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Optical reflectometric measurement of SARS-CoV-2 (COVID-19) RNA based on cationic cysteamine-capped gold nanoparticles

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ABSTRACT

The coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged as a major public health outbreak in late 2019 and was proclaimed a global pandemic in March 2020. A reflectometric-based RNA biosensor was developed by using cysteamine-stabilized gold nanoparticles (cysAuNPs) as the colorimetric probe for bioassay of COVID-19 RNA (SARS-CoV-2 RNA) sequence. The cysAuNPs aggregated in the presence of DNA probes via cationic and anionic electrostatic attraction between the positively charged cysteamine ligands and the negatively charged sugar-phosphate backbone of DNA, whilst in the presence of target RNAs, the specific recognition between DNA probes and targets depleted the electrostatic interaction between the DNA probes and cysAuNPs signal probe, leading to dispersed particles. This has rendered a remarkable shifting in the surface plasmon resonance (SPR) on the basis of visual color change of the RNA biosensor from red to purplish hue at the wavelength of 765 nm. Optical evaluation of SARS-CoV-2 RNA by means on reflectance transduction of the RNA biosensor based on cysAuNPs optical sensing probes demonstrated rapid response time of 30 min with high sensitivity, good linearity and high reproducibility across a COVID-19 RNA concentration range of 25 nM to 200 nM, and limit of detection (LOD) at 0.12 nM. qPCR amplification of SARS-CoV-2 viral RNA showed good agreement with the proposed RNA biosensor by using spiked RNA samples of the oropharyngeal swab from COVID-19 patients. Therefore, this assay is useful for rapid and early diagnosis of COVID-19 disease including asymptomatic carriers with low viral load even in the presence of co-infection with other viruses that manifest similar respiratory symptoms.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) began to spread around the globe in December 2019. The coronavirus disease of 2019 or named as COVID-19 was first appeared in the Wuhan city of Hubei Province in China and was declared a global health emergency by World Health Organization (WHO) [1–2]. The clinical spectrum of SARS-CoV-2 infection appears to be wide encompassing asymptomatic infection, mild upper respiratory tract illness, severe viral pneumonia with respiratory failure and even death [3]. SARS-CoV-2 is an enveloped and spherical viral particle, approximately 120 nm in diameter, containing a positive-sense single-stranded RNA [4]. Novel SARS-

CoV-2 exhibits a significant similarity with previous coronaviruses, such as SARS-CoV in 2002 and Middle East respiratory syndrome coronavirus (MERS-CoV) in 2015. It was found that genome of COVID-19 has 80 % similarities with SARS-CoV and MERS-CoV at the nucleotide level [5]. The mechanism for COVID-19 infection required the binding of the virus to the angiotensin-converting enzyme 2 (ACE2) receptor [6].

COVID-19 infection can be detected in multiple ways, such as from the genetic materials of the virus, viral antigen and serological antibodies. Current methods for the diagnosis of COVID-19 infection include quantitative reverse transcription polymerase chain reaction (qPCR), antigen test kit and serological assay for plasma antibodies [6]. The

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qPCR method is thus far the primary method to diagnose COVID-19 infection by direct detection of SARS-CoV-2 RNA at specific region serving as the target, which includes the RdRp gene (RNA-dependant RNA polymerase), E gene (Envelope Protein) or the N gene (Nucleo-capsid) [7]. Meanwhile, the serological test examines the production of specific IgM and IgG antibodies against SARS-CoV-2 in response to the infection, whereby the SARS-CoV-2 antibodies can be detected five days after symptom onset, and IgG was detectable 14 days after symptom onset, thus providing a longer period window for active and past infection [8]. However, they sustained some disadvantages, such as required expensive equipment and reagents; high level of antibody concentration is necessary for a detectable result following exposure and infection; and risk of false positive results is high as a result of contamination by amplicons from previous nucleic acid amplifications [9]. In particular, qPCR is the gold standard for the diagnosis of COVID-19 infection, as recommended by the World Health Organization (WHO) and the American Centre for Disease Control (CDC). However, the testing workflow of qPCR consists of several steps including the reverse transcription of viral RNA to complementary DNA (cDNA), followed by PCR-based amplification of specific regions from the cDNA, which is tedious, time-consuming and error-prone task.

The failure to rapidly and accurately diagnose contagious respiratory viruses results in an increased number of patients and duration of infection. Therefore, there is a great need for an affordable, robust, rapid, accurate, flexible and simple point-of-care (POC) testing in order to control the unpredictable pandemic. This has important implications for both patient isolation decision making and guidance around the length of antiviral treatment to improve the patient's outcome as well as allows appropriate use of antibiotics and facilitates cost-effective care [10]. The cell culture method remains the gold standard in many laboratories and hospitals owing to the lack of an alternative high sensitivity technique. Although cell culture is a time-consuming and laborious method, it can generally detect over 90 % of the viruses within 48 h [9]. Rapid antigen direct tests (RADT) and direct fluorescent antibody testing (DFA) have been developed as alternative techniques, which are simple, cheap, with a rapid turnaround time of 15–30 min. However, these methods are limited by the availability of antibodies against newly found viruses and sub-families of pathogens [11]. Alternatively, real-time RT-PCR/qPCR is the current gold standard because of its superior sensitivity, rapid turnaround time (2–4 h), and ability to identify multiple types of pathogens in a single test. However, these are not well implemented in all hospitals, as they require a molecular diagnostic laboratory with specialized personnel, equipment and time to validate the products with large clinical samples [12]. In order to overcome the limitations of qPCR, the viral RNA-based biosensor with rapid, simple and specific detection method is highly desirable in the healthcare system.

In recent years, colorimetric sensor arrays based on gold nanoparticles (AuNPs) have received more and more attention due to their simplicity, rapidity, cost-effective and provides naked-eye visualization of assay strategy [13,14]. In view of the fascinating optical properties of the AuNPs associated to their strong surface plasmon resonance (SPR) absorption with colour of red or blue corresponding to their respective dispersion and aggregation states [15], they have been widely used in the fabrication of biosensors especially for the identification of micro-organisms in many fields of interest including clinical diagnostics, biology and food safety [6,16]. The colour alteration in the colorimetric sensing arrays is associated with the aggregation of AuNPs with rapid visual assessment that does not involve any complex instrumentation or trained personnel. However, the salt-aging process for DNA attachment to the mainstream citrate-capped AuNPs requires the addition in small increments of sodium chloride (NaCl) or trisodium citrate (TSC) buffer solution with pH matching that of the nanoparticle suspension to avoid irreversible aggregation of AuNPs, which is laborious and time-consuming due to isolation of the undesired electrostatic repulsion between both negatively charged citrate-capped AuNPs and DNA [17].

Previous studies revealed that by coupling of ligand compound to the AuNPs, it enhanced the AuNPs interaction with hydroxyl and amine groups on the small molecules [18,19], thereby would improve the aggregation behaviour of AuNPs for target RNA. Due to the excellent physicochemical properties and simple preparation procedure, cysteamine has been successfully applied in the detection of various analytes, e.g. drug, antibiotic, alcohol and mycotoxin upon incorporation with AuNPs [20–22].

In this study, we demonstrated reflectometric bioassay of positively-charged cysteamine-capped AuNPs (cysAuNPs) for detection of target RNA of COVID-19 based on direct electrostatic interaction between cysAuNPs, unmodified DNA probe and target RNA without undergoing NaCl-salted aged AuNP aggregates process. Herein, cysteamine ligand offers both thiol and amine groups, which enables strong chemical binding to AuNPs via thiol chemistry, and leaving the other free amine group protruding outwards to render the AuNPs a net positive surface charged, which is critical for the efficient receptor immobilization via electrostatic interaction between negatively charged phosphate sugar backbone of DNA probe and positively charged amine-terminated AuNPs. By manipulating the aggregation and dispersion behaviour of cysteamine cation-stabilized AuNPs based on nucleic acid loading, we have demonstrated a colorimetric biosensor that is able to semi-quantify COVID-19 RNA at low levels with a visual change in color as well as reflectance spectra intensities. The working principle of the proposed colorimetric-based RNA biosensor is illustrated in the schematic diagram in Fig. 1.

2. Methodology

2.1. Instrumentation

UV–vis absorption spectra of the cysAuNPs nanoparticles dispersion were measured by using a UV–vis spectrophotometer (Varian-Cary Win UV 50). The optical reflectance measurement was carried out by using Ocean Optics USB4000 spectrometer coupled to a bifurcated optical fiber with a DH-2000-BAL UV–vis-near-infrared (NIR) light source in the wavelength range of 200–1099 nm. The average size of the cysteamine-coated gold nanoparticles (cysAuNPs) was characterized by transmission electron microscopy (TEM). FTIR was carried out using Perkin Elmer Paragon 1000 FTIR spectrometer. qPCR analysis was performed on an iQ5 Bio-Rad thermal cycler (Bio-Rad, USA) interfaced with iQ5 optical system software (Bio-Rad, USA). RNA concentration was determined by using Thermo Scientific NanoDrop UV–vis 2000 spectrophotometer (USA).

2.2. Chemicals and reagent

Chloroauric acid (HAuCl_4), 2-aminoethanethiol or cysteamine, sodium borohydride (NaBH_4), Ammonium citrate dibasic, boric acid and L-ascorbic acid were obtained from Fisher Scientific (M) Sdn. Bhd. Ultrapure water (18 mΩ cm) was used for the preparation of the buffer solution. The qPCR reaction was performed using TOYOBO Thunderbird SyBR green master mix.

2.3. Oligonucleotide preparation

The oligonucleotides of target RNA and single-base mismatch (1-bp mismatch) used in this study were designed at the specific region at N gene based on the SARS-CoV-2 genome isolated from a patient in Wuhan, China. All the oligonucleotides were synthesized and procured from Apical Scientific (M) Sdn. Bhd. (Selangor, Malaysia) The oligonucleotide was resuspended in 0.1 M potassium phosphate buffer (pH 7.0) and diluted to the desired concentration by using the same buffer solution. The purified oligonucleotide sequences used in this study are listed in Table 1.

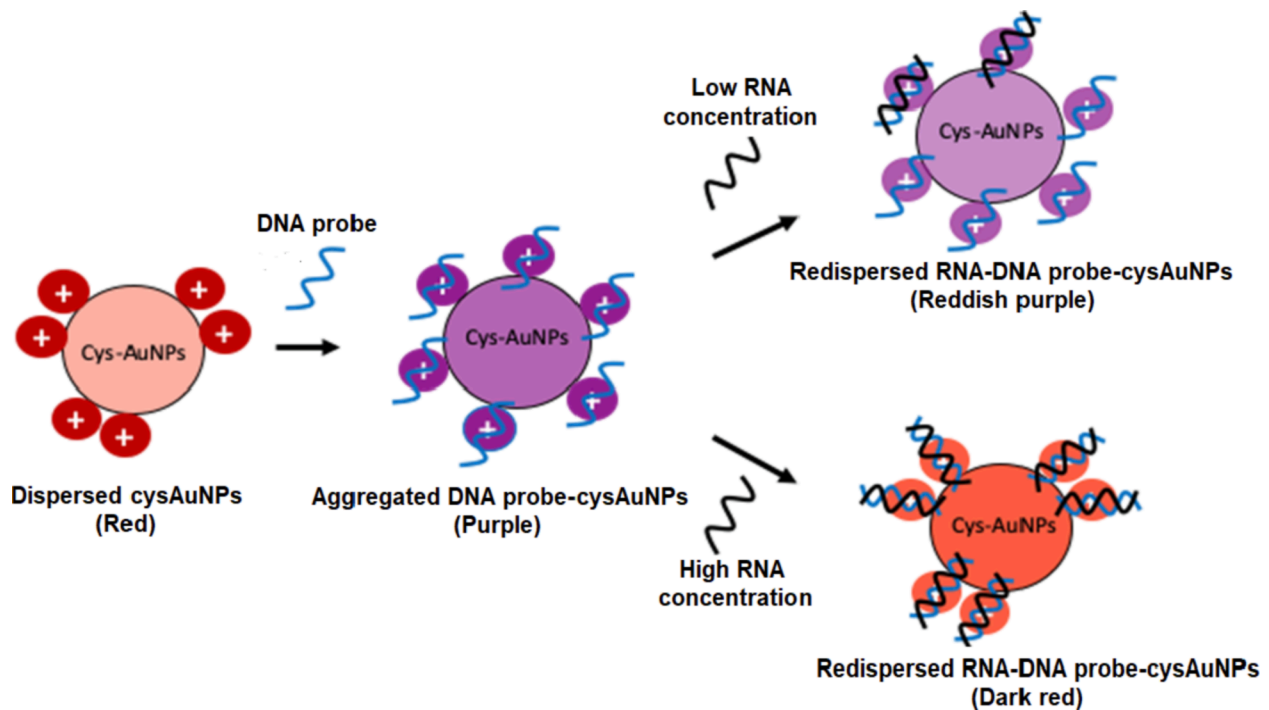


Fig. 1. Schematic representation of the working principle of the proposed reflectometric-based RNA biosensor based on cysAuNPs colorimetric agent for optical detection of target RNA.

Table 1

The list of oligonucleotides used in this study.

Oligonucleotide	Base sequence	Region
DNA Probe	5'-AGTTCCTGGTCCCCAAAATTCCTTGGGTT-3'	–
Target RNA	5'-AACCCAAGGAAUUUUGGGACCAGGAACU-3'	N Gene
1-bp mismatch	5'-AACCCAAGGAAUUUUGGGACCAGGACU-3'	N Gene
MERS-CoV	5'-AACCCUGUGUACUUCUUCGGUACAGUGGA-3'	N Gene
SARS-CoV	5'-GAGGGAGCCUUGAAUACACCCAAAGACAC-3'	N Gene

2.4. Synthesis of cysteamine-capped gold nanoparticles

Cysteamine-stabilized AuNPs (cysAuNPs) were prepared according to the previously reported study with slight modifications [23]. In brief, 400.0 μL of cysteamine (213 mM) was mixed with a freshly prepared 40.0 mL of HAuCl_4 (1.42 mM), and gently stirred in the dark until a yellowish solution formed. After that, 10.0 μL of 10 mM NaBH_4 was added in a dropwise manner under vigorous stirring for 10 min and continued with mild stirring for another 30 min. The resulting red ruby-colored solution was kept in the dark at 4 $^\circ\text{C}$ in the refrigerator overnight before used.

2.5. Preparation of DNA probe-modified cysAuNPs optical bioprobe

The optical RNA biosensor was prepared by dispensing 95.0 μL of the as-synthesized colloidal cysAuNPs in a 500.0 μL microcentrifuge tube followed by adding 2.5 μL of the single-stranded DNA probe at 300 nM, and incubated for 5 min for electrostatic immobilization of DNA probe on the cysAuNPs to take place. Hybridization between the immobilized DNA probe and target RNA was carried out by depositing 2.5 μL of 200 nM target RNA into the DNA probe-modified cysAuNPs (DNA probe-cysAuNPs) colloids, and incubated for 30 min. Optical reflectance intensities of the cysAuNPs colloidal suspension and DNA probe-cysAuNPs before and after hybridization with target RNA were taken in the wavelength range of 300 nm to 900 nm.

2.6. Optimization of the reflectometric RNA biosensor response

The dynamic linear range of the cysAuNPs-based reflectance biosensor was studied by preparing a series of target RNA concentrations from 25 nM to 300 nM and hybridized with 300 nM DNA probes, which have been pre-coated on the cysAuNPs colloids, and analysed with reflectance spectrophotometer at 765 nm. The response time of the optical RNA biosensor was determined by reacting 100 nM target RNA with DNA probe-modified colloids for 5 min, 10 min, 30 min, 40 min, 50 min and 60 min with a constant DNA probe loading at 300 nM, and the reflectometric response was recorded at 765 nm. Selectivity of the optical RNA biosensor was investigated by using different viral RNA sequences, such as 1 base-pair mismatch RNA and both MERS-CoV and SARS-CoV viral RNAs at N gene region. The reflectance response obtained was then compared with the response attained with the target RNA from the specific region at N gene of the SARS-CoV-2 (COVID-19) RNA.

2.7. Detection of SARS-CoV-2 RNA in clinical samples

Three RNA samples of the oropharyngeal swab namely PC1, PC2 and PC3 from COVID-19 patients were obtained from Malaysia Genome Institute (MGI, Selangor, Malaysia). The RNA samples were prepared for qPCR quantification of viral RNA transcript by measuring their respective Ct values. The RNA concentration was determined by using Nano-Drop Spectrophotometer (USA). 2000 viral copies per μL of 2019-nCoV_N_Positive Control (Cat. 10006625, IDT) were spiked into the respective RNA samples. The positive control spike-in samples without nucleic acid amplification was then directly used for SARS-CoV-2 RNA detection by using the proposed colorimetric RNA biosensor, whilst for qPCR analysis the positive control spike-in samples containing 50 ng RNA were converted into cDNA by using ReverTra Ace qPCR RT Master Mix Kit (Toyobo, Japan). The obtained cDNA was further used for qPCR analysis. The primers of N2 gene namely nCoV_N2 forward: 5'-TTA-CAAACATTGGCCGCAAA-3' and nCoV_N2 reverse: 5'-GCGCGA-CATTCCGAAGAA-3' (IDT, USA) at 300 nM were added into every PCR reaction mixture containing SYBR green polymerase (Thunderbird SYBR

qPCR Master Mix, Toyobo, Japan) and nuclease free water with a final volume of 10 μ L. No template control (NTC) was included in every PCR assay as a negative control. The amplification program was started with an initial denaturation step at 95 $^{\circ}$ C for 10 min, followed by 40 amplification cycles each at 95 $^{\circ}$ C for 15 s, 56 $^{\circ}$ C for 30 s and 72 $^{\circ}$ C for 30 s. The Ct value produced by qPCR analysis was later compared with the results acquired by the proposed colorimetric-based RNA biosensor. This study was approved by the National University of Malaysia Research Ethics Committee (UKMREC) with ethic approval number JEP-2021-029.

3. Results and discussion

3.1. Characterization of the as-prepared colloidal cysAuNPs

The average size of the as-prepared cysAuNPs was characterized by transmission electron microscopy (TEM) and UV-vis spectrophotometry analysis. The cysAuNPs was synthesized by the reduction of gold salt (HAuCl_4) with sodium borohydride (NaBH_4) as the reducing agent in the presence of cysteamine dihydrochloride. The UV-vis spectrophotometric spectrum of the cysAuNPs colloids in Fig. 2a exhibits a maximum absorption peak at 542 nm, which indicates the formation of small monodisperse gold nanoparticles. The surface plasmon resonance phenomenon causes an absorption of light in the blue-green portion of the spectrum (~ 450 nm) whilst red light (~ 700 nm) is reflected, and yielding a red ruby colloidal gold [24,25]. TEM analysis revealed that the as-synthesized cysAuNPs were spherical in shape, uniformly distributed and had an average particle size of 20.9 ± 4.5 nm (Fig. 2b). The particle size of cysAuNPs synthesized by pulsed laser ablation has been reported in the range 5–50 nm with a large amount of cysAuNPs with particle sizes (median) of ~ 20 nm was observed [26]. FTIR was carried out using Perkin Elmer Paragon 1000 FTIR spectrometer in order to identify the possible biomolecules responsible for capping the gold nanoparticles. Fig. 2c displays the FTIR spectrum of the as-obtained cysteamine-coated AuNPs. The existence of broad absorption band at 3305.03 cm^{-1} indicates the presence of OH- group of hydroxides due to the hydroxyl stretching vibration. Stretching vibration happened when there is continuous change in the interatomic distance along the axis of the bond between two atoms [27]. Furthermore, the prominent peak of 1534.87 cm^{-1} is interpreted as N-H bending vibration due to the gold nanoparticles in the colloid. The peaks at 939 cm^{-1} and 1072 cm^{-1} correspond to the bending vibrations of NH_2 of cysteamine moiety [28]. The obtained results confirmed the formation of cysAuNPs, which is stable for a week without aggregate formation.

3.2. The principle of cysAuNPs-based reflectometric biosensor for RNA detection

Fig. 3 shows the reflectance spectra of the cysAuNPs colloids and DNA probe-immobilized cysAuNPs (DNA probe-cysAuNPs) before and after hybridization with 200 nM target RNA. The as-prepared red ruby colloidal cysAuNPs gave a maximum reflectance signal at the wavelength of 639 nm, which was attributed to the electrostatic repulsion between cationic cysAuNPs, whereby each AuNPs was repelled from the other by an electrostatic force and that forming colloidal stable monodisperse AuNPs. Upon introduction of 300 nM non-modified DNA probes, the cysAuNPs colloidal suspension turned from red to purple hue based on electrostatic attraction-induced aggregation of the AuNPs with anionic DNA probe without the need of salt aging procedure, which normally applied in the citrate-stabilized AuNPs with weak Au-carboxylate binding energy that is susceptible to ionic of pH changes, resulting in unstable colloids. A remarkable reflectance response attenuation was observed in the direct interaction between anionic DNA probe and cationic cysAuNPs was ascribed to the purple-colored DNA probe-cysAuNPs bioprobe that possessed a darker coloration compared to the red-colored colloidal cysAuNPs, thereby considerably reduced the

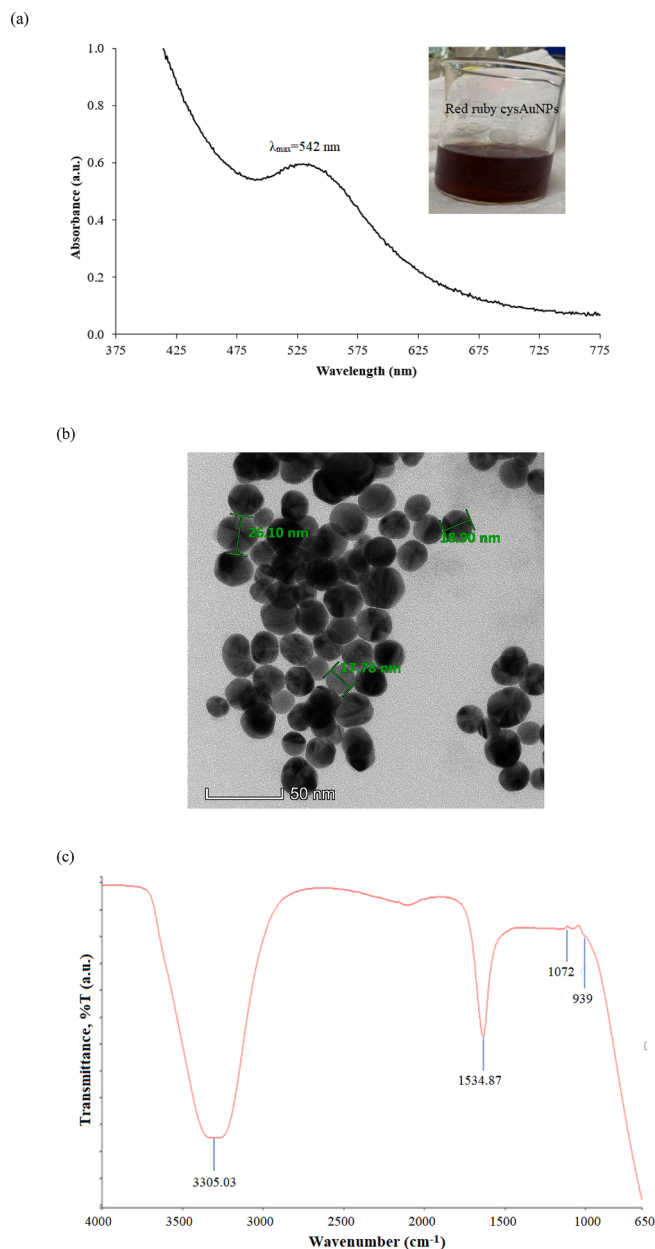


Fig. 2. (a) The UV-vis absorption spectrum of the cysAuNPs with maximum absorption peak at 542 nm. The inset shows the resulting red ruby cysAuNPs colloidal suspension. (b) The TEM image of the cysAuNPs colloids at 100 kV accelerating voltage and 100 kx magnification. (c) The FTIR spectrum of the as-synthesized cysAuNPs.

reflected light intensity. In view of the affinity of the immobilized DNA probe towards binding with its target RNA is higher than the cationic cysAuNPs, hybridization between DNA probe and target RNA neutralized the electrostatic interaction between anionic DNA probe and cationic cysAuNPs, leading to redispersion of the cysAuNPs and heightening the reflectance response. However, the level of reflectance intensity enhancement of the optical RNA biosensor based on cysAuNPs signal probe was depending on the loading of the target RNA. The purple-colored DNA probe-cysAuNPs bioprobe changed to reddish purple hue upon reaction with low concentration of target RNA due to only small number of negatively charged immobilized DNA probes were being detached from the positively charged cysAuNPs for hybridization with the target RNA, and the remaining large amount of anionic DNA probes were remained electrostatically bound to the cationic cysAuNPs colorimetric probe to induce aggregation of the DNA probe-coated

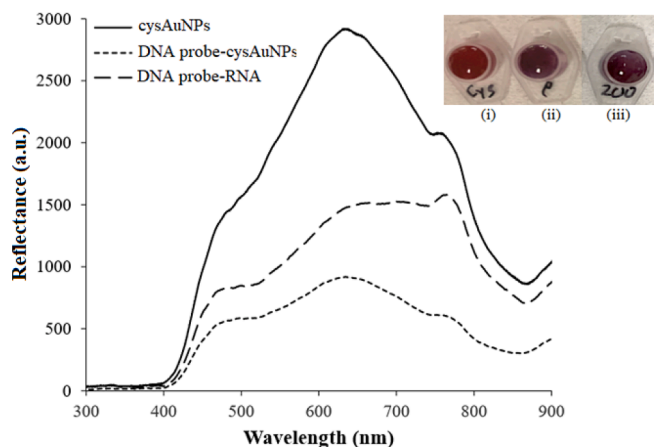


Fig. 3. Reflectance spectra of cysAuNPs and DNA probe-cysAuNPs before and after hybridization with 200 nM target RNA in the wavelength range of 300 nm to 900 nm. The inset displays (i) the original red ruby cysAuNPs and the colour change of the DNA probe-cysAuNPs optical bioprobe (ii) before and (iii) after hybridization with 200 nM target RNA.

colloids. However, further loading of high RNA concentration in the DNA probe-cysAuNPs colloids, a distinct color change from reddish purple to dark red was perceived as most of the immobilized DNA probes were detached from the cysAuNPs as a result of competitive binding of the DNA probe to the cysAuNPs and target RNA. Because of the specific interaction between DNA probes and target RNAs, it negated the electrostatic attraction between DNA probes and cysAuNPs optical probe. The positive surface charge of the cysAuNPs repelled each other and rendering redispersion of the cysteamine cation-stabilized AuNPs.

3.3. Establishing calibration curve for the determination of viral RNA

Different concentrations of target RNA were prepared between 25 nM and 300 nM and analysed with the optical RNA biosensor in order to determine the linear concentration response range of the cysAuNPs-based reflectance biosensor. The reflectance spectra of the optical RNA biosensor towards reaction with various target RNA concentrations is demonstrated in Fig. 4a. The purple coloration of the cysAuNPs-based RNA biosensor has the lowest reflectance intensity at 639 nm compared to its original unmodified red ruby cysAuNPs. Hybridization between DNA probe and target RNA between 25 nM and 75 nM has heightened the reflectance response at 639 nm due to electrostatic repulsion-induced redispersion of the AuNPs, and turned to reddish purple that conferred a brighter coloration. However, loading of the target RNA concentration above 75 nM i.e., between 100 nM and 250 nM, the RNA biosensor's maximum reflectance signal shifted to 765 nm as the DNA-coated colloids changed to dark red hue when like charges repelled each other. Over loading of the target RNA into the DNA probe-cysAuNPs colloids at 300 nM RNA, however, has shifted back the maximum reflectance intensity at 639 nm as the presence of a large amount of RNAs, which consist of ribose sugar phosphate backbones have induced an electrostatic attraction between the highly negatively charged RNAs and highly positively charged cysAuNPs. In view of the reflectance peaks of the cysAuNPs-based RNA biosensor at 765 nm provided a wide RNA concentration detection range with well-defined optical reflectance signal separation towards changes of RNA concentration, therefore it was opted as the working reflectance wavelength in the determination of viral RNA. The linear response range of the optical RNA biosensor (represented as $R-R_0$, where ' R_0 ' and ' R ' are the reflectance intensities of the DNA-stabilized AuNPs colloids at 765 nm before and after the introduction of target RNA, respectively.) was determined to be between 75 nM and 300 nM target RNA at 765 nm by using 30 nM DNA probe in the presence of 95.0 μ L colloidal cysAuNPs (Fig. 4b). The limit of

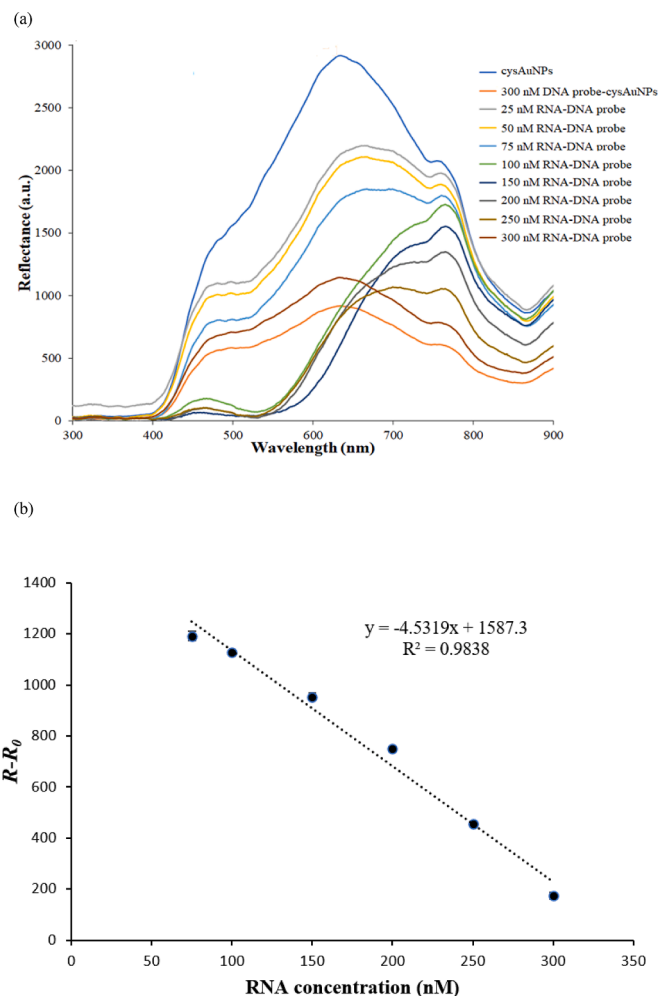


Fig. 4. (a) The reflectance spectra of the optical RNA biosensor based on cysAuNPs in different target RNA concentrations from 25 to 300 nM and (b) linear concentration response range of the optical RNA biosensor between 75 nM and 300 nM target RNA at 765 nm with DNA probe concentration was held constant at 300 nM and 95.0 μ L colloidal cysAuNPs ($n = 3$).

detection (LOD) of the RNA biosensor based on DNA probe-cysAuNPs colloids calculated according to the equation of $LOD = 3.3S/M$, where ' S ' is the average standard deviation of blank and ' M ' is the slope of the calibration curve [29] was estimated at 0.12 nM RNA. The high sensitivity and simplicity of the cysAuNPs-based colorimetric array could serve as a profoundly promising signal probe platform for labelling of nucleic acid for application in clinical diagnosis of genetic or infectious diseases, food-contaminating organisms, environmental science and pollution research, biomedicine or in criminal investigations.

3.4. Response time and selectivity of the reflectometric RNA biosensor

Response time of the reflectometric RNA biosensor determines the length of analysis duration for probe-target hybridization. The cysAuNPs-based RNA biosensor was in purple coloration prior to the introduction of target RNA with the lowest reflectance response at 765 nm due to the electrostatic attraction-induced aggregation of the AuNPs with anionic DNA probe. The response time curve in Fig. 5 shows that the reflectometric RNA biosensor for assay of 100 nM target RNA changed to reddish purple after 5 min of DNA-RNA hybridization time, and remained reddish purple for the first 10 min with colloidal cysAuNPs colorimetric agent stayed in the dispersed form. This increased the reflectance response of the DNA-stabilized AuNPs colloids (R) relative to the purple-colored cysAuNPs-based RNA biosensor before

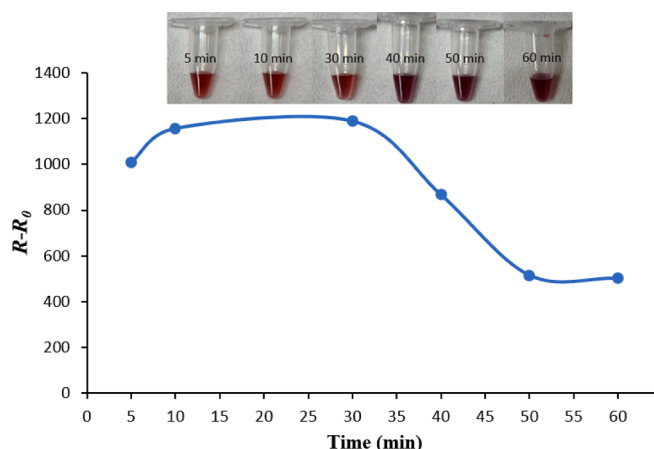


Fig. 5. Response time of the reflectometric RNA biosensor for assay of 100 nM target RNA between 5 min and 60 min incubation time by using 300 nM DNA probe in the presence of 95.0 μ L colloidal cysAuNPs ($n = 3$). (Inset) Photographic images of the corresponding colloidal cysAuNPs colorimetric bioprobe at different incubation times.

the hybridization reaction (R_0) at 765 nm. The DNA-modified colloids turned to purple coloration after 30 min of DNA hybridization reaction as a result of electrostatic interaction between highly cationic cysAuNPs and highly anionic DNA-RNA duplexes. The reflectance reading in terms of $R-R_0$ decreased from 40 min and onwards of the accumulation duration as the difference between the reflectance responses of the DNA-stabilized AuNPs colloids before and after the hybridization with target RNA was getting smaller at 765 nm due to no significant color change of the RNA biosensor was perceived before and after nucleic acid hybridization. RNA is relatively unstable at room temperature and extremely sensitive to RNase and pH alteration [30]. However, as the RNA bound to the immobilized DNA probe, it formed a stable DNA-RNA duplex structure within 30 min of the incubation period. Since the DNA-stabilized colloids started to demonstrate a visual color change from red to reddish purple as fast as 30 min of reaction time that can be measured with an optical reflectance spectrophotometer, the proposed optical reflectance biosensor exhibited diagnostic potential in nucleic acid test for viral RNA.

The selectivity of the optical RNA biosensor was examined by using several viral RNA sequences including the perfectly complementary target RNA from the specific region at N gene of the SARS-CoV-2, 1 base-pair mismatch RNA and non-complementary RNAs (ncRNAs), which comprised of N gene from both MERS-CoV and SARS-CoV viral RNAs. The reflectance response of the cysAuNPs-based nucleic acid biosensor towards different viral RNA sequences in terms of percent (%) selectivity

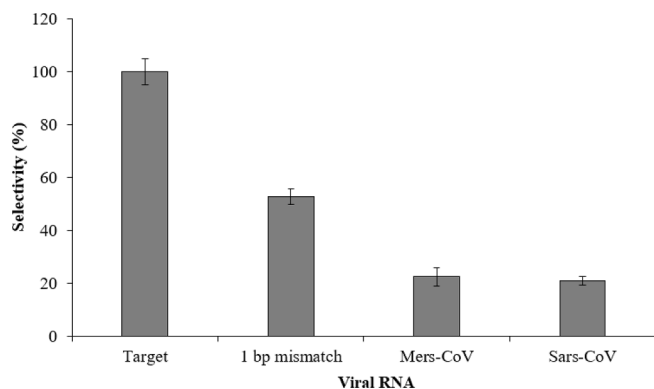


Fig. 6. Selectivity study of the reflectometric-based RNA biosensor towards determination of target RNA i.e., SARS-CoV-2 and ncRNAs such as 1-bp mismatch RNA, MERS-CoV and SARS-CoV RNAs ($n = 3$).

is presented in Fig. 6. The selectivity of the optical RNA biosensor herein is defined as the $R-R_0$ of mismatch RNA or ncRNA divided by the $R-R_0$ of target RNA. The term above can be expressed as a percent by multiplying 100 to the value. The formula used to calculate the % selectivity is as follow,

$$\% \text{ Selectivity} = \frac{R - R_0 \text{ of mismatch RNA or ncRNA}}{R - R_0 \text{ of target RNA}} \times 100\%$$

The optical biosensor exhibited 100 % reflectance response at 765 nm towards complementary target RNA. The reflectometric biosensor showed an appreciable decrease in reflectance signal with 1 base-pair mismatch RNA with a ~ 50 % reflectance response was afforded compared to the target RNA. A considerably diminishing optical reflectance response was obtained for the non-complementary viral RNA sequences of MERS-CoV and SARS-CoV with only 13 % and 23 % reflectance response at 765 nm was acquired, respectively. This affirms that the redispersion of DNA-capped colloids was only occurred in the presence of SARS-CoV-2 (COVID-19) viral RNA containing the viral N gene, and demonstrated single-base mismatch selectivity. Thus, the developed cysAuNPs-based reflectometric nucleic acid biosensor showed excellent selectivity towards COVID-19 detection. Molecular detection of SARS-CoV-2 mainly relies on the highly conserved sequence of RNA-dependant RNA polymerase gene (RdRP gene), nucleocapsid protein gene (N gene) and envelope protein gene (E gene). N gene is reported to be the most sensitive target for SARS-CoV-2 detection because of the N protein, which holds higher RNA genome of the virus as compared to the other structural proteins of spike (S), envelope (E) and membrane (M) [30,31]. Most recently, nanopore-based transcriptome analysis has shown that N gene RNA is the most abundantly expressed transcript in SARS-CoV-2 infected cells [32].

3.5. Validation study of the cysAuNPs colorimetric bioprobe for the determination of COVID-19 viral RNA

To investigate the practical application of the cysAuNPs optical sensing probe-based reflectometric biosensor for nucleic acid, the detection of SARS-CoV-2 viral RNA of the oropharyngeal swab from COVID-19 patients were carried out. The nature of nucleic acid is a crucial aspect to be considered in this study. One of the challenges for detecting viral RNA in the clinical specimen is the easily degradable RNA molecules by RNase enzyme. No amplification was detected in the initial qPCR analysis with the RNA samples may be due to no cross reactivity between the primer and the host or the possibility of degradation of nucleic acid in the samples. Spiking of 2000 viral copies μ L⁻¹ of the 2019-nCoV_N_Positive Control into the respective RNA samples was later resulted in detection of N2 gene with Ct value of < 40 (Table 2). All the spiked RNA samples demonstrated sensitive and reliable amplification with detection of Ct value approximately 30 cycles per reaction. The three positive samples showed Ct value of ≥ 30 , indicating low COVID-19 viral load with detected status, and the NTC sample gave a Ct value at 37.06, signifying not target RNA detected by the standard qPCR method.

To allow direct comparison between colorimetric RNA biosensor and qPCR, the reflectance response at 765 nm and colour change of the cysAuNPs colorimetric bioprobe in the presence of SARS-CoV-2 target RNA in positive control spike-in samples without nucleic acid amplification and negative control sample or no template control (NTC) were observed. Based on the results tabulated in Table 2, the positive samples showed obvious color change from purple to ruby red whilst NTC sample remained purple in color due to electrostatic attraction-induced aggregation of the AuNPs with anionic DNA probe in the absence of target RNA. The observation and status obtained with RNA biosensor based on DNA probe-cysAuNPs colloids were in consistent with the results produced by qPCR analysis of the COVID-19 positive samples and NTC sample in terms of Ct value. This suggests that the results obtained by cysAuNPs signal probe-based reflectometric biosensor are perfectly agreeable to the qPCR gold standard method for detection of SARS-CoV-

Table 2

Detection of SARS-CoV-2 viral RNA of the oropharyngeal swab from three COVID-19 patients (i.e., PC1, PC2, PC3) and no template control (NTC) sample by qPCR gold standard and colorimetric biosensor based on cysAuNPs.

Sample ID	qPCR		Biosensor	
	Ct value	Status	Observation	Status
PC1	30.02 ^b	Detected		Detected
PC2	30.25 ^b	Detected		Detected
PC3	31.86 ^b	Detected		Detected
NTC	37.06 ^c	Not detected		Not detected

^aCt value ≤ 25 : high viral load.

^b Ct value from 26 to 36: low viral load.

^c Ct value from 37 to 40: not detected.

2 viral RNA in the clinical samples. Although qPCR provides highly specific and sensitive detection of COVID-19 viral RNA, it is not appropriate for point-of-care (POC) settings. Nanomaterials-based colorimetric sensing approach such as the DNA-stabilized AuNPs colloids would enable the diagnostic test to be done at the POC to permit convenient acquisition of information about viral presence in non-laboratory setting with rapid turnaround time [33].

3.6. Comparison of nucleic acid- and serological-based assays to optically detect SARS-CoV-2 virus

We have summarized a comparative diagnostic potential of nucleic acid and serological assay systems used to detect COVID-19 infection in Table 3. Contemporary literature on COVID-19 diagnostic strategies revealed that optical approaches have shown great potential to be used in the detection of SARS-CoV-2. In particular, nucleic acid detection of SARS-CoV-2 is currently under intensive investigation. Qiu et. al [34] suggested the detection of SARS-CoV-2 RdRp gene by using two-dimensional gold nanoisland (AuNIs) functionalized with complementary DNA receptor provides a sensitive nucleic acid hybridization. Their dual-functional localized surface plasmon resonance (LSPR) biosensor exhibited lower detection limit at 0.22 pM. However, thermoplasmonic heat is required to be generated on the AuNIs chip when illuminated at

Table 3

Comparison of nucleic acid- and serological-based optical biosensors for SARS-CoV-2 virus detection.

Assay type	Strategy	Target	LOD	Advantage	Disadvantage	Reference
Nucleic acid assay	Reflectometric RNA biosensor based on DNA probe-cysAuNPs colloids on the basis of visual color change as a result of competitive binding of DNA probe to the cysAuNPs and target RNA.	RNA SARS-CoV-2 (N gene)	0.12 nM	Simple, rapid, cost effective, high throughput and non-invasive method for early diagnosis of COVID-19 disease including asymptomatic carriers with low viral load even in the presence of co-infection with other viruses without complex instrument.	30 min DNA hybridization reaction is required.	Present Study
	Dual-functional plasmonic biosensor combining plasmonic photothermal (PPT) effect and localized surface plasmon resonance (LSPR) sensing transduction with DNA receptor functionalized gold nanoisland (AuNIs) for nucleic acid hybridization detection.	RNA SARS-CoV-2 (RdRp gene)	0.22 pM	Specific genomic detection system; capable to differentiate RdRp gene sequences from SARS-CoV and SARS-CoV2.	Thermoplasmonic heat is required to be generated on the AuNIs chip when illuminated at their plasmonic resonance frequency in order to elevate in-situ hybridization.	[34]
	SPR structure incorporating layers of silicone and BaTiO ₃ on top of Ag using thiol-tethered DNA as ligand. Numerical analysis based on transfer matrix theory and finite-difference time-domain (FDTD) technique is integrated to characterize the sensor response.	RNA SARS-CoV-2 (RdRp gene)	–	Highly sensitive, non-invasive biosensing and label free detection.	The results are primarily numerical and experimental validation study is required.	[35]
Serological assay	CRISPR/Cas12a-based-detection with naked eye readout. A ssDNA reporter labelled with a quenched green fluorescent molecule, which will be cleaved by Cas12a when there is nucleic acid of SARS-CoV-2 in the detection system. Green fluorescence can be seen with the naked eye under 485 nm light.	RNA SARS-CoV-2 (E gene)	1.00–1000.00 copies RNA	Portable, simple, sensitive and specific detection of SARS-CoV-2 virus.	Required amplification step of RT-RAA. to obtain enough DNA before a CRISPR/Cas12a reaction could be implemented at 37 °C.	[36]
	Microfluidic chip-based biosensor for spike protein detection with LSPR and electrodeposited gold nanospikes coupled with an optical probe.	Antibody (S protein)	0.50 pM	Rapid assay.	Invasive as blood plasma is required for the detection of anti-SARS-CoV-2 spike protein antibodies.	[37]
	Plasmonic fiber-optic platform for one step wash free detection of N-protein for COVID-19 confirmation in saliva sample.	Antibody (N protein)	–	Handy and sensitive diagnostic with minimal samples pre-processing.	Delayed immune response; false positive/negative results due to low level of antibody IgM/IgG.	[38]

their plasmonic resonance frequency in order to elevate the in-situ hybridization. A SPR-based sensing platform based on Kretschmann configuration was constructed by Uddin et al. [35] by incorporating layers of silicone and BaTiO₃ on top of silver (Ag) substrate with thiol-tethered DNA as ligand for detection of RNA SARS-CoV-2 RdRp gene. The SPR sensor was integrated with an extensive numerical analysis, and achieved some 7 times enhanced sensitivity compared to the basic Kretschmann configuration. Anyhow, the analysis results are primarily numerical and experimental validation of such enhancements are required. Other than that, an advanced nucleic acid detection strategy based on CRISPR/Cas12a system by Wang et al. [36] is capable to detect as few as 10 copies of the viral gene in 45 min without sophisticated instrument and reported to have a good consistency with qPCR assay. This system proposed a ssDNA reporter labelled with a quenched green fluorescent molecule, which will be cleaved by Cas12a protein when there is a nucleic acid of SARS-CoV-2 in the detection system. Green fluorescence can be seen with the naked eye under 485 nm light. Nonetheless, the CRISPR/Cas12a-based detection requires reverse transcript recombinase-aided amplification (RT-RAA) step so as to obtain enough DNA for subsequent Cas12a-mediated detection.

On the other hand, optical platform of serological assay showed great potential to improve COVID-19 diagnosis. An optical microfluidic chip-based biosensor was developed to detect antibodies against SARS-CoV-2 spike protein [37]. The sensing mechanism relies on the LSPR involving electrodeposited gold nanospikes in a microfluidic device coupled with an optical probe. The antigen-antibody binding causes local refractive index to change that is correlated with the LSPR wavelength peak shift of gold nanospikes. The developed sensing platform achieved a detection limit of 0.5 pM in 30 min. However, the proposed opto-microfluidic device is invasive as blood plasma is required for the detection of anti-SARS-CoV-2 spike protein antibodies, which is a common practice in serological assays. Meanwhile, Murugan et. al [38] described a plasmonic fiber-optic platform for one-step, wash-free detection of SARS-CoV-2 virus particles in saliva samples with minimal sample pre-processing. The fiber-optic absorbance biosensor, which is based on a U-bent optical fiber sensor system, is a handy and sensitive diagnostic platform that has been used to detect a variety of biomolecular analytes for a long time. The plasmonic immunoassays based on fiber-optic platform could be a good option for diagnosing 10^6 particles per mL in 15 min, but the limitation for serological assay includes the delayed in immune response and false positive or negative results due to low level of antibody IgM/IgG.

The proposed optical RNA biosensor based on cysAuNPs colorimetric bioprobe via reflectometric transduction provides an alternative simple/easy solution for rapid and early diagnosis of COVID-19 with an observable visual color-change signals acquired in 30 min by virtue of the well-dispersed nature of the cysAuNPs colloid and their aggregation behaviour. The facile configuration of the DNA probe-cysAuNPs colloids would enable high throughput COVID-19 diagnostic screening in non-invasive human fluids without the need for peripheral equipment. This proposed research could improve a better screening and detection strategies particularly to be established into POC testing systems.

4. Conclusions

The optical RNA biosensors based on cysteamine cation-stabilized AuNPs demonstrated good potential as colorimetric biosensor with high sensitivity and selectivity as well as high speed of viral RNA detection. The colour alteration of the cysteamine ligand-capped AuNPs based on immobilized anionic nucleic acid loading can be a favourable colorimetric probe and a versatile nucleic acid biosensing system without requiring any aid of complex instruments. The cysAuNPs-based colorimetric array showed colour change with nucleic acid in the visible region, which can be visualized by naked eyes, permits measurement by using simple instruments e.g., smart phone camera and portable scanner devices for interpretation of results. The proposed cysAuNPs-based RNA

biosensor would serve as a promising signal probe platform for early diagnosis and monitoring as well as surveillance of COVID-19 infection in human for rapid and real-time assessment even on the first day of disease onset.

CRediT authorship contribution statement

Nur Diyana Jamaluddin: Investigation, Validation, Writing – original draft. **Nadiah Ibrahim:** Validation, Writing – review & editing. **Nurul Yuziana Mohd Yusof:** Project administration, Supervision, Funding acquisition. **Choo Ta Goh:** Supervision, Visualization, Methodology. **Ling Ling Tan:** Conceptualization, Funding acquisition, 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

Data will be made available on request.

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