Rapid Electrochemical Detection of Coronavirus SARS-CoV-2.

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Research Article

Keywords: COVID-19, electrochemical detection, multiplexing, rolling circle amplification

Posted Date: October 7th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-86933/v1

Abstract

COVID-19 is a highly contagious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Diagnosis of COVID-19 depends on quantitative reverse transcription PCR (qRT-PCR), which is time-consuming and requires expensive instrumentation. Here, we report an ultrasensitive electrochemical biosensor based on isothermal rolling circle amplification (RCA) for rapid detection of SARS-CoV-2. The assay involves the hybridization of the RCA amplicons with probes that were functionalized with redox active labels that are detectable by an electrochemical biosensor. The one-step sandwich hybridization assay could detect as low as 1 copy/mL of N and S genes, in less than 2 hours. Sensor evaluation with 105 clinical samples, including 40 SARS-CoV-2 positive and 9 samples positive for other respiratory viruses, gave a 100% concordance result with qRT-PCR, with complete correlation between the biosensor current signals and quantitation cycle (Cq) values. In summary, this biosensor could be used as an on-site, real-time diagnostic test for COVID-19.

Introduction

The global outbreak of coronavirus disease (COVID-19) is caused by the rapid spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[1]. SARS-CoV-2 has been classified as a beta coronavirus with high nucleotide sequence homology to two severe acute respiratory syndrome (SARS)-like bat coronaviruses and moderate homology with the Middle East respiratory syndrome coronavirus (MERS) CoV[2, 3]. The virus genome is a single positive stranded RNA of approximately 30,000 bases in length, which encodes for 10 genes, including the 5′ untranslated region, replicase complex (orf1ab), spike (S), envelope (E), membrane (M), and nucleocapsid (N) structural proteins, 3′ untranslated region, and several unidentified non-structural open reading frames[4].

The mode of virus transmission includes droplet, contact, airborne, fomite, fecal-oral and bloodborne transmissions which exacerbates the rapid spread of the virus[5]. A person infected with SARS-CoV-2 can either remain asymptomatic or show non-specific clinical symptoms such as fever, cough, or shortness of breath. Even during the incubation period, an infected person is highly contagious and can spread the virus to a non-infected person[6]. Thus, rapid diagnostic testing for SARS-CoV-2 at a large scale is crucial for virus detection, surveillance and swift management of outbreaks[7].

There are two types of diagnostic tests for COVID-19, serological and viral nucleic acid tests. Serological testing detects the presence of antibodies produced by an individual due to exposure to the virus or detection of antigenic proteins in the infected individuals. Serological tests include enzyme-linked immunosorbent assays (ELISA) and lateral flow immunoassays. However, these assays have been shown to give false-positive and false-negative results and are not recommended by the World Health Organization (WHO) to diagnose an active COVID-19 infection [8].

Therefore, for the accurate diagnosis of an active COVID-19 infection, viral nucleic acid testing should be used. Currently, quantitative reverse transcription PCR (qRT-PCR) is the gold standard for the diagnosis and confirmation of SARS-CoV-2 infection. Examples of commercially available qRT-PCR test kits are the Xpert Xpress SARS-CoV-2 test[9], CDC 2019- Novel Corona- virus Real-Time RT-PCR Diagnostic Panel[10], ExProbeTM SARS-CoV-2 Testing Kit[11], Abbott RealTime SARS-CoV-2 RT-PCR Kit[12], PerkinElmer® New Coronavirus Nucleic Acid Detection Kit[13] and TaqPath COVID-19 Combo Kit[14]. However, there are disadvantages with these methods as they are time-consuming, requiring a specialized laboratory setting with expensive instruments and trained personnel[15].To overcome these drawbacks, various isothermal nucleic acid amplification assays such as recombinase polymerase amplification (RPA)[16] and loop-mediated isothermal amplification (LAMP) [17-20], DNA nanoscaffold-based hybrid chain reaction (DNHCR)[21] and nucleic acid sequence-based amplification (NASBA)[22], have been developed to overcome the need for sophisticated thermal cycling equipment associated with qRT-PCR.

An isothermal amplification method known as rolling circle amplification (RCA) has also been widely used for nucleic acid testing[23]. The RCA assay involves the amplification of DNA or RNA primers that are annealed to a circular DNA template using DNA or RNA polymerases[24]. The RCA amplicon is a concatemer containing multiple repeats of sequences that are complementary to the circular template. RCA is able to produce amplicons about 10^9-fold within 90 min with minimal reagents[25]. Due to its isothermal nature, RCA can be performed using a simple water bath or heating block. A significant advantage of RCA is that the detection of the amplicons can be undertaken using an electrochemical biosensor, which enables rapid, quantitative results to be obtained either in the laboratory or more importantly, in a field setting. This allows for rapid, widespread deployment of testing kits to outbreak areas or remote regions where laboratory facilities do not exist or are difficult to access. This approach will be of particular benefit to developing countries. An RCA assay for the 2003 SARS-CoV has been reported, however detection of the RCA amplicons relied on cumbersome and time-consuming gel electrophoresis[26]. To our knowledge, this is the first report of an RCA assay for SARS-CoV-2.

In this study, we describe an ultrasensitive electrochemical biosensor based on multiplex RCA for the rapid detection of the N and S genes of SARS-CoV-2 (Scheme 1). The assay involves sandwich hybridization of RCA amplicons with probes that are functionalized with redox active labels, which are subsequently detected by differential pulse voltammetry (DPV). The assay could detect as low as 1 copy/mL of viral N or S genes in less than 2 hours. Clinical samples were also used to evaluate the performance of the assay, which was found to be in agreement with qRT-PCR results

Results

Characterization of the silica core and redox dye-incorporated silica nanoparticles

Two redox dyes, methylene blue and acridine orange were coated onto silica nanoparticles (SiNPs) through surface-reactive functional groups. Figure 1 displays the scanning electron micrographs (SEM) of silica core particles (Fig. 1a) and silica with a redox–dye layer, denoted as SiMB (Fig. 1b) and SiAO (Fig. 1c), respectively. The size of the SiNPs after coating with the redox-dye increased compared to the silica core which is similar to previous report by Cheeveewattanagul[27]. In addition, we performed layer-by-layer modification with two polyelectrolytes, which are positively charged PAA and negatively

charged PSS, onto SiMB and SiAO particles. The absorbed PSS left a net negative charge on the surface of the SiNPs, which facilitated the binding with the avidin linker.

Detection of RCA amplicons by agarose gel electrophoresis

In the presence of the target gene, the circular DNA template hybridizes to the target gene, causing the 5' end of the circular DNA template to be juxtaposed to its 3' end (Fig. 1d). Following ligation, the Padlock DNA serves as a template for amplification by phi29 DNA polymerase to produce RCA amplicons, which are long DNA amplicons containing hundreds of tandem repeats of the Padlock DNA complementary sequence. This sequence lies the universal capture probe and gene specific Si-RP binding regions. Figure 1e shows the successful amplification of the N and S genes visualized using agarose gel electrophoresis. Within 30 min of amplification, high molecular weight RCA amplicons were formed, which did not migrate out of the wells because of their large size.

Performance of the sandwich and one-step hybridization

The performance of the stepwise sandwich and one-step hybridization strategies were compared using 1 pM of linear target for the N gene. Stepwise sandwich hybridization involved the sequential hybridization of CP-MNB to Target and CP-MNB-Target to SiRP, with washing steps between each hybridization. In the one-step strategy, CP-MNB, SiRP and the target were mixed in a single hybridization step, followed by a single washing step (Fig. 2a). We found no significant difference ($p > 0.05$) in the current signal between the step-wise sandwich and the one-step hybridization strategies (Fig. 2b), Therefore, the one-step hybridization strategy was used for the optimized assay as it was easier, faster, and reduced pipetting steps. This also minimizes contamination, which is a key concern for field-based testing. The use of magnetic beads coated with streptavidin enables easy manipulation of the target nucleic acid during isolation and purification using a magnetic field [28, 29]. The cDNA or RNA target will be specifically hybridized to the biotinylated probe on the magnetic beads via hydrogen bonding, permitting their precise targeting. Moreover, this approach has become a valuable method because of their low cost, compatibility with liquid sample, and high surface area for hybridization[30].

Assay sensitivity

For both the S and N genes, the increase in the electrochemical signals were positively correlated to the increase in the gene copy number (Fig. 2 (c, d, e)). different redox dyes (MB for N gene and AO for S gene), the strength of the current signals obtained from both genes were very similar. The detection limit of both the N and S genes was 1 copy/mL, with a linear range of 1 to 1 x 10^{10} copies/mLThe correlation of current signal for N and S genes was 99%.

Assay specificity

COVID-19 and influenza and are both contagious respiratory illnesses. Although they are caused by different viruses, the symptoms of COVID-19 and influenza are very similar and may be hard to distinguish based on clinical symptoms alone. Therefore, a diagnostic test that can distinguish the influenza virus from COVID-19 is necessary to avoid misdiagnosis. In the specificity testing, target sequences of Influenza A (IAV) and Influenza B (IVB) viruses were included as non-complementary targets, where the sequence alignment is shown in Fig. 2f. In addition, linear targets with two bases mismatch were also included. A current signal that was equal or greater than +3 standard deviations (3 SD) above the mean of the blank (background) signal was considered a positive result. All complementary targets for both the N and S genes yielded positive results, while the non-complementary targets (IAV, IBV, IAV+IBV, mismatch) were all negative (Fig. 2g). Moreover, the discrimination between perfectly complementary targets and two bases mismatch targets demonstrates that the assay is highly specific. Therefore, this assay could be useful for detecting SARS-CoV-2, even in the presence of co-infection with other viruses that manifest similar respiratory symptoms.

Performance of the assay for detection of SARS-CoV-2 from clinical samples

RNA and cDNA samples prepared from clinical samples were used as the template in RCA, using the one-step hybridization method and electrochemical detection. All 40 samples (10 RNA and 30 cDNA) prepared from SARS-CoV-2 positive clinical samples yielded positive results, while the 65 samples (40 RNA, 25 cDNA) prepared from SARS-CoV-2 negative clinical samples recorded negative results (Fig. 3). A comparison of the current signals with the Cq values from qRT-PCR showed a good correlation. Samples with low Cq values (indicating higher viral titer) also recorded higher current signals. The DPV data for each of these tests is shown in Fig. S1.

Discussion

RCA is a robust and technically simple, isothermal technique for *in vitro* DNA amplification. RCA uses strand displacing phi29 DNA polymerase to continuously amplify the circular nucleic acid template[31]. The unique concatenated DNA strand that is produced as the amplification product has multiple binding sites for the reporter probe to hybridize[32] (approximately 10^2 -10^3 repeats of complementary sequence, and even up to 10^5 in some cases can be achieved on the RCA amplicons)[23]. In addition, the circular DNA can bind both DNA and RNA targets which advantageously eliminates the need to perform a reverse transcription step, unlike other isothermal methods such as RPA and LAMP. The use of T4 DNA ligase enables the joining of adjacent blunt end termini of DNA/RNA hybrids[33].

In comparison with PCR-based assays, RCA can be performed under isothermal conditions with minimal reagents and avoids the generation of false-positive results. RCA assay is also less complicated compared to other isothermal amplification methods, such as a transcription-based system, strand displacement approach, invasive-cleavage reaction, or loop-mediated technology. RCA can be performed with a minimal pre-optimization step, hence it can be readily employed by non-skilled users[23].

Target amplification by RCA followed by electrochemical biosensor detection requires three steps of target recognition and hybridization to its complementary sequence. First, the gene target must hybridize with the complementary sequence in the circular DNA, followed by ligation of the circular DNA to produce a Padlock DNA. After amplification of the Padlock DNA by phi29 DNA polymerase, RCA amplicons are produced, which are long repeats of the complementary sequence of the Padlock DNA. Next, the RCA amplicons are bound by the reporter and capture probes, followed by electrochemical detection of the redox-active dye. This strategy ensures high specificity is achieved with the assay. Moreover, utilization of magnetic capture and separation of targets from non-targets reduces the chances of carry-over contamination and pipetting error.

The World Health Organization (WHO) recommends that for laboratory confirmation of cases by nucleic acid testing in areas with no known COVID-19 virus circulation, the test must be positive for at least two different targets on the COVID-19 virus genome. In areas where COVID-19 virus circulation have been established, nucleic acid testing for a single discriminatory target is considered sufficient[34]. Therefore, in this study, we performed multiplex RCA for the simultaneous amplification of 2 genes, which are the N and S genes encoding the nucleocapsid and spike proteins, respectively. We designed circular DNA templates with the same capture probe binding sequence for both N and S genes so that the CP-MNB could bind to the RCA amplicons of both genes. The RCA amplicons containing both N and S genes were electrochemically detected using the respective redox-labeled RP.

Other assays for SARS-CoV-2 detection also targeted the N and S genes using qRT-PCR and conventional RT-PCR [35-37]. Additionally, some diagnostic tests have been designed to target the E gene to broadly detect coronavirus infections (bat SARS-like coronavirus and SARS-CoV) [20, 38, 39]. Besides these genes, other commonly used gene targets for detection of SARS-CoV-2 are the ORF1ab[17]and RdRp[40] genes.

RNA viruses such as SARS-CoV-2 have a high mutation rate, which contributes to its adaptation[41] . SARS-CoV-2 genomic variations have been associated with differences in the mortality rate of COVID-19. Mutations in the RdRP [41] ORF1ab[42], N[43] and S[44] genes have been identified. Therefore, it is recommended that at least 2 targets are included in a SARS-CoV-2 diagnostic test to reduce the possibility of false-negatives due to mutations in the target genes. On the other hand, the ability of the assay to discriminate 2 bases mismatch could be useful for viral mutation studies. Therefore, wild-type and mutant strains could in principle be distinguished using probes tagged with different redox dyes as demonstrated in this study. Another advantage of our assay is the limit of detection (LoD), which showed that it is highly sensitive and comparable to commercially available test kits and existing biosensors (Table 2). Our assay's LoD of 1 copy/µL for the N gene is comparable, if not slightly better, than CDC's RT-PCR assay of 3.2 copies/µL[10, 15].

The application of the optimized assay for the diagnosis of COVID-19 was evaluated with 105 clinical samples. This included samples that were SARS-CoV-2 negative but positive for other respiratory viruses. The ability of a test to distinguish between SARS-CoV-2 and other respiratory viruses is important because the clinical symptoms of COVID-19 and influenza are very similar, however, the approach in management and control of the disease is different. Clinical diagnosis is further complicated in cases of co-infection with SARS-CoV-2 and influenza[45]. A multiplex test that can detect SARS-CoV-2, influenza and other respiratory viruses simultaneously would save time and provide more accurate information to clinicians and public health officials [46]. Currently, we have only tested multiplex RCA with 2 genes, however, it is possible to include additional gene targets for influenza and other respiratory viruses with additional optimization.

A recent study reported that the viral loads of SARS-CoV-2 in clinical samples by RT-PCR ranged from 641 copies/mL to 1.34×10^{11} copies/mL, with a median of 7.99×10^{4} in throat samples and 7.52×10^{5} in sputum samples, and 1.69×10^{5} copies/mL for nasal swab samples taken on day 3 of post-onset of symptoms. The average viral load after early onset was $>1 \times 10^{6}$ copies/mL. In death cases, the viral load was 1.34×10^{11} copies/mL detected from sputum samples collected on day 8 of post-onset of symptoms[47]. Therefore, our assay's LoD of 1 copy/µL is lower than the viral load in clinical samples. This shows that our assay fulfills the requirement for sensitivity and could potentially be used to diagnose COVID-19 in the early stages of the illness when the viral load is still low. It is known that many factors can affect test performance and cause false negative results. Several studies have shown that the viral load varies from specimen types, collection methods and time of collection [5, 48]. Therefore, a robust diagnostic test with high sensitivity could reduce the chances of false negatives caused by low recovery of the virus from real samples.

The detection of RCA amplicons using a USB or battery-powered, portable Palmsens4 potentiostat makes it easy to conduct the test in the field or in a community setting. Indeed, electrochemical based detection using techniques such as DPV, is highly sensitive, quantitative, cost-effective and compatible with multiplexing. The test can be easily set-up in places with limited resources such as in developing countries or in community centers where an outbreak has occurred. We are currently optimizing the RNA extraction process and exploring the use of a smartphone-based biosensor device that will further simplify the test procedure and reduce the sample-to-result turnaround time.

Table 2: Sensitivity of commercially available test kits and existing biosensors for COVID-19 testing.

Detection method	Sample	Targets	LOD	Ref.
CRISPR technology	plasma, serum, and throat and nasal swab	-	-	49
	nasal swab	ORF1ab and N gene	2 copies/sample	50
	nasopharyngeal swabs	E gene and N gene	10 copies/μL	20
	-	S, N and Orf1ab genes	42 copies/reaction	51
Colorimetric assay	oropharyngeal swab	N gene	0.18 ng/μL	52
Localized surface plasmon resonance	synthetic cDNA	RdRp	2.26×10^4 copies	40
RT-LAMP	swabs and bronchoalveolar lavage fluid	orf1ab	20 copies/reaction	18
		S genes	200 copies/reaction	
	throat swabs	orf1ab	20 copies /25uL	17
		S gene	2 copies/25uL	
		N gene	2 copies/25uL	
	nasal swabs	N gene	100 copies	19
DNA nanoscaffold-based hybrid chain reaction	synthetic RNA	conserved region	0.96 pM	21
RCA	cultured material	SARS-CoV	-	26
	Synthetic DNA	RdRp	0.4 fM	53
	nasopharyngeal and/or throat swab	N gene and S gene	1 copy/μL	**This work**

Conclusion

In summary, we have developed an electrochemical biosensor coupled with RCA for the highly sensitive and specific detection of SARS-CoV-2. The high amplification capability of RCA and sensitivity of the electrochemical detection method enabled the detection of the viral N and S genes in synthetic linear targets as well as clinical samples. The whole assay took under 2 hours to complete, from RNA extraction to the detection step, and does not require the use of a thermal cycler. The performance of the assay with clinical samples was comparable to that of RT-qPCR, which is currently the standard for SARS-CoV-2 detection, while also demonstrating zero false positive results This approach may have a significant impact in places where rapid detection is required to minimize emerging SARS-CoV-2 outbreaks.

Methods

Chemicals, reagents and instrumentation

Tetraethyl orthosilicate (TEOS), acridine orange (AO), poly(allylamine)hydrochloride (PAA, MW ~56,000), poly(sodium 4-styrene) sulfonate (PSS, MW ~70,000), avidin, were purchased from Sigma-Aldrich, USA. Methylene blue (MB), 25% ammonia solution, 2-propanal were purchased from Merck, Germany. Dynabeads^{TM} MyOne^{TM} Streptavidin T1 was purchased from Thermo Fisher Scientific, USA. RCA reagents (dNTPs, phi29 DNA polymerase) were purchased from Integrated DNA technologies, USA. MagLEAD Consumable Kit and magLEAD 12gC instrument were purchased from Precision System Science, Chiba, Japan. For electrochemical measurements, 2-electrode screen printed carbon electrodes (SPCE) were obtained from Quasense, Thailand and the PalmSens4 potentiostat with PSTrace software was obtained from Palmsens, The Netherlands. All the reagent and buffer solutions were prepared with DI water (18.2 MΩ).

Design of circular DNA, primers and probes

Circular DNA, primers and probe sequences were designed to target the N and S genes of SARS-CoV-2 (accession number MN908947.3). The oligonucleotide sequences are listed in Table 1. The universal capture probe (RCA-CP) is able to bind the RCA amplicons of both the N and S genes. The capture, reporter and blocking probes were tagged with a biotin moiety at either the 3’ or the 5’ end. The mismatched targets for N and S genes contain 2 mismatched bases (shown underlined in Table 1). Oligonucleotides were synthesized by Integrated DNA Technologies Pte. Ltd., Singapore.

Table 1. Sequences of circular DNA, primers and probes used in this study.

Type	Name	Sequence (5’ à 3’)	Length
Universal Capture Probe	CP	CGCAACTGAACTACTTGTCG - Biotin	20
Blocking probe	BP	Biotin-TTTTTTTTTT	10
For N gene			
Forward primer	Ng_SARS2_F	TCATCACGTAGTCGCAACAG	20
Reverse primer	Ng_SARS2_R	CAAAGCAAGAGCAGCATCAC	20
Circular DNA	RCA-Ng	TACGTGATGACGCAACTGAACTACTTGTCGCTGTAGTTCAAGATATCGCGTCCTACCTGTTGCGAC	66
Reporter probe	Ng-RP	CAAGATATCGCGTCCTAC - Biotin	18
Linear target	Ng-LT	GTTCCTCATCACGTAGTCGCAACA GTTCAA	30
Mismatched target	Ng-MM	GTTCCTCATCACGTTCTCGCAACA GTTCAA	30
For S gene			
Forward primer	Sg_SARS2_F	TACCCATTGGTGCAGGTATATG	22
Reverse primer	Sg_SARS2_R	AGTGTAGGCAATGATGGATTGA	22
Circular DNA	RCA-Sg	ACCAATGGGTCGCAACTGAACTACTTGTCGCTGTAGTTATTCTGTCATGCGCTCACATATACCTGC	66
Reporter probe	Sg-RP	ATTCTGTCATGCGCTCAC – Biotin	18
Linear target	Sg-LT	GACATACCCATTGGTGCAGGTATATGCGCT	30
Mismatched target	Sg-MM	GACATACCCATTGGACCAGGTATATGCGCT	30

Synthesis of monodisperse silica microspheres

Monodisperse silica microspheres were prepared by the modified Stöber process[54].

Firstly, 1.375 mL of TEOS was added to 9.6 mL of 2-propanol under slow stirring. The solution was heated to 50°C and a mixture of 0.5 mL of 25% (v/v) ammonia and 1.025 mL of DI water were added with constant stirring for 1 h to form the silica seed. After that, 5 mL TEOS, 227.5mL of 2-propanol and 44.5 mL of 8.29 % (v/v) ammonia were added into the silica seed solution. Then, 45 mL of TEOS was added to the mixture at a rate of 0.5 ml/min, and the reaction was allowed to continue for an additional 30 min with fast stirring. The total volume and total reaction time were 335 mL and 1 h, respectively. The silica microsphere particles were isolated by centrifugation at 10,000 rpm for 10 min. The pellet was washed with DI water and centrifuged at 8,000 rpm for 5 min. The washing step was repeated 4 times. The silica pellet was dried at 105 °C in an oven.

Incorporation of redox dye onto the silica microspheres

The redox active dye was incorporated into the silica particles by a modified method from previous work[27]. First, 0.3 g of silica particles was mixed with 10.9 mL of 2-propanol containing 1.5 x 10^{-5} mol of the redox dye. Methylene blue (MB) dye was used for N gene detection, while acridine orange (AO) was used for S gene detection. The mixture was sonicated at 50°C for 60 min to ensure good dispersion. Next, under stirring conditions at 40°C, 0.55 mL of TEOS, 1.5 mL of 25% (v/v) ammonia and 12 mL of DI water were added separately. The reaction was allowed to proceed for another 2 h. The unbound dye was separated by discarding the supernatant after centrifugation at 8,000 rpm for 5 min. The pellet was washed with DI water and centrifuged at 8,000 rpm for 5 min. The washing step was repeated 4 times. The dye-incorporated silica (referred to as (Si) pellet was dried at 105 °C in an oven. The silica core and dye-incorporated silica were characterized by scanning electron microscopy (JSM-6610LV, JEOL Ltd., Tokyo, Japan).

Preparation of avidin-coated Si-PAA-PSS particles

SiMB and DNA conjugation was performed according to Cheeveewattanagul et al. [27]. First, 0.2 mL of PAA (10 mg/mL) was added into a solution containing Si (10 mg/ml) and mixed well via ultrasonication. The mixture was incubated at room temperature for 30 min. Then, the excess polyelectrolyte was removed by centrifugation at 8,000 rpm for 5 min and washed 3 times with 0.1 M PBS (pH 7.0). The pellet containing Si-PAA was redispersed in 1 mL DI water. Subsequently, 0.2 mL of PSS (10 mg/mL) was added to the Si-PAA solution, mixed well and incubated at room temperature for 30 min. After the centrifugation and washing step, the pellet containing Si-PAA-PSS was reconstituted with 1 mL of 10 mM PB. Next, 10 mL of avidin (21.14 mg/mL) was added and incubated at 37°C for 90 min. The avidin-coated Si-PAA-PSS particles were isolated by centrifugation at 8,000 rpm for 5 min. The pellet was washed with 0.1M PBS (pH7.0) and centrifuged at 8,000 rpm for 5 min. The washing step was repeated 3 times. The pellet containing avidin-coated Si-PAA-PSS particles (referred to as Si-Avidin) was resuspended in 2 mL of 0.1M PBS.

Functionalization of Si-Avidin with the reporter probe

The conjugation of Si-Avidin with the reporter probe (RP) was achieved via the avidin/biotin interaction. The solution containing 0.1 mL of Si-Avidin and 0.3 mL of 0.1 M PBS (pH 7.0) was mixed with 1 mL of 10 mM RP and 99 mL of 10 mM blocking probe (BP). Both RP and BP contain a biotin moiety at the 3' end, which can bind to avidin. The mixture was incubated at room temperature for 30 min. The DNA-conjugated Si-Avidin was recovered by centrifugation at 8,000

rpm for 5 min. The pellet was washed with 1 M PBS (pH 7.0) and centrifuged at 8,000 rpm for 5 min. The washing step was repeated 3 times. The pellet (referred to as Si-RP) was redispersed with 250 mL of 1 M PBS and kept at 4°C until use.

Immobilization of the capture probe on magnetic beads

The immobilization of biotinylated capture probe (CP) on magnetic beads (Dynabeads^{TM} MyOne^{TM} Streptavidin T1) was performed according to the manufacturer's instructions. 4 mL of 100 mM CP was mixed with 100 mL of magnetic beads (10 mg/mL) and 12 mL of 100 mM of poly T-blocking probe (BP). The mixture was incubated at room temperature for 30 min. The CP-conjugated magnetic bead particles (CP-MNB) were separated from the unbound CP by magnet separation, followed by a washing step of 3 times with 20 mM PBS (pH 7.0). Finally, the CP-MNB were redispersed with 100 mL of 20 mM PBS and stored at 4°C until use.

Multiplex RCA assay

The RCA assay consists of two steps, which are padlock ligation and RCA amplification [55]. For the ligation reaction, 0.6 mL of 1 mM circular DNA template for each gene and target DNA/cDNA/RNA were added to the ligation solution that contained 4mL of 10X DNA ligase buffer and 5U/mL T4 DNA ligase. The reaction mixture was incubated at room temperature for 10 min followed by a heat inactivation step at 65°C for 5 min. The ligation product, called Padlock DNA, served as the template for amplification. For the RCA amplification, the reaction mixture contained 3mL Padlock DNA, 1 mL dNTPs, 1 mL of 10X phi29 polymerase buffer, 0.1mL of phi29 DNA polymerase (10U/mL) in a final volume of 10 mL. The mixture was incubated at 30°C for 30 min, followed by heat inactivation at 95°C for 5 min. The mixture containing the RCA amplicons was resolved by agarose gel electrophoresis. Briefly, 2 mL of the RCA amplicons were resolved using 0.8% agarose gel in Tris-acetate-EDTA (TAE) buffer. The resolved gel was visualized under UV light.

Target hybridization and electrochemical detection

Firstly, 2 mL of CP-MNB and 18 mL of RCA amplicons were mixed and incubated at 50°C for 30 min. Then, a magnet was applied to the side of the tube to allow the solid-solution phase separation, followed by washing once with 20 mM PBS-Tween and 3 times with 20 mM PBS[56]. 20 mL of Si-RP was added to the CP-MNB-Target mixture and incubated at 50°C for 30 min to allow sandwich hybridization to occur. After that, the pellet was washed 3 times with 20 mM PBS-Tween and facilitated by magnet separation. Finally, the pellet was re-suspended with 0.1 M PB/KCl (pH 7.0). This solution was pipetted onto the screen-printed carbon electrode. Differential pulse voltammetry (DPV) was performed by scanning from -0.8 to -0.05 V, with a step potential of 0.01 V, modulation amplitude of 0.1 V with an interval time of 0.01 sec, and 0.1V/s scan rate.

Another hybridization strategy, called one-step hybridization, was also tested to shorten the assay time. All components (CP-MNB, Si-RP and target) were mixed together with the same volume as previously described, and incubated at 50°C for 30 min. Afterwards, the washing step was performed with 400 mL of 20 mM PBS (pH 7.0) and the pellet was resuspended in 150 mL of 0.1M PB/KCl (pH7.0). This solution was used for DPV measurements.

Sensitivity and specificity of the assay

The assay sensitivity was determined using pGEM-T Easy Vector plasmids (Promega, Madison, WI) containing the target sequence of the N (961 bp) and S (1119 bp) genes of SARS-CoV-2. Ten-fold serial dilutions ($1x10^{10}$ to 0.1 copies/µL) of each plasmid was used as the template in RCA, followed by hybridization and electrochemical detection. The assay specificity was evaluated with complementary and non-complementary targets. Non-complementary targets comprised of short gene fragments of Influenza A (IAV) and Influenza B (IVB) viruses, and 2-bases mismatch linear targets.

Detection of SARS-CoV-2 from clinical samples

A total of 105 anonymized respiratory clinical samples were used to evaluate the performance of the assay. The samples include SARS-CoV-2 positive samples and samples that tested positive for other respiratory viruses such as the influenza virus and a respiratory syncytial virus. The SARS-CoV-2-positive samples were previously tested and confirmed using the Allplex 2019-nCoV multiplex qRT-PCR assay (Seegene, Seoul, Republic of Korea) with primers and probes specifically targeting the N and S genes.

RNA was extracted from a 200 µL of nasopharyngeal and/or throat swab sample using a magLEAD Consumable Kit with the magLEAD 12gC instrument (Precision System Science, Chiba, Japan) following the manufacturer's protocol. cDNA synthesis was achieved with the ImProm-II Reverse Transcription System (Promega, Madison, WI) and random hexamer primers according to the manufacturer's protocol. Fifty RNA samples (10 positive and 40 negative for SARS-CoV-2) and 55 cDNA samples (30 positive and 16 negative for SARS-CoV-2, 9 negative for SARS-CoV-2 but positive for other respiratory viruses) were used as templates in RCA, followed by electrochemical detection.

Statistics

Statistic significances were calculated by Microsoft Excel version 16.30 and all the data were shown as mean ± s.d. The two-tailed Student's t test was used to compare differences between two groups with a P value < 0.05 as a threshold for significance. Five technical replicates were performed to improve the statistics.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files or from the corresponding author upon reasonable request. Sequence information used in this study was from National Center for Biotechnology Information (NCBI) with GenBank accessions of MN908947.3 (https://www.ncbi.nlm.nih.gov/). Source data are provided with this paper.

Declarations

Acknowledgements

This work was supported by the National Research Council of Thailand, the Research Chair Grant from the National Science and Technology Development Agency (P–15–5004), The authors would like to thank the staffs and nurses of the Institute of Urban Disease Control and Prevention (IUDC), Department of Disease Control, Ministry of Public Health, Thailand for the support and valuable specimens' collection.

Author contributions

BL, LSY, NB, TN and TC contributed to designing the study, writing and editing the manuscript. BL, NB and TC performed experiments. BL, TN and PA analyzed data and prepared figures. AOM, SV and YP conceived and supervised the study and wrote the manuscript. All authors participated in manuscript editing and approved the manuscript.

Competing interests

The authors declare no competing interests.

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Figures

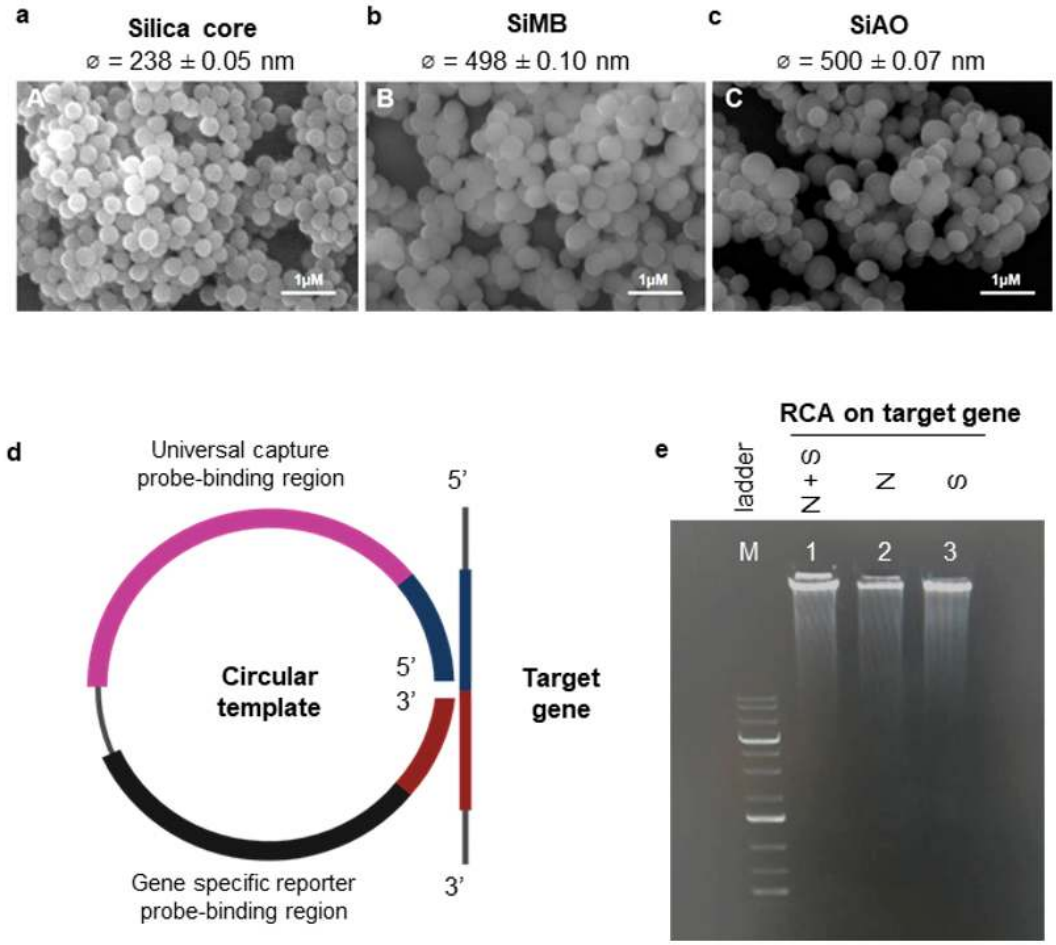

Figure 1

Visualization of the silica-redox dye and RCA amplicons. SEM image of (a) silica core, (b) silica methylene blue (SiMB) and (c) silica acridine orange (SiAO) composite particles with diameter size (n=10). (d) Target gene, universal capture probe, and gene-specific reporter probe binding regions on the circular DNA template. (e) Gel representation of RCA amplicons visualized on 0.8% agarose gel electrophoresis. Lane M indicates the 1kb DNA ladder, while lanes 1, 2 and 3 indicate the RCA amplicons of both N and S genes, N gene only, and S gene only, respectively.

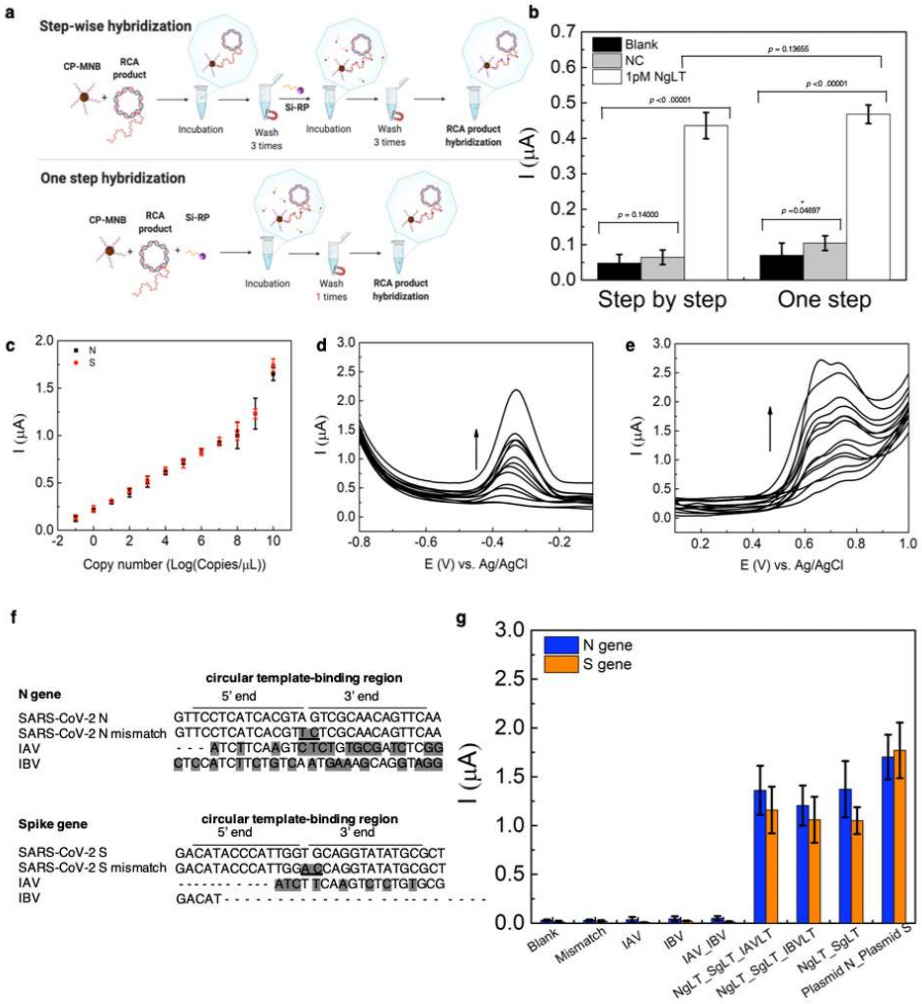

Figure 2

Assay performance. (a) Comparison of the step-wise (step-by-step) and one-step sandwich hybridization procedures using the N gene as the target. (b) Step-wise and one-step hybridization strategies produce current signals that are not significantly different ($p > 0.05$, $n = 5$). (c) Sensitivity assay for N and S genes shows a positive correlation to the copy number for both genes. Differential pulse voltammograms showing the increase in the current signal as the concentrations of N (d) and S genes (e) increased. (f) Multiple sequence alignment of the N and S gene target sequences with mismatch and non-complementary target sequences. Dark shaded areas are the non-complementary sequences to target gene, while the underlined bases are the mismatch bases. (g) The specificity of the assay with N (blue bar) and S (orange bar) genes, tested with perfect complementary targets (Ng, Sg, Plasmid N, Plasmid S), mismatch DNA target (Mismatch), and non-complementary targets (IAV, IBV). The bar charts represent the mean of 5 replications of DPV measurement.

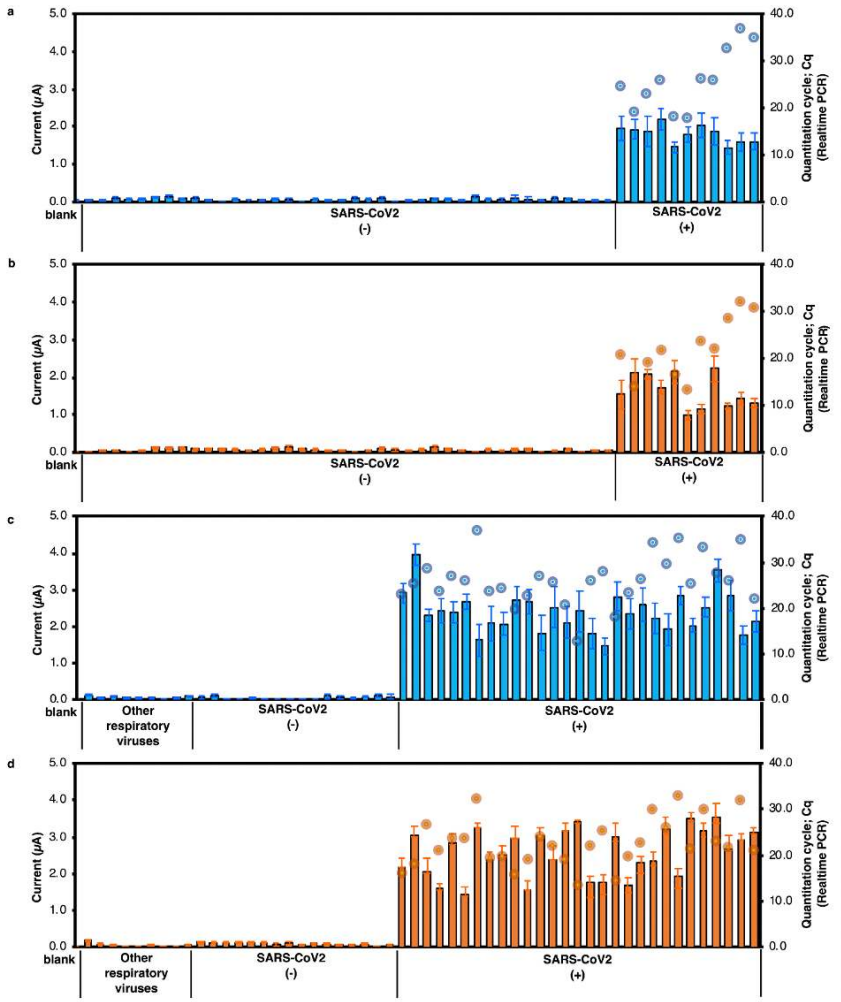

Figure 3

RCA with rapid electrochemical detection for detection of N and S genes of SARS-CoV-2 in clinical samples. A total of 50 RNA (a, b) and 55 cDNA (c, d) samples prepared from nasopharyngeal and throat swab samples were used for the evaluation. The current signals from electrochemical measurements of N gene (blue bar graph) and S gene (orange bar graph) are shown compared with the quantitation cycle result from qRT-PCR (blue dots for N gene and orange dots for S gene).

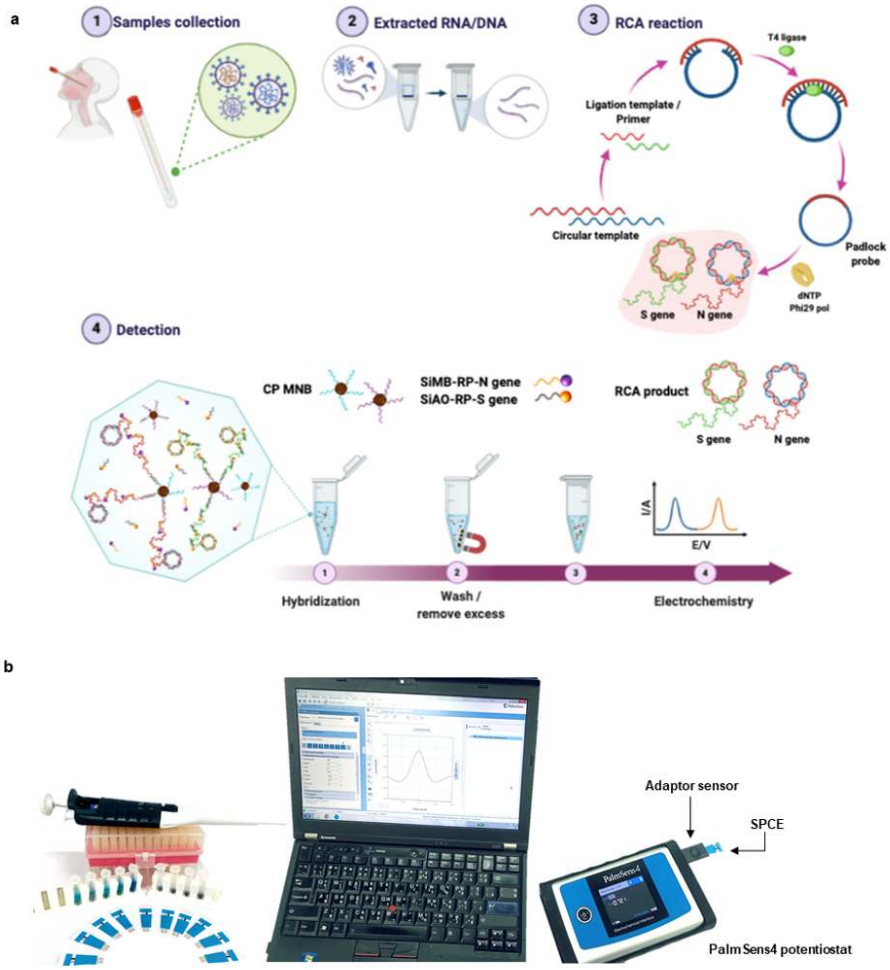

Figure 4

Scheme 1: Overview of the detection platform. (A) Detection workflow of SARS-CoV-2 from clinical samples using our electrochemical biosensor with RCA of the N and S genes. (B) The detection setup for electrochemical analysis by using a portable potentiostat device connected to a laptop.

Supplementary Files

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- Covid19RapidECSI.pdf