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PAM-free cascaded strand displacement coupled with CRISPR-Cas12a for amplified electrochemical detection of SARS-CoV-2 RNA

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ABSTRACT

The early diagnosis of coronavirus disease 2019 (COVID-19) is dependent on the specific and sensitive detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA. Herein, we develop a highly sensitive and specific electrochemical biosensor for SARS-CoV-2 target RNA detection based on the integration of protospacer adjacent motif (PAM)-free cascaded toehold-mediated strand displacement reaction (TSDR) and CRISPR-Cas12a (PfTSDR-CRISPR). In this study, each target is transformed into multiple DNA substrates with bubble structure in the seed region by the cascaded TSDR, which can directly hybridize with guide RNA (gRNA) without PAM requirement and then activate CRISPR-Cas12a′s *trans*-cleavage activity. Subsequently, the hairpin DNA modified with methylene blue (MB-HP) is cleaved by activated CRISPR-Cas12a. Therefore, as MB leaves the electrode surface, a decreased current signal is obtained. With the involvement of PAM-free cascaded TSDRs and CRISPR-Cas12a amplification strategy, the PfTSDR-CRISPR-based electrochemical biosensor achieves the detection of target RNA as low as 40 aM. The biosensor has high sequence specificity, reliability and robustness. Thanks to the PAM-free cascaded TSDR, the biosensor can achieve universal detection of different target RNA without redesigning gRNA sequence of CRISPR-Cas12a. In addition, this biosensor successfully detects SARS-CoV-2 target RNA in complex samples, which highlights its potential for diagnosing COVID-19.

1. Introduction

Since 2019, coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread worldwide with a lot of deaths and significant economic impact [1,2]. Although the COVID-19 vaccine has been developed, specific and sensitive detection of SARS-CoV-2 remains an effective way to control the spread of COVID-19 and monitor treatment, given the effectiveness of the vaccine and the rapid mutation of SARS-CoV-2 (termed as Omicron/Delta/Alpha) [3,4]. Therefore, the development of specific and sensitive detection methods for COVID-19 has always been the focus of research.

The RNA genome of SARS-CoV-2 consists of the open reading frame 1 ab (ORF1ab), the RNA-dependent RNA polymerase (RdRp), spike S nucleocapsid (N) and envelope (E) fragment, with a total length of about 30 KB [5]. Compared with antibody and antigen tests, nucleic acid-based test is highly sensitive and not easy to cause false-negative diagnosis [6]. Therefore, nucleic acid detection can provide more effective information for the diagnosis of COVID-19. The gold-standard method for detecting SARS-CoV-2 is called real-time reverse transcription polymerase chain reaction (qRT-PCR), which directly quantifies the SARS-CoV-2 target RNA [7]. However, qRT-PCR requires thermocycling, complex protocols and expensive instrument, not applicable to small clinics or community health facilities. Recently, the clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) technology has led to a technological revolution in the construction of isothermal nucleic acid diagnostic systems [8,9]. In the CRISPR/Cas-based systems, most of them rely on the *trans*-cleavage activity of protein, such as SHERLOCK (Cas13a), DETECTR (Cas12a), Cas14-DETECTR (Cas14) and etc [10–12]. The Cas12a protein is able to specifically *cis*-cleave a double-strand DNA (dsDNA) substrate or a single-strand DNA (ssDNA) substrate and then *trans*-cleave any single

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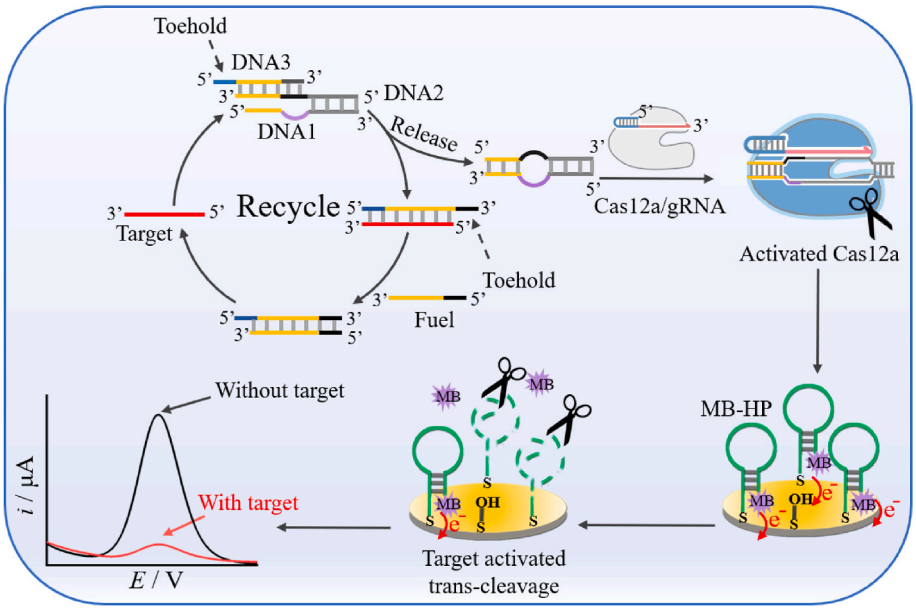

Scheme 1. Schematic illustration of the PfTSDR-CRISPR system for SARS-CoV-2 target RNA detection.

stranded DNA (ssDNA) with the assistance of guide RNA (gRNA) [11, 13]. Due to Cas12a activated by dsDNA substrate has a more efficient *trans*-cleavage activity than ssDNA substrate, most researchers employ dsDNA as activation substrates [11]. However, the recognition of dsDNA substrate by CRISPR-Cas12a system depends on protospacer adjacent motif (PAM) sequence.

To avoid the dependence of dsDNA substrate on PAM sequence, Nie's group reported a PAM-free CRISPR-Cas12a system for biosensing in live-cells. In this system, a dsDNA substrate was designed, which has a bubble structure in the seed region of CRISPR-Cas12a [14]. The dsDNA substrate can directly hybridize with the guide RNA (gRNA) of CRISPR-Cas12a system without the help of PAM sequence, and then activate the Cas12a′s *trans*-cleavage activity. The advantage of no PAM requirement and the bubble structure is to distinguish the strand replacement region of the DNA substrate from the gRNA recognition region. However, whether this method can be applied to electrochemical biosensors and coupled with isothermal signal amplification to construct a highly sensitive, specific and universal detection system remains to be explored.

The toehold-mediated strand displacement reaction (TSDR) occurs when the shorter single-stranded DNA or RNA in a double strand is invaded with the assistance of an unpaired region (toehold) [15]. By designing cascaded TSDRs, capable of achieving target recycling amplification without any enzymes, avoiding the issues of rigorous experimental conditions and high-cost. Thus, the cascaded TSDRs as isothermal signal amplification technology shows tremendous potential in biosensing [16–18]. Based on regulating PAM sequence, Yan's group reported a dual signal amplification strategy coupled with cascade TSDR and Cas12a for miRNA detection with high sensitivity and specificity [19]. While, this biosensor's strand displacement region intersects with the recognition region of Cas12a/gRNA complex. This makes it necessary to redesign the gRNA of CRISPR-Cas12a for the developed biosensor to detect different targets, which limits universality and increases the cost of the biosensor. The electrochemical biosensors possess great prospects due to its simplicity, high sensitivity, cost-effective and portability [20]. Therefore, it is urgent to develop an electrochemical biosensor based on PAM-free cascaded TSDRs and CRISPR-Cas12a for SARS-CoV-2 target RNA detection.

Herein, we develop a PAM-free cascaded TSDR coupled with CRISPR-Cas12a electrochemical biosensor system (PfTSDR-CRISPR) to detect SARS-CoV-2 with specificity and sensitivity. To verify the performance of the PfTSDR-CRISPR, we choose RdRp fragment of SARS-CoV-2 RNA as the model target. The target RNA is able to initiate two cascaded TSDRs, resulting in recycling of target and forming numerous dsDNA substrates. The dsDNA substrate is designed to contain a 6-nt bubble in the seed region, which activated CRISPR-Cas12a′s *trans*-cleavage activity without the help of PAM sequence. The hairpin DNA (MB-HP) modified by methylene blue on the sensing surface is cleaved by activated CRISPR-Cas12a, which led to disassociate MB from the surface, resulting in a significantly amplified electrochemical current signal. In such design, the PfTSDR-CRISPR system achieves specific, sensitive and universal detection of SARS-CoV-2 target RNA in complex samples, which provides a promising method for diagnosing COVID-19.

2. Results and discussion

2.1. Principle of the PfTSDR-CRISPR system

In PfTSDR-CRISPR system, the effective coupling between PAM-free TSDR and CRISPR-Cas12A is used to construct the PfTSDR-CRISPR system by recognizing the dsDNA with bubble structure output in TSDR (Scheme 1). The PfTSDR-CRISPR consists of three parts: a target RNA recycling isothermal amplification system, a CRISPR-Cas12a *trans*-cleavage activation system and a detection sensing system. In the absence of the target RNA, CRISPR-Cas12a′s *trans*-cleavage activity is not activated due to DNA3 partially hybridized with DNA1/DNA2 probe to produce a DNA1/DNA2/DNA3 complex. When the target RNA is added, it first hybridizes with the toehold region at the 5′-terminus of DNA3 and release DNA1/DNA2 substrate from DNA1/DNA2/DNA3 complex through the first TSDR. Next, the fuel probe hybridizes with the exposed toehold region at the 3′-terminus of DNA3 and trigger the second TSDR to form a DNA3/fuel probe, leading to the recycling of target RNA. After many cycles, a large amount of DNA1/DNA2 substrate is formed due to two cascaded TSDRs. In particular, an artificial bubble structure is ingeniously designed into the DNA1/DNA2 substrate, which can directly hybridize with gRNA without PAM requirement and then be cleaved by the CRISPR-Cas12a′s *cis*-cleavage activity. After that, the *trans*-cleavage activity of Cas12a is effectively activated. To realize the coupling of the above activated CRISPR-Cas12a and electrode interface to construct electrochemical biosensor, a nonspecific hairpin DNA modified with methylene blue (MB) (MB-HP) is assembled on electrode via the Au-S interaction. When activated Cas12a appears on the sensing

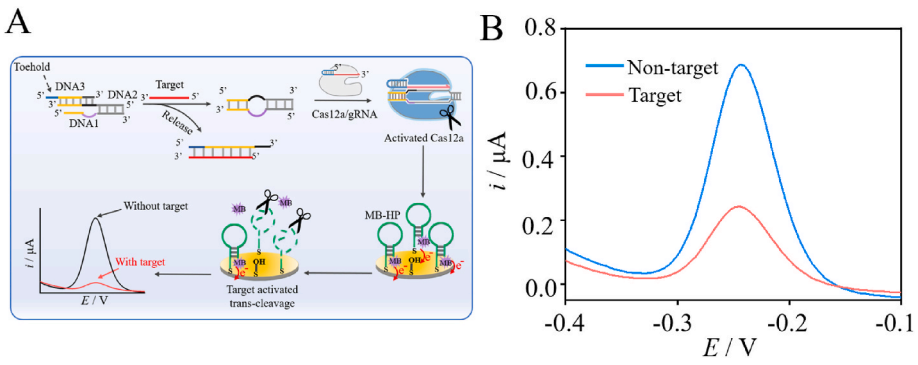

Fig. 1. (A) Schematic showing application of PAM-free CRISPR-Cas12a system in electrochemistry. (B) Typical square wave voltammetry (SWV) responses with or without target RNA (10 nM).

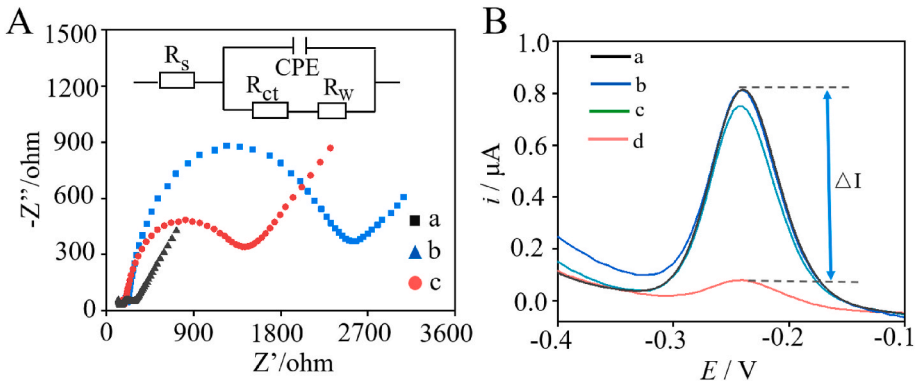

Fig. 2. (A) Typical electrochemical impedance spectroscopy (EIS) responses for the modification procedures of PfTSDR-CRISPR system: (a) unmodified electrode, (b) MCH/MB-HP/electrode, (c) (b) after *trans*-cleavage in test solution containing 0.1 M KCl and 5.0 mM $[Fe(CN)_6]^{3-/4-}$. R_{ct}, R_s, Z_w, and CPE represents the resistance of electron-transfer, the resistance of solution, the Warburg impedance and the constant phase element, respectively. (B) SWV signal response to explore the feasibility of PfTSDR-CRISPR system.

surface, the loop of MB-HP is cleaved into many small segments. Meanwhile, MB is detached from the electrode surface due to thermodynamic instability. Finally, the obtained current signal decreases. Because of target recycling and Cas12a′s *trans*-cleavage activity, the PfTSDR-CRISPR system has successfully realized the detection of target RNA.

2.2. Application of PAM-free CRISPR-Cas12a system in electrochemistry

To construct the PfTSDR-CRISPR system by coupling PAM-free TSDR and CRISPR-Cas12a, we firstly explored the application of PAM-free CRISPR-Cas12a system in electrochemistry. Compared to an unmodified electrode, the electrode shows a high SWV signal near −0.25 V without the presence of PAM-free dsDNA substrates (DNA1/DNA2 complexes) after the MB-HP probe is assembled on the electrode. When PAM-free dsDNA substrates are added (10 nM), a significant decrease in SWV signal is observed (Fig. S1), which implies that DNA1/DNA2 complexes can activate the CRISPR-Cas12a′s *trans*-cleavage to construct electrochemical biosensors. Then, DNA3 was employed to partially hybridize with DNA1/DNA2 to form DNA1/DNA2/DNA3 complexes. The significantly enhanced SWV signal is obtained without target RNA, suggesting that the DNA1/DNA2/DNA3 complex can't activate Cas12a′s *trans*-cleavage. While, the significantly decreased SWV signal is obtained with the presence of RdRp RNA, due to the TSDR between RdRp RNA and the DNA1/DNA2/DNA3 complex, which releases PAM-free dsDNA substrates and target/DNA3 complexes. Hence, we confirm that PAM-free CRISPR-Cas12a system can be applied to electrochemistry (see Fig. 1).

2.3. Feasibility investigation of target RNA detection by PfTSDR-CRISPR system

We then investigated the modification procedures of PfTSDR-CRISPR system through EIS measurements. The semicircle in the Nyquist diagram is proportional to the resistance of electron transfer (R_{et}). As shown in Fig. 2B, a small semicircle of unmodified electrode is observed (118.8 Ω, curve a), indicating low impedance and good conductivity of the unmodified electrode. However, the modification of the MB-HP and MCH on the AuE surface, the R_{et} is significantly increased (2508 Ω, curve b) due to that the negatively charged phosphate backbones of the MB-HP inhibited the charge transfer. After the incubation of the MCH/MB-HP/AuE with activated Cas12a, numerous MB-HPs are *trans*-cleaved, leading to significantly decrease in Ret (1401 Ω, curve c). Thus, these EIS results demonstrate that the biosensor is successfully constructed.

After testify the successful fabrication of the biosensor, SWV measurements were also employed to test the feasibility of PfTSDR-CRISPR for target RNA detection (Fig. 2B). Dropping the fuel probe onto the MB-HP-modified electrode generates a high SWV signal (curve a), demonstrating that the PAM-free cascaded TSDR cannot be triggered without the target RNA. In the presence of the RNA (10 pM), a strong current signal is still observed (curve b), owing to the lack of fuel probe resulting in a low concentration of DNA1/DNA2 complexes. By prolonging TSDR and CRISPR-Cas12a reaction times to 24 h, we observe a slightly decreased current signal (curve c). However, when the MCH/MB-HP/electrode is reacted with the target RNA and fuel probes, about a 90% rate of SWV signal change (represented by ΔI%, the calculation formula is $\Delta I\% = [(I_0\text{-}I)/I_0] \times 100\%$, where I and I_0 represent the presence or absence of target RNA, respectively.) can be observed (curve d). This is owing to a large number of DNA1/DNA2 substrate with bubble structure is produced and then activated Cas12a′s *trans*-cleavage. The *trans*-cleavage activity of Cas12a degrades MB-HP reporter, making MB away from the electrode surface. Therefore, these experimental results show the excellent feasibility of the PfTSDR-CRISPR system for target RNA detection.

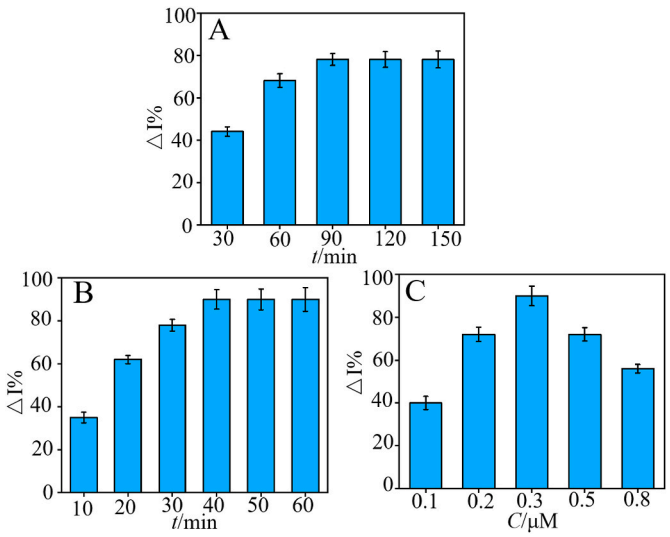

Fig. 3. The effects of (A) the reaction time of TSDR, (B) the cleavage time of CRISPR-Cas12a, (C) the concentration of MB-HP. (Error bars: SD, n = 3).

2.4. Optimization of experimental conditions

To obtain ideal analytical performance of PfTSDR-CRISPR system, three experimental parameters: the reaction time of TSDR, the cleavage time of CRISPR-Cas12a and the concentration of MB-HP probes were optimized. Reaction time as an important parameter of TSDR was first optimized. Fig. 3A displays the ΔI% increases with the TSDR reaction time until it stabilized at 90 min. Therefore, we choose 90 min as the best time for TSDR reaction. Another key factor for PfTSDR-CRISPR performance is the cleavage time of CRISPR-Cas12a. The ΔI% increases from ranging 10 min–40 min and will not increase after 40 min (Fig. 3B). Considering the time cost factor, we choose 40 min as the best cleavage time for CRISPR-Cas12a. The efficiency of Cas12a cutting the MB probe on the electrode surface also needs to be considered. Hence, we finally optimized the concentration of MB-HP probes. As long as the incubated MB concentration does not exceed 0.3 μM, the ΔI% increases with the increase of MB-HP concentration. Incubation of MB probes with concentrations greater than 0.3 μM may increase the cleavage hindrance of Cas12a, resulting in ΔI% reduction (Fig. 3C). Therefore, the concentration of MB-HP at 0.3 μM is chosen for the following experiments.

2.5. Sensitivity, reproducibility and robustness of the PfTSDR-CRISPR system

Under optimized conditions, the analytical performance of PfTSDR-CRISPR system for target RNA detection was evaluated. The SWV signal decreases by degrees with the concentration of RNA arising from 0 to 10.0 pM (Fig. 4A). Fig. 4B shows that the ΔI% is linearly related to the logarithm of target RNA concentration in the range from 100.0 aM to 10.0 pM. The regression equation is $\triangle$I% = 16.417lg C + 271.931 (C is the concentration of the RdRp RNA, R^2 = 0.997). The detection limit is calculated based on the ΔI% value generated by 40 aM is higher than the ΔI% value produced in the background plus 3 times of the standard deviation. Therefore, the detection limit is estimated to be 40 aM. Compared with previous reported methods, the PfTSDR-CRISPR system shown comparable or more sensitive detection limits (Table 1), which contribute to the two-stage signal amplification by the TSDR and Cas12a's *trans*-cleavage activity and electrochemical signal output excellent performance. Six repeated experiments with target RNA concentration at 1.0 pM were employed to verify the reproducibility of the PfTSDR-CRISPR. Fig. S3 shows that the experimental results produce a relative standard deviation (RSD) of 2.3%, demonstrating a suitable reproducibility of the PfTSDR-CRISPR system. Moreover, to evaluate the robustness of the PfTSDR-CRISPR system, we constructed three different biosensors and then employed to quantify the target (1.0 fM) on three different days. Table S2 exhibits that the average target RNA obtained was 1.03 fM with an RSD of 4.3%, suggesting the robustness of the PfTSDR-CRISPR system.

2.6. Selectivity and universality of the PfTSDR-CRISPR system

Specific detection of target RNA is another major challenge in achieving accurate analysis of target RNA. Herein, the RdRp RNA and the interfering sequences, including the random sequence (ORF1ab fragment) and single to four-base mismatched RdRp RNA, were investigated by the PfTSDR-CRISPR system. As displayed in Fig. 5A, single-base mismatched sequence shows a significant decrease in the ΔI%

Table 1
Comparison of different methods for SARS-CoV-2 detection.

Amplification strategy	Method	Linearity	LOD	Ref
CHA and TdT	Electrochemical	0.1 pM–1000 pM	26 fM	[21]
Exo III and CRISPR-Cas12a	Electrochemiluminescence	10 aM-10 pM	43.7 aM	[22]
EDA and CRISPR-12a	Electrochemiluminescence	0 aM-1 fM	32.8 aM	[23]
DSN	Electrochemical	50 aM–400 aM	21.69 aM	[24]
TSDR and CRISPR-Cas12a	Electrochemical	100 aM-10 pM	40 aM	This work

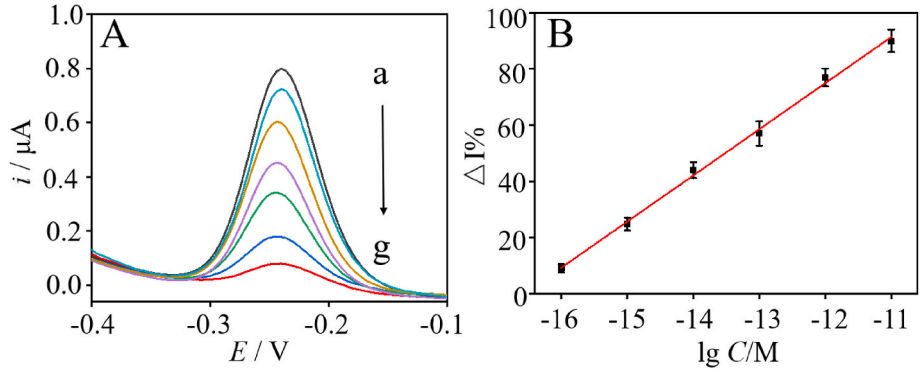

Fig. 4. (A) SWV signals of the target RNA at different concentrations (0 aM–10.0 pM). (B) Calibration curve of lg c vs. ΔI% for target RNA in the concentration range from 100.0 aM to 10.0 pM. (Error bars: SD, n = 3).

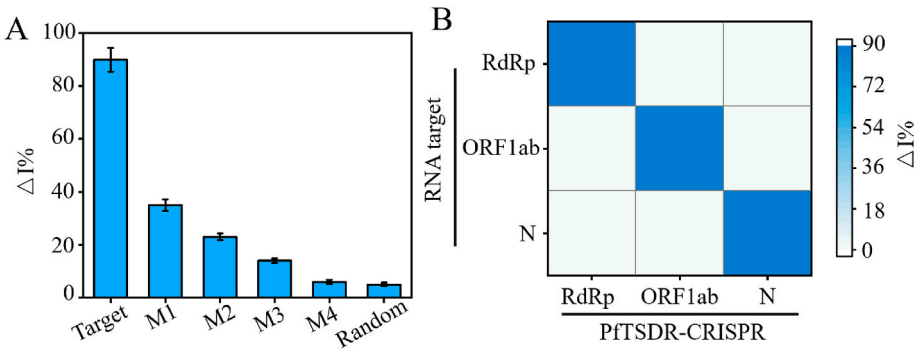

Fig. 5. (A) Specificity investigation of the biosensor for the RdRp RNA sequence, single to four-base mismatched RdRp and random sequence at the concentrations of 10.0 pM. (B) Heat map of multiplex detection of SARS-CoV-2 RNA fragment. The concentration of each target RNA and nonspecific RNA were 10 pM. (Error bars: SD, n = 3).

Table 2
Recovery tests for target in wastewater and saliva (n = 6).

Sample numbers	Samples	Added (fM)	Found (fM)	Recovery (%)	RSD (%)
1	Wastewater	1.0	0.991	99.1	3.7
2	Wastewater	10	10.1	101	4.2
3	Wastewater	100	99.6	99.6	2.8
4	Saliva	1.0	0.986	98.6	4.0
5	Saliva	10	10.2	102	2.9
6	Saliva	100	100.3	100.3	3.9

value (2.5 fold) compared to the target sequence. With the increasing of the number of mismatched bases, the ΔI% value also decreases. When four-base mismatched occurs in the target RNA, the ΔI% value is similar to random sequence. These results indicate that the high selectivity of the PfTSDR-CRISPR system, which is related to the Watson-Crick base pair of TSDR. Moreover, we chose three different SARS-CoV-2 target fragments (RdRp, ORF1ab and N) to validate the universality of the PfTSDR-CRISPR system. As shown in Fig. 5A, almost no cross-response is obtained in the non-target tests. The result suggest that the developed biosensor can achieve universal detection of different RNA fragments of SARS-CoV-2 without redesigning gRNA sequence, owing to the separation of TSDR and gRNA recognition region.

2.7. Complex sample analysis and detection of SARS-CoV-2 genome

To further investigate the PfTSDR-CRISPR system for target RNA detection in complex samples, 1.0 fM, 10 fM and 100 fM target RNA was separately spiked in wastewater and saliva samples to perform recovery tests. As shown in Table 2, the recovery of wastewater is between 99.1% and 101% with the RSD ranging from 2.8 to 4.2. And the recovery of saliva is between 98.6% and 102% with the RSD ranging from 2.9 to 4.0. This result provides that the PfTSDR-CRISPR system has the excellent application value for SARS-CoV-2 RNA detection in complex samples. In addition, Fig. S4 exhibits that the PfTSDR-CRISPR system can detect the actual SARS-CoV-2 genomic RNA. The above results demonstrate that the PfTSDR-CRISPR system possesses significant practicability.

3. Conclusions

We have developed a dual-amplified electrochemical biosensor, termed as PfTSDR-CRISPR, for the sensitive and specific electrochemical detection of the SARS-CoV-2 target RNA. This biosensor features three major advantages. First, an electrochemical biosensor based on the ability of the dsDNA substrate with bubble structure in the seed region to activate CRISPR-Cas12a's *trans*-cleavage activity without PAM requirement is successfully constructed. Second, due to the effective combination of PAM-free cascaded TSDR and CRISPR-Cas12a, the dual-amplified electrochemical biosensor exhibits high sensitivity detection SARS-CoV-2 RdRp RNA as low as 40 aM and shows high specificity detection of even target with a single-base mismatch. Third, the strand displacement region can be separated from the Cas12a/gRNA complex recognition region by combining PAM-free cascade displacement reaction and CRISPR-Cas12a, making it possible to generically detect different targets without the need to redesign gRNA sequence. The developed biosensor successfully monitors the spread of SARS-CoV-2 in complex samples, which exhibits a great potential for early diagnosing COVID-19. Consequently, our study extends a new avenue for biomarkers analysis by the integration of PAM-free DNA circuit and CRISPR-Cas12a.

CRediT author statement

Kai Shi: designed the study, performed experiments, analyzed the data, drafted the manuscript.
Zhigang Yi: performed experiments and analyzed the data.
Yaoxia Han: performed experiments.
Jiaxuan Chen. analyzed the data.
Yu Hu: analyzed the data.
Ying Cheng: analyzed the data.
Sujun Liu: analyzed the data.
Wei Wang: reviewed the literature.
Jiuhua Song: Supervision.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2023.115046.

References

[1] C.L. Huang, Y.M. Wang, X.W. Li, L.L. Ren, J.P. Zhao, Y. Hu, et al., Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China, Lancet 395 (2020) 497–506.

[2] E. Janik, M. Bartos, M. Niemcewicz, L. Gorniak, M. Bijak, SARS-CoV-2: outline, prevention, and decontamination, Pathogens 10 (2021) 114.

[3] S.S. Mahshid, S.E. Flynn, S. Mahshid, The potential application of electrochemical biosensors in the COVID-19 pandemic: a perspective on the rapid diagnostics of SARS-CoV-2, Biosens. Bioelectron. 176 (2021), 112905.

[4] D. Campos-Ferreira, V. Visani, C. Cordula, G.A. Nascimento, L.M.L. Montenegro, H. C. Schindler, et al., COVID-19 challenges: from SARS-CoV-2 infection to effective point-of-care diagnosis by electrochemical biosensing platforms, Biochem. Eng. J. 176 (2021), 108200.

[5] N. Kirtipal, S. Bharadwaj, S.G. Kang, From SARS to SARS-CoV-2, insights on structure, pathogenicity and immunity aspects of pandemic human coronaviruses, Infect. Genet. Evol. 85 (2020), 104502.

[6] P. Rai, B.K. Kumar, V.K. Deekshit, I. Karunasagar, I. Karunasagar, Detection technologies and recent developments in the diagnosis of COVID-19 infection, Appl. Microbiol. Biotechnol. 105 (2021) 441–455.

[7] V.M. Corman, O. Landt, M. Kaiser, R. Molenkamp, A. Meijer, D.K.W. Chu, Euro Surveill. 25 (2020), 2000045.

[8] X.L. Zuo, C.H. Fan, H.Y. Chen, Biosensing: CRISPR-powered diagnostics, Nat. Biomed. Eng. 1 (2017), 0091.

[9] K. Shi, S. Xie, R. Tian, S. Wang, Q. Lu, D. Gao, et al., A CRISPR-Cas autocatalysisdriven feedback amplification network for supersensitive DNA diagnostics, Sci. Adv. 7 (2021), eabc7802.

[10] J.S. Gootenberg, O.O. Abudayyeh, J.W. Lee, P. Essletzbichler, A.J. Dy, J. Joung, Nucleic acid detection with CRISPR-Cas13a/C2c2, Science 356 (2017) 438–442.

[11] J.S. Chen, E. Ma, L.B. Harrington, M.D. Costa, X. Tian, J.M. Palefsky, et al., CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity, Science 360 (2018) 436–439.

[12] L.B. Harrington, D. Burstein, J.S. Chen, D. Paez-Espino, E. Ma, I.P. Witte, Programmed DNA destruction by miniature CRISPR-Cas14 enzymes, Science 362 (2018) 839–842.

[13] S.Y. Li, Q.X. Cheng, J.K. Liu, X.Q. Nie, G.P. Zhao, J. Wang, CRISPR-Cas12a has both *cis*- and *trans*-cleavage activities on single-stranded DNA, Cell Res. 28 (2018) 491–493.

[14] S.Y. Chen, R.J. Wang, S. Peng, S.Y. Xie, C.Y. Lei, Y. Huang, et al., PAM-less conditional DNA substrates leverage trans-cleavage of CRISPR-Cas12a for versatile livecell biosensing, Chem. Sci. 13 (2022) 2011–2020.

[15] B. Yurke, A.J. Turberfield, A.P. M Jr., F.C. Simmel, J.L. Neumann, A DNA-fuelled molecular machine made of DNA, Nature 406 (2000) 605–608.

[16] X.L. Li, W.J. Zhou, L. Liao, Y. Xiang, B.Y. Jiang, R. Yuan, Target-triggered autocatalytic sequence recycling for sensitive and simultaneous detection of microRNA and mRNA *via* multi-donor iFRET signal amplification, Sensor. Actuator. B Chem. 345 (2021), 130351.

[17] H. Zhou, J.S. Zhang, B.X. Li, J. Liu, J.J. Xu, H.Y. Chen, Dual-mode SERS and electrochemical detection of miRNA based on popcorn-like gold nanofilms and Toehold-mediated strand displacement amplification reaction, Anal. Chem. 93 (2021) 6120–6127.

[18] S. Peng, Z. Tan, S.Y. Chen, C.Y. Lei, Z. Nie, Integrating CRISPR-Cas12a with a DNA circuit as a generic sensing platform for amplified detection of microRNA, Chem. Sci. 11 (2020) 7362–7368.

[19] X.R. Li, D.C. Zhang, X.F. Gan, P. Liu, Q.Y. Zheng, T.T. Yang, et al., A cascade signal amplification based on dynamic DNA nanodevices and CRISPR/Cas12a trans-cleavage for highly sensitive microRNA sensing, ACS Synth. Biol. 10 (2021) 1481–1489.

[20] M.H. Wang, Y.X. Lin, S. Wu, Y. Deng, Y.Y. Zhang, J. Yang, G.X. Li, An electrochemical biosensor for PD-L1 positive exosomes based on ultra-thin two-dimensional covalent organic framework nanosheets coupled with CRISPR-Cas12a mediated signal amplification, Sensor. Actuator. B Chem. 362 (2022), 131813.

[21] Y. Peng, Y.H. Pan, Z.W. Sun, J.L. Li, Y.X. Yi, J. Yang, G.X. Li, An electrochemical biosensor for sensitive analysis of the SARS-CoV-2 RNA, Biosens. Bioelectron. 186 (2021), 113309.

[22] K. Zhang, Z.Q. Fan, Y.D. Ding, M.H. Xie, A pH-engineering regenerative DNA tetrahedron ECL biosensor for the assay of SARS-CoV-2 RdRp gene based on CRISPR/Cas12a *trans*-activity, Chen. Eng. J. 429 (2022), 132472.

[23] K. Zhang, Z.Q. Fan, Y.D. Ding, S. Zhu, M.H. Xie, N. Hao, Exploring the entropy-driven amplification reaction and trans-cleavage activity of CRISPR-Cas12a for the development of an electrochemiluminescence biosensor for the detection of the SARS-CoV-2 RdRp gene in real samples and environmental surveillance, Environ. Sci.: Nano 9 (2022) 162–172.

[24] K. Zhou, J. Dai, A duplex-specific nuclease based electrochemical biosensor for the assay of SARS-CoV-2 RdRp RNA, Anal. Biochem. 661 (2023), 114983.