
RCA-based electrochemical biosensor	DPV	S and N genes: 1 copy/ μ l	$1-1 \times 10^{10}$ copies/ μ l	< 2 h	Highly sensitive, accurate, and real-time diagnostic test	Single detection, sample amplification is needed.	[7]
CRISPR-based electrochemical biosensor	DPV	ORF gene: 4.4×10^{-2} fg/mL S gene: 8.1×10^{-2} fg/mL	10^{-1} – 10^5 fg/mL	1.5 h	Sensitive, selective, and amplification-free	Single detection, sample preparation.	[43]
RT-LAMP based electrochemical biosensor	SWV	N and Orf1ab genes: 0.038×10^{-3} ng/ μ l	0.001 – 10000 $\times 10^{-3}$ ng/ μ l	–	Accurate, low-cost, multiplexed detection.	NA amplification is required, sample preparation.	[44]
4WJ-based electrochemical biosensor	SWV	S gene: 52 copies/ μ l Orf1ab gene: 80 copies/ μ l	1×10^{-16} – 1×10^{-11} M	40 min	Rapid, sensitive, amplification-free, and multiplexed detection	Pretreatment of real samples and sample preparation.	[9]
Electrochemical biosensor	EIS	N gene: 0.59 fg/mL	0.1 fg/mL – 1 ng/mL	50 min	Rapid, highly selective, and high stability.	Single detection, sample preparation.	[45]
Capacitive biosensor	EIS (Capacitance changes)	RdRp gene: 10 nM	10 nM – 5 μ M	1 h	Rapid, low-cost, highly recycle, specific.	Single detection, low sensitive.	[46]
ECL biosensor	ECL	RdRp gene: 7.8 aM	1×10^{-16} – 1×10^{-11} M	–	Highly sensitive and specific detection strategy	Single detection, NA amplification is required.	[47]
ECL biosensor	ECL	ORF1ab gene: 514 aM	50 fM – 100 nM	–	Sensitive and NA amplification-free	Single detection, complex preparation step.	[48]
CRISPR/Cas12a-based ECL biosensor	ECL	RdRp gene: 43.7 aM	0 – 2000 aM	3 h	Highly sensitive, accurate, and specific detection strategy	Single detection, NA amplification is required, multi-step procedure.	[49]
HCR-based ratiometric ECL biosensor	ECL	RdRp gene: 59 aM	0 – 3000 aM	3 h	Highly sensitive, stable, and specific detection strategy	Single detection, NA amplification is required.	[50]
CRISPR technology	Colorimetry	Orf1ab gene: 1 copy/test N gene: 1 copy/test	1–5 pM	–	Sensitive and specific detection strategy	Narrow dynamic range, NA amplification is required.	[5]
CRISPR technology	Fluorescence	Orf1ab and N genes: 2 copy/sample	–	–	Highly sensitive, selective, rapid, low-cost, multiplexed detection	NA amplification is required.	[51]
CRISPR/Cas13a-based electrochemical biosensor	SWV	S gene: 26 copies/ μ l Orf1ab gene: 53 copies/ μ l	1×10^{-17} – 1×10^{-11} M	3 h	Highly sensitive, accurate, specific, amplification-free, and multiplexed detection	Sample preparation and clinical sample pretreatment	This work

4WJ: Four-way junction, RCA: rolling circle amplification, CRISPR: clustered regularly interspaced short palindromic repeats, SWV: square wave voltammetry, DPV: differential pulse voltammetry, NA: nucleic acid, ECL: electrochemiluminescence, EIS: Electrochemical Impedance Spectroscopy, HCR: Hybridization chain reaction, RT-LAMP: reverse transcription loop-mediated isothermal amplification.

signal, with the majority of them having a $J/J_0 < 0.7$. The cleavage of MB/Fc-labeled reRNA in the presence of Cas13a-crRNA-target triplex and the removal of redox labels from the biosensing surface cause a significant drop in the signal. The cleavage phenomenon is controlled by the stage of the disease and the viral RNA load of clinical samples. In the advanced stage of the disease, the higher viral RNA load leads to more cleavage of MB/Fc-labeled reRNA and eventually lower electrochemical signal. Our CRISPR-powered biosensing system's LODs of 2.5 and 4.5 ag/ μ L for the S and Orf1ab genes, respectively, are lower than the reported viral RNA load in clinical samples [52]. In addition to adequate LOD, the relatively short turnaround time and multiplexed detection of our proposed biosensor may make it a suitable candidate for point-of-care applications. Eventually, the CRISPR-powered biosensing system's results were compared to the results of a gold standard, RT-qPCR. The electrochemical signals correlated well with the Ct values, indicating that the biosensor can be used as an alternative for COVID-19 detection assays.

3.5. Specificity and control test

To investigate the CRISPR-powered biosensor's specificity, various target RNAs, differing from the S and Orf1ab genes by a single nucleotide or several nucleotides, as well as synthetic Influenza-A RNA, were tested (Fig. 4a). For this, a concentration of 5×10^{-13} M of each strand was used. A variety of RNA strands were also used, including the complementary S and Orf1ab genes (Sg-Og); Sg-Og, and single-mismatched S and Orf1ab genes (Sg-Og_SMM); Sg-Og, and multi-mismatched S and Orf1ab genes (Sg-Og_MMM); Sg-Og, and Influenza-A (IAV) (Sg-Og_IAV). The final concentration of each strand in solution was 5×10^{-13} M. It is worth noting that all sample solutions contained the Cas13a and the crRNA, complementary to S and Orf1ab strands. For both S and Orf1ab genes containing single or multi-base mismatches, the Cas13a collateral activity will not be triggered because the mismatched targets do not sufficiently hybridize to the crRNA, consequently the enzyme's activity cannot be activated.

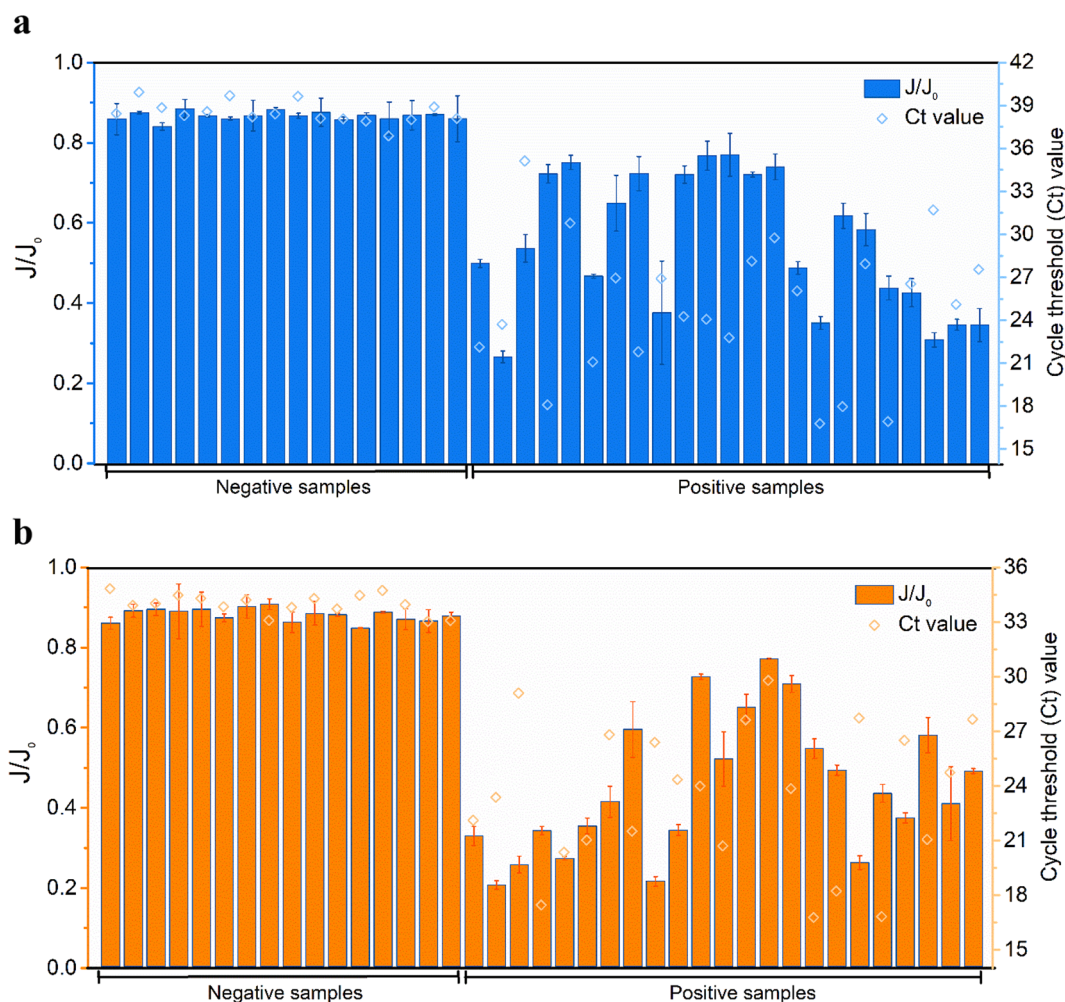


Fig. 3. The determination of the (A) S gene and (B) Orf1ab gene in 39 clinical specimens using SWV and RT-PCR. The samples were collected using nasopharyngeal swabs. The electrochemical signals are depicted in bar charts for the S gene (blue bar chart) and the Orf1ab gene (orange bar chart). The RT-PCR Ct values are represented in the blue hollow diamond (S gene) and the orange hollow diamond (Orf1ab gene). All electrochemical responses are represented as mean values $\pm$ SD ($n = 3$), and RT-PCR Ct values are from single measurements. SWV measurements were recorded in SWV measuring buffer at a potential range from -0.6 to $+0.5$ V, a frequency of 15 Hz, amplitude of 2.5×10^{-2} V, and a step potential of 5×10^{-3} V. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

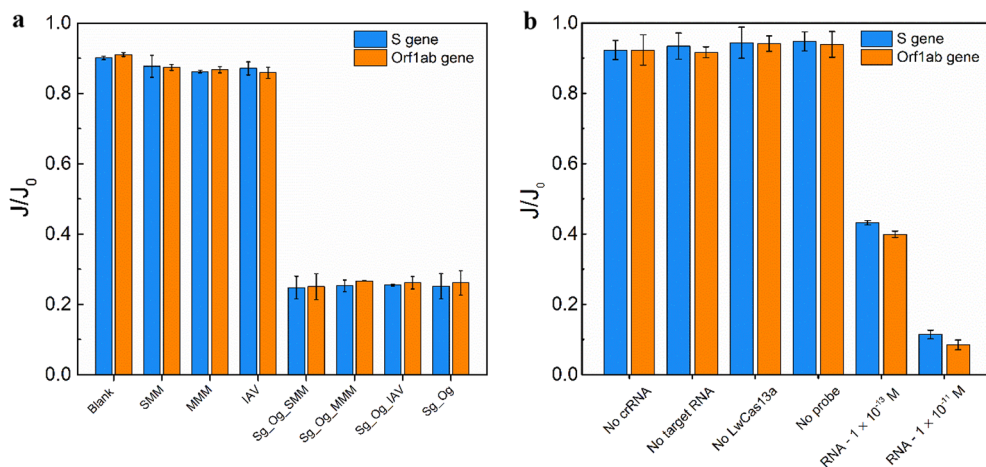


Fig. 4. (a) The specificity of the biosensor with S (blue bar) and Orf1ab (orange bar) genes was examined using 5×10^{-13} M perfect complementary targets (Sg and Og), 5×10^{-13} M single mismatched (SMM) and multi mismatched (MMM) RNA targets, and non-complementary synthetic Influenza-A (IAV) RNA targets. (b) Biosensor response with one or more of the following components: crRNA, RNA target, LwCas13a, and reRNA. The fixed concentrations of reRNA, crRNA, and LwCas13a were 2×10^{-6} , 6.25×10^{-8} , and 1.25×10^{-7} M, respectively. RNA targets concentrations were fixed at 1×10^{-13} M or 1×10^{-11} M, respectively. All the measurements were carried out in triplicate ($n = 3$), and the error bars represent the standard deviation for independent measurements. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

No signal changes were also observed for IAV RNA because non-complementary bases prevent hybridization of the IAV target strand and crRNA, and thus the enzyme's cleavage activity cannot be initiated. Alternatively, the S and Orf1ab genes produced low electrochemical signals. The signal reduction is caused by perfect hybridization of the S and Orf1ab genes with the corresponding crRNAs, which then activates the enzyme's cleavage activity. The presence of non-or partially complementary (i.e., IAV, SMM, and MMM) strands in samples containing S and Orf1ab genes showed a similar pattern and yielded a low electrochemical signal, demonstrating the high specificity of the CRISPR platform.

Control experiments were also carried out to validate the E-CRISPR performance in the absence of target RNA and/or other biosensing assay components. As shown in Fig. 4b, if one or more components (e.g., target RNA, crRNA, Cas13a) are missing, Cas13a's *trans*-cleavage activity is not triggered and it cannot cleave rRNA, resulting in no changes in electrochemical signal. This evidence reveals that the E-CRISPR platform can detect SARS-CoV-2 RNA sequences only when all of the components are present (Fig. 4b).

4. Conclusions

The CRISPR/Cas13a -powered electrochemical biosensor was developed for highly sensitive, specific, rapid, multiplexed, and nucleic acid amplification-free of SARS-CoV-2 RNA fragments. The exciting figures of merit namely sensitivity, specificity, relatively short turnaround time, as well as expandable feasibility for clinical samples were experimentally demonstrated. The CRISPR platform delivered an ultra-low limit of detection of 2.5 and 4.5 ag/ μ L for S and Orf1ab genes, respectively, which meets the sensitivity requirement. Its excellent specificity provided the capability of differentiating target strands among related RNA target sequences, and we foresee this technology will be a powerful tool in detecting SARS-CoV-2 RNA targets in the early-stage of the disease. Therefore, we are working to improve the proposed method's specifications for point-of-care applications (e.g., replacing the CHI-660E potentiostat with a portable one, integrating a microfluidic device with the biosensor to continuously accumulate the RNA strands onto the sensing surface, etc.) so that it can be used not only in central laboratories but also as a near-patient test anywhere.

CRediT authorship contribution statement

Leila Kashefi-Kheyraadi: Conceptualization, Methodology, Visualization, Formal analysis, Writing – review & editing. **Huynh Vu Nguyen:** Methodology, Visualization, Formal analysis, Writing – review & editing. **Anna Go:** Methodology. **Min-Ho Lee:** Funding acquisition, Project administration, Supervision, Writing – review & editing.

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

No data was used for the research described in the article.

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Appendix A. Supplementary data

The supporting information includes additional experimental details, methods, photographs, and result figures from S1 to S7. Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioelechem.2023.108364>.

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