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Ultrasensitive SARS-CoV-2 diagnosis by CRISPR-based screen-printed carbon electrode

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HIGHLIGHTS

• The ssDNA reporter randomly distributed on the electrode could be cleaved by activated Cas12a protein as well as the ssDNA fixed upright on the surface of the electrode which could greatly shorten the time of ssDNA reporter immobilization.
• CRISPR-SPCE enabled ultra-sensitivity SARS-CoV-2 detection, with a detection limit as low as 0.27 copy/μL.
• CRISPR-screen-printed carbon electrode system is fast, high specific and low cost, thus providing a simple and faster path way for SARS-CoV-2 detection.

ABSTRACT

Early and accurate diagnosis of SARS-CoV-2 was crucial for COVID-19 control and urgently required ultra-sensitive and rapid detection methods. CRISPR-based detection systems have great potential for rapid SARS-CoV-2 detection, but detecting ultra-low viral loads remains technically challenging. Here, we report an ultra-sensitive CRISPR/Cas12a-based electrochemical detection system with an electrochemical biosensor, dubbed CRISPR-SPCE, in which the CRISPR ssDNA reporter was immobilized onto a screen-printed carbon electrode. Electrochemical signals are detected due to CRISPR cleavage, giving enhanced detection sensitivity. CRISPR-SPCE enables ultrasensitive SARS-CoV-2 detection, reaching as few as 0.27 copies μL⁻¹. Moreover, CRISPR-SPCE is also highly specific and inexpensive, providing a fast and simple SARS-CoV-2 assay.
1. Introduction

The fast-spreading severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is endangering human health and the world’s economies, lowering production and limiting travel [1–3]. The detection of SARS-CoV-2 is crucial for isolating infected individuals and preventing the virus from spreading in the general population [4,5]. Digital PCR and real-time reverse transcription PCR have been applied for SARS-CoV-2 detection [6–8]. However, these methods require special instruments, skilled personnel and are time-consuming [9–11]. Recently, new methods requiring no particular instrumentation have been developed based on the CRISPR/Cas system (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein) [12,13].

CRISPR/Cas system, which normally plays the role of a bacterial immune systems [14,15], has been developed into powerful tools for genome editing [16,17], genome imaging [18], genome editing [16,17], genome imaging [18], cell imaging [19–21], and disease treatment [22–24]. The CRISPR/Cas system is also a promising platform for next-generation molecular detection technologies with high sensitivity and specificity that require no special instrumentation [25,26]. The trans-cleavage activity of Cas effector proteins (such as Cas12, Cas13, Cas14, etc.) [27–30] combined with signal amplification [31,32] has achieved ultrasensitive, specific and cheaper DNA or RNA detection in applications such as SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) and DETECTR DNA (endonuclease-targeted CRISPR trans reporter) [33–36].

Electrochemical sensors are undergoing rapid development due to their unique and attractive advantages, including high sensitivity, rapid signal readout, low cost, and portability [37–39]. Dai et al. reported a CRISPR/Cas12a (cpf1)-based electrochemical biosensor (ECRISPR) for the detection of human papillomavirus 16 (HPV-16) and parvovirus B19 (PB-19), which is more cost-effective and portable than biosensors based on optical-transduction [40]. Sheng et al. integrated the CRISPR/Cas13a system and a catalytic hairpin DNA circuit (CHDC) on a reusable electrochemical biosensor to detect non-small-cell lung carcinoma-related RNA [41]. Chaibun et al. reported an ultrasensitive electrochemical biosensor based on isothermal rolling circle amplification (RCA) for rapid detection of SARS-CoV-2 [42]. Ali et al. constructed a 3D nano-printed three-dimensional electrodes, coated with reduced-graphene-oxide and gold film combined with antigen and antibody for the detection of COVID-19 [43]. The electrochemical CRISPR/CHDC system is a fast, inexpensive and highly accurate tool for early cancer diagnosis.

To achieve the trans-cleavage of the ssDNA reporter through the CRISPR/Cas system on electrochemical sensors, ssDNA was fixed onto the working gold electrode through Au-SH bonds [44–46]. Although stable, gold electrodes or screen-printed gold electrodes are more expensive than carbon-glass electrodes or screen-printed carbon electrodes, and the formation of Au-SH bond takes as long as 12 h [47–52]. To solve this problem, we employed CeO2 nanorods and poly (allylamine hydrochloride) (PAH) to randomly immobilize the ssDNA onto a screen-printed carbon electrode (SPCE). PAH is a positively-charged weak polyelectrolyte, and the positively charged PAH layer could attract negatively charged ssDNA through electrostatic adsorption [53,54]. The CeO2 nanorods then immobilize ssDNA through the affinity of cerium to phosphate-containing ligands. The combination of PAH and CeO2 enabled the facile immobilization of reporter ssDNA onto the electrode [55]. We demonstrated that ssDNA randomly distributed in the CeO2/PAH complex on the electrode could be cleaved by activated Cas12a protein, and the ssDNA was fixed upright on the surface of the electrode. Based on this, we established a CRISPR ssDNA reporter immobilizing onto SPCE, dubbed CRISPR-SPCE, enabling the rapid diagnosis of COVID-19.

2. Materials and methods

2.1. Reagents and materials

The LbCas12a protein was expressed in Escherichia coli and purified as described previously [56]. The E gene fragment of SARS-CoV-2 (Wuhan-1 strain, GenBank: MN908947) was synthesized by GenScript (Nanjing, China) and was used as the target gene fragment template for the experiments [57]. A 228-bp fragment (Fig. 3) was amplified by PCR using the forward primer (PCR-F) and reverse primer (PCR-R). Reverse-transcription recombinase polymerase amplification was conducted using the GenDx ERA Kit (Suzhou GenDx Biotech, China). The RT-RPA for the E gene was performed using forward primer (RT-RPA-F) and reverse primer (RT-RPA-R). The crRNAs (Fig. 3) targeting E gene and ssDNA reporter (Fc-labeled ssDNA) were used for electrochemical detection. All oligonucleotides were synthesized by GenScript (Nanjing, China) and the detailed sequences are listed in Table S1. Pseudoviruses based on 4 coronavirus species, including SARS-CoV-2, hCoV-HKU1, MERS-spF and SARS-CoV-1 were purchased from Cobioer Biotechnology Company (Nanjing, China).

2.2. Synthesis of CeO2 nanorods

CeO2 nanorods were synthesized based on a previous report [58], with minor modifications as follows: 0.4 g of CeCl3⋅7H2O was dissolved in 15 mL ultrapure water, mixed with 20 mL of a 600 g L−1 NaOH solution and stirred for 30 min. Then, the mixture was transferred into a 50 mL high-pressure reaction vessel and kept at 140 °C for 24 h. After cooling the reaction vessel to room temperature, the product was washed with double distilled water until the pH became neutral and rinsed with ethanol several times. The resulting product was dried at 80 °C in an oven overnight.

2.3. Biosensor fabrication

The SPCE consisted of a silver pseudo-reference electrode, a carbon working electrode with a diameter of 3 mm, and a carbon counter electrode. To obtain a homogeneous dispersion, one mg of CeO2 nanorods and 5 mg polyethylenimine were dissolved in 1 mL water and sonicated for 5 min. Then, the CeO2/PAH suspension and 2 μL ssDNA were mixed at equal proportion. The carbon working electrode was modified with 3 μL of the CeO2/PAH/ssDNA mixture and left to dry at room temperature. The crRNA mixture (1 nM) contained crRNA1, crRNA2, crRNA3 and crRNA6 (0.25 nM, respectively, Fig. 3). The CRISPR/Cas12a reaction mix consisted of 2 μL 10 × Buffer, 1 μL CRISPR/Cas12a (200 ng μL−1), 1 μL crRNA mixture (1 nM), 1 μL RNase Inhibitors (40 U μL−1, Novoprotein, China), 1 μL the synthesized E gene fragment or RT-RPA product and nuclease-free water up to 20 μL. The buffer consisted of Tris-HCl (pH 7.5, 2 mM), glycerin (1%, v/v), and NaCl (0.5 mM). Then 20 μL of the CRISPR/Cas12a reaction mix was dropped on the surface of SPCE for trans-cleavage of ssDNA at 37 °C for 60 min.

2.4. Recombinase polymerase amplification

Isothermal amplification was conducted using the GenDx ERA Kit (Suzhou GenDx Biotech, China). Briefly, the RT-RPA was performed at 37 °C for 20 min in a 50 μL reaction comprising 25 μL of reaction buffer, 2.5 μL of RT-RPA-F (10 μM), 2.5 μL of RT-RPA-R (10 μM), 5 μL sample, 2.5 μL of magnesium acetate (280 mM), and ddH2O to 50 μL. Reverse transcription of RNA was accomplished by the reverse transcriptase in the RT-RPA system at the same time. After amplification, 5 μL of the product was transferred to the Cas12a reaction system for electrochemical detection.
2.5. Electrochemical detection

Electrochemical measurements including cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed in 100 mM Tris-HCl buffer pH 7.4. DPV with a potential range from −0.2 to 0.7 V, a scan rate of 0.05 V s\(^{-1}\), an amplitude of 0.05 V and pulse width of 0.05 s was applied before and after the treatment with the CRISPR/Cas12a system to obtain the electrochemical signal change of ferrocene.

2.6. RT-qPCR assay

The RT-qPCR detection of the SARS-CoV-2 E gene was carried out according to the WHO recommended procedure [59]. The reaction system had a volume of 20 μL, consisting of 10 μL of 2 × Probe Master Mix (AceQ U+, Vazyme Biotech Co., Ltd., Nanjing, China), 0.4 μL of PCR-F, 0.4 μL of PCR-R, 0.2 μL of probe, 1 μL of template, and 8 μL of double distilled water. The reaction was carried at 37 °C for 2 min and 95 °C for 5 min, followed by 60 cycles at 95 °C for 10 s, and 60 °C for 30 s in a Bio-Rad CFX96 Touch RT-PCR system (Bio-Rad, USA). Reverse transcription of RNA was accomplished by the reverse transcriptase in the RT-PCR system.

3. Results and discussion

3.1. Working principle

The working principle of the biosensor is illustrated in Scheme 1. The RPA amplification reaction mixture containing primer and reverse transcriptase was used to convert amplified RNA from the samples into sufficient DNA. In the presence of the synthetic target gene fragment and crRNA, the Cas12a protein could be activated to exert its trans-cleavage activity on non-specific ssDNA. When the mixture containing the synthetic target gene fragment, Cas12a protein and crRNA was added onto the surface of SPCE modified with CeO\(_2\)/PAH/Fc-labeled ssDNA, the Fc-labeled ssDNA was cleaved by the activated Cas12a protein to induce a decrease of the electrochemical signals, which could be used for the detection of SARS-CoV-2.

3.2. Trans cleavage of CeO\(_2\)/PAH/Fc-labeled ssDNA on the surface SPCE by the CRISPR/Cas12a system

PAH binds DNA onto the SPCE through the electrostatic interactions between amine groups and the phosphate backbone [60]. Cerium is a heavy metal with a strong affinity to phosphate, which can also be used to adsorb DNA. As a result, PAH and CeO\(_2\) nanorods were both used to immobilize Fc-labeled ssDNA onto SPCE.

Moreover, CeO\(_2\) nanorods have excellent electrocatalytic properties, which also enhance the sensitivity [61]. The Ce\(^{3+}\) and Ce\(^{4+}\) oxidation states can easily gain and lose electrons to facilitate the electrochemical reaction. Finally, the oxygen vacancies in CeO\(_2\) nanorods are also beneficial for improving oxygen mobility and enhancing the electrocatalysis ability [61].

Fig. 1A shows a TEM image of PAH-dispersed CeO\(_2\) nanorods, indicating good dispersion. The length of the nanorods ranged from 150 to 220 nm, with an average value of approximately 200 nm. The XPS survey spectrum of the CeO\(_2\) nanorods (Fig. 1B) identified the binding energies of C1s, Ce3d and O1s, indicating the chemical composition and valence states of CeO\(_2\).

Cyclic voltammetry was used to investigate the performance of different SPCE. PAH/Fc-labeled ssDNA modified SPCE sensors (Fig. 2A, curve a). The curves showed a couple of weak redox peaks at 0.297 V and 0.247 V, indicating the successful immobilization of Fc-labeled ssDNA. In the presence of CeO\(_2\) nanorods, the redox peaks became stronger, well defined and symmetric. These peaks occurred at 0.303 V and 0.273 V (ΔE = 30 mV) indicating a higher electron transfer rate (Fig. 2A, curve b). CeO\(_2\) nanorods combined with PAH were used to not only enhance the immobilization of ssDNA, but also accelerate electron transfer.

Then, we investigated the cleavage performance of the activated Cas12a-crRNA system on ssDNA immobilized on SPCE through DPV. The CeO\(_2\)/PAH/ssDNA modified SPCE showed a peak current value of 13.34 μA at 0.24 V (Fig. 2B curve a). After incubation with the Cas12a-crRNA system without the synthetic target gene fragment, the peak current practically did not exhibit any change at all (Fig. 2B, curve b), indicating that the Fc-labeled ssDNA was stably immobilized on the
When the SPCE was incubated in the Cas12a-crRNA system with the synthetic target gene fragment for 60 min, the peak current significantly decreased from 13.34 to 1.869 μA due to the activated Cas12a protein cleaving the ssDNA on the SPCE surface, resulting in the Fc-labeled ssDNA fragment falling off. Taken together, the random DNA attached to the SPCE surface, as well as the ssDNA strands immobilized in an upright orientation on the electrode, could be cleaved by activated Cas12a protein, demonstrating the feasibility of the simple DNA immobilization method for the CRISPR/Cas system.

The trans-cleavage ability of CRISPR was also validated by polyacrylamide gel electrophoresis (Fig. 2C). The bands of the synthetic target gene fragment and Fc-labeled ssDNA complex are shown in lane 1. In the presence of Cas12a (lane 2), there was no change in the bands of the synthetic target gene fragment and ssDNA. In the presence of crRNA (lane 3), an additional upper band belonging to crRNA was observed. In the absence of the synthetic target gene fragment, the Cas12a/crRNA/ssDNA complex showed independent strips of crRNA and ssDNA, respectively (lane 4), indicating that Cas12a was inactive. In the presence of the synthetic target gene fragment, Cas12a was activated and indiscriminately cleaved ssDNA, leading to the disappearance of crRNA, synthetic target gene fragment and ssDNA (lane 5). These results indicated that trans-cleavage was only successful in the presence of Cas12a protein.

3.3. Optimization of reaction condition

The binding of crRNA to target sites could trigger the trans-cleavage of ssDNA. The CRISPR detection readout signal relies on the crRNA-dependent targeting cleavage efficiency, which is affected by the secondary structure and spacer sequence of the crRNA [62,63].

Six different crRNAs (crRNA1-crRNA6, Fig. 3) binding to different target sites were designed and evaluated under the optimized conditions for the detection of the synthetic target gene fragment (insert of Fig. 3). The ΔI (%) on the insert of Fig. 3 represents the percentage decrease of the ferrocene electrochemical signal before and after incubation with the activated CRISPR/Cas12a system. The six crRNAs showed obvious responses in the presence of the synthetic target gene fragment, indicating that all six crRNAs could bind to target sites and trigger the trans-cleavage of ssDNA. Furthermore, crRNA1-crRNA3 and crRNA6 showed more sensitive responses compared with crRNA4 and crRNA5. Thus, we chose the complex (1 nM) of crRNA1, crRNA3, crRNA3 and crRNA4 (0.25 nM respectively) for further experiments to obtain a strong detection signal, thus ensuring the stability of detection by avoiding the deactivation of crRNA recognition due to single nucleotide polymorphisms in the target region.

To achieve the best performance, experimental conditions such as the concentrations of CeO$_2$ and PAH as well as the incubation time of Fc-labeled ssDNA were investigated (Fig. 4A). The addition of CeO$_2$ nanorods increased the sensitivity of the electrochemical signal, while a
higher concentration might increase the background signal and decrease the ΔI (%), which was defined as the percentage decrease of ferrocene the electrochemical signal before and after incubation with the activated CRISPR/Cas12a system. Along with the increase of CeO$_2$ nanorod concentration from 0.5 to 4 mg mL$^{-1}$, the ΔI value reaches the maximal value at the concentration of 1 mg mL$^{-1}$ and decreased in the range of 1.0–4.0 mg mL$^{-1}$. Therefore, 1.0 mg mL$^{-1}$ was selected as the optimal concentration of CeO$_2$ nanorods.

Considering that the concentration of PAH would affect the amount of immobilized Fc-labeled ssDNA, ΔI gradually increased with the increasing of PAH concentration and reached its maximum value at 5.0 mg mL$^{-1}$ (Fig. 4B). Accordingly, 5.0 mg mL$^{-1}$ of PAH was selected for...
sensitive detection.

It is well-known that the concentration of Fc-labeled ssDNA is also one of the critical factors affecting the sensitivity of the biosensor [64–66]. An excess of Fc-labeled ssDNA may result in shedding from the SPCE and impact the electrochemical signal. An appropriate concentration is beneficial to maintain stability and increase sensitivity. The results showed that 2 μmol L⁻¹ Fc-labeled ssDNA resulted in maximal ΔI (Fig. 4C). The ΔI curve reached saturation in the range of 3.0–4.0 μmol L⁻¹, indicating that Fc-labeled ssDNA tended to be over-saturated. Thus, 2 μmol L⁻¹ was chosen as the optimal concentration of Fc-labeled ssDNA.

In addition, the incubation time of SPCE in the CRISPR/Cas system which had a profound effect on the biosensor was optimized in the range from 10 min to 2 h. As depicted in Fig. 4D, the peak current increased from 20 min and tended to level off after 1 h, indicating the completion of trans-cleavage. Hence, 1 h was selected as the optimal incubation time for the CRISPR/Cas system with Fc-labeled ssDNA on SPCE.

Additionally, magnesium ions can promote the trans-cleavage process of the CRISPR/Cas12a system [67]. Consequently, the concentration of magnesium ions was also investigated, and 15 mM was selected as the optimal concentration (Fig. 4E). Also, the effect of the length of the Fc-labeled ssDNA fragments on the trans-cleavage activity was investigated (Fig. 4F). Fc-labeled ssDNA with a length of 5 nt had a lower response, probably due to the distance hindering the trans-cleavage of Cas12a, while lengths from 10 to 30 nt had comparable results. Therefore, an ssDNA length of 10 nt was selected for this system.

3.4. Sensitivity, detection limit and stability

The sensitivity of the system was investigated under the optimized conditions. With increasing amounts of the E gene fragment of SARS-CoV-2, the peak current of ferrocene decreased and ΔI increased (Fig. 5A). The current response showed a linear relationship with the amount of the synthetic target gene fragment in the concentration range of 2 × 10⁻⁸ to 5 × 10⁻⁵ ng μL⁻¹ and the detection limit was calculated to be 5.0 × 10⁻⁹ ng μL⁻¹.

By utilizing RPA, the sensitivity of the CRISPR/Cas system was enhanced more than 100-fold, resulting in a limit of detection (LOD) of 5.0 × 10⁻¹¹ ng μL⁻¹ (0.27 copies μL⁻¹). The comparison shown in results Table 1 indicates that the proposed method has higher sensitivity than previously reported methods [68–76].

The increased sensitivity was a result of the electrochemical method and the immobilization method. Electrochemical methods are generally recognized as highly sensitive. Fc-labeled ssDNA was immobilized on the electrode through PAH and CeO₂ nanorods. The electrostatic interactions of PAH [53,54] and the adsorption affinity of CeO₂ [55] resulted in the immobilization of large amounts of ssDNA, which improved the sensitivity.

To investigate the stability of the proposed biosensor, the fabricated biosensor was stored at room temperature and used for measurements after 7 and 10 days, respectively. The biosensor retained 99.35% and 98.67% of the initial electrochemical signal after 7 days and 10 days, respectively. These results demonstrated that Fc-labeled ssDNA was stably conjugated onto the surface of SPCE, and remained functional for the detection of SARS-CoV-2 after prolonged storage.

3.5. Specificity and application in sample analysis

To assess the specificity of the SPCE sensors, pseudovirus corresponding to hCoV-HKU1-E, MERS-uPE/E and SARS-CoV-1 were used as interfering analytes. The concentration of interfering pseudovirus was 10 times higher than that of SARS-CoV-2. As shown in Fig. 6, no significant changes were found after the addition of interfering pseudoviruses, indicating excellent specificity.

We also applied the CRISPR/Cas system to detect SARS-CoV-2 in different samples. Human throat swabs, frozen food and frozen food packaging materials were collected to validate the practical applicability of the sensor. Throat swab samples were collected from volunteers,

| Assays | Target | Limit of detection | Refs. |
|--------|--------|--------------------|-------|
| DNA nanoscaffold-based hybrid chain reaction colorimetric assay | SARS-CoV-2 RNA | 0.96 pM (5.78 × 10⁹ copies μL⁻¹) | [68] |
| CRISPR/Cas12a fluorescence detection | N gene | 3 copies μL⁻¹ | [69] |
| CRISPR/Cas12a lateral flow visual detection | ORF1ab and N genes | 7 copies μL⁻¹ | [70] |
| CRISPR-Cas12a lateral flow visual detection | E and N genes | 10 copies μL⁻¹ | [71] |
| CRISPR-Cas13a SHERLOCK | S, N and Orf1ab genes | 42 copies reaction⁻¹ (2.1 copies μL⁻¹) | [72] |
| CRISPR-Cas12a LAMP lateral flow visual detection | N gene | 2 copies μL⁻¹ | [73] |
| Paper based electrochemical biosensor | SARS-CoV-2 RNA | 6.9 copies μL⁻¹ | [74] |
| CRISPR/Cas13a electrochemical biosensor | ORF and S genes | 0.14 copies μL⁻¹ and 0.75 copies μL⁻¹ | [75] |
| 4WJ-based electrochemical biosensor | S gene and Orf1ab gene | 2 copies μL⁻¹ and 3 copies μL⁻¹ | [76] |
| CRISPR-SPCE | E gene | 0.27 copies μL⁻¹ | This work |

Fig. 5. (A) DPV with different concentrations of target DNA; (B) The linear calibration curve in the range of 2.0 × 10⁻⁸ to 5.0 × 10⁻⁵ ng μL⁻¹.
while frozen food and frozen food packaging were purchased from Yonghui supermarket. The samples were preprocessed according to the standard TCAQ1159-2020 “Sampling and real-time RT-PCR assay for detection of SARS-CoV-2 in food and food packaging surfaces”. Insert the swab into the sampling tube with 3.0 mL sampling solution and 1.0 mL 0, 10, 20, 200 copies standard TCAQ159-2020.

Yonghui supermarket. The samples were preprocessed according to the curation.

Different concentrations of each ingredient were used to verify the detection ability of the system at different concentration levels. The spiked samples were prepared and used to extract genomic RNA, which was reverse-transcribed and used as template. RT-qPCR and the CRISPR-SPCE system were used to test the samples in parallel to verify the consistency of the two methods. The RT-qPCR method is a standard method commonly used in clinical practice. As shown in Table S2, a total of 50 samples were used in qPCR and CRISPR-SPCE tests, including 26 human throat swab samples, 16 frozen shrimp samples, and 8 frozen food packaging materials samples. Among the 50 samples, 37 samples were determined to be positive and 13 samples were negative by both qPCR and CRISPR-SPCE methods. And the results of the two methods qPCR and CRISPR-SPCE were 100% consistent, demonstrating that the great potential of the CRISPR-SPCE system in quantifying SARS-CoV-2 in real samples. The CRISPR-SPCE sensor offers a cost-effective strategy for the quantification of disease biomarkers.

4. Conclusions

In this study, we successfully demonstrated that ssDNA randomly immobilized on the electrode, as well as upright immobilized ssDNA could be cleaved by the CRISPR/Cas system. This result demonstrated the feasibility of applying a carbon-based electrode instead of a gold-based electrode for CRISPR/Cas detection, which could reduce the cost and shorten the detection time. CeO₂ nanorods and PAH were used for random immobilization of the ssDNA on SPCE. Finally, CRISPR-SPCE was successfully used for the detection of SARS-CoV-2 pseudoviruses. The proposed method only requires minimal equipment, indicating its great potential for point-of-care diagnosis. The detection of SARS-CoV-2 in throat swabs, frozen food, and frozen food packaging materials demonstrates the feasibility of using this system in clinical diagnosis and frozen food monitoring.

CRediT authorship contribution statement

Lina Wu: Data curation, Writing – original draft. Xinjie Wang: Writing – review & editing, Supervision. Chengyuan Wu: Data Collection, Data curation, Formal analysis. Xizhong Cao: Methodology, Data curation. Taishan Tang: Writing – review & editing. He Huang: Conceptualization, Supervision. Xingxu Huang: Conceptualization, Supervision, Funding acquisition.

Declaration of competing interest

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

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Appendix A. Supplementary data

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