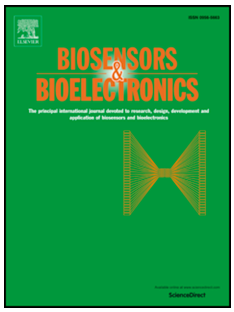
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A Versatile CRISPR/Cas12a-Based Biosensing Platform Coupled with a Target-Protected Transcription Strategy

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Abstract:

Besides the critical role in gene editing, CRISPR/Cas system also brings a new signal amplification mechanism to the development of next generation biosensing technologies. Herein, we have developed a versatile CRISPR/Cas12a sensing platform by combining a target protection-based transcription amplification strategy with the Cas12a-based signal amplification mechanism, which allows for the sensitive detection of both nucleic acid and non-nucleic acid targets. In this design, a rationally designed transcription template sequence is able to avoid Exonuclease I (Exo I) degradation only in the existence of the target-mediated binding events including either nucleic acid hybridization or protein-based affinity interactions. This target binding-induced protection effect can facilitate the subsequent transcription amplification to generate crRNA and activate the subsequent Cas12a *trans*-cleavage signal amplification mechanism to yield target dosage-responsive fluorescence signal. In contrast, if the target is absent, the protection-free transcription template will be completely digested by Exo I, thus no fluorescence response is produced. This new strategy well eliminates the T7 polymerase-associated non-specific transcription background and realizes the sensitive detection of various kinds of biomolecules including microRNA, protein, as well as exosome, broadening the application scenarios of CRISPR/Cas system in the field of bioanalysis and biosensing.

Keywords: CRISPR/Cas12a, target protection, microRNAs, transcription, exosomes

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1. Introduction

CRISPR/Cas system is an RNA-guided adaptive immune system found in most bacteria and all archaea. It can specifically recognize and cleave foreign genetic elements from invading plasmids and bacteriophages and thus has been extensively used in gene editing (Barrangou et al., 2007; Liu et al., 2019; Mali et al., 2013; Marraffini and Sontheimer, 2008; Zetsche et al., 2017). Besides its powerful gene editing capabilities through the intrinsic *cis*-cleavage, CRISPR/Cas system, particularly Cas12a and Cas13a, is also becoming a hot spot in the fields of biosensing and clinical diagnosis due to their efficient *trans*-cleavage ability (Dai et al., 2020; Li et al., 2019; Nguyen et al., 2022; Sun et al., 2020; Swarts et al., 2019; Tian et al., 2021; Wang et al., 2020). For such applications, both Cas12a and Cas13a need to be combined with the corresponding guide RNA (crRNA) for specific target recognition and thus activate their *trans*-cleavage ability upon the formation of the ternary complex. After then, the activated Cas proteins can cut surrounding ssDNA or RNA reporters nonspecifically to lead to efficient fluorescence signal amplification (Chen et al., 2018; Liu et al., 2017). Due to the precise nucleic acid targeting and isothermal signal amplification, CRISPR diagnostics based on Cas12a/Cas13a system have been widely applied to the analysis of nucleic acid-associated targets including virus detection, SNP detection and genotyping, pathogen identification, as well as liquid biopsy, etc (Choi et al., 2021; Ding et al., 2020; Fozouni et al., 2021; Gootenberg et al., 2017; Yu et al., 2021; Moon et al., 2022; Myhrvold et al., 2018; Shi et al., 2021; Tian et al., 2021; Wang et al., 2020). It should be noted that most of the existing Cas12a/Cas13a system-based sensing methods are mainly focused on the detection of nucleic acid markers owing to the nucleic acid recognition and cleavage nature of the Cas proteins. Up to now, only a few pioneering works are attempting to apply Cas12a/Cas13a to the detection of non-nucleic acid targets (Chen et al., 2022; Huang et al., 2021; Huang et al., 2022; Ivanov et al., 2022; Li et al., 2021; Zhao et al., 2020). In the

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early exploration, Liang's group developed a platform called CaT-Smelor for the detection of small molecules (e.g., tetracycline, p-hydroxybenzoic acid, and uric acid) by bacterial allosteric transcription factors (aTFs)-mediated CRISPR/Cas12a signal output (Liang et al., 2019). Other groups have reported functional DNA (fDNA) molecules such as aptamers and DNAzymes to modulate the *trans*-cleavage activity of the CRISPR/Cas system to detect non-nucleic acid targets (Xiong et al., 2020). Although such works have made some progress in broadening the target species of the CRISPR/Cas systems, they still suffer from several shortcomings, such as poor universality and sophisticated/tedious design. Therefore, there is still of great significance in developing a simple, efficient, and general biosensing strategy based on CRISPR/Cas system to enlarge the detectable target range.

Herein, we wish to propose a versatile biosensing platform by integrating target binding-protected transcription amplification with the CRISPR/Cas12a-based *trans*-cleavage signal amplification mechanism (denoted as TPCas assay), which has realized the sensitive detection of various biomarkers including microRNA (miRNA), protein, and exosome. In this study, the transcription template containing a target binding sequence, a complementary sequence of T7 promoter and a Cas12a crRNA-generating sequence is rationally designed. In the absence of the target molecules, the whole transcription template will be completely digested by Exonuclease I (Exo I), and thus no fluorescent response will be produced. On the contrary, upon the target binding through either nucleic acid hybridization or protein-ligand interaction, the template can be effectively protected from Exo I degradation. As such, the intact transcription template can execute the T7 transcription process to produce a large number of crRNA products. Finally, the Cas12a will assemble with the amplified crRNA and DNA activators to activate its *trans*-cleavage activity, resulting in the digestion of the fluorophore/quencher-labeled ssDNA reporters and the release of amplified fluorescence signal that

can faithfully reflect the target dosage. Theoretically, due to the characteristic property of Exo I for specifically hydrolyzing the 3' end of ssDNA, this system can be used to detect target substances such as nucleic acids, proteins, and small molecules, as long as they can bind to the 3' end of the template sequence, or specifically bound to the receptors labeled at the 3' end of the template. Therefore, the platform provides a more general solution to expand the target diversity of the CRISPR/Cas-based detection systems.

2. Experimental section

2.1 Reagents and Materials

Exo I and 10× CutSmart buffer, T7 RNA polymerase and 10× T7 RNA polymerase reaction buffer, Cas12a and 10× NEBuffer 2.1 were purchased from New England BioLabs (USA). Recombinant RNase Inhibitor and RNAiso for Small RNA were purchased from Takara Biotechnology Co. Ltd. (Dalian, China). NTP mixture and 4S Red Plus Nucleic Acid Stain was supplied by Sangon (Shanghai, China). Streptavidin (SA) was bought from Sigma-Aldrich (USA). Dynabeads™ M-270 Streptavidin (2.8 μm), 20× TE buffer RNase-free (pH 7.5), and nuclease-free water were obtained from Thermo Fisher Scientific (USA). All nucleic acid sequences used in this study were synthesized by either Thermo Fisher Scientific or Sangon, and listed in Table S1.

2.2 Standard procedures for target-protected Exo I digestion

Taking let-7a miRNA analysis as a model system. The target-based protection reaction was conducted in 4 μL mixture containing 1× CutSmart buffer (50 mM KAc, 20 mM Tris-Ac, 10 mM $Mg(Ac)_2$, 100 μg/mL BSA, pH 7.9), 1.25 nM let-7a-specific transcription template, and different amounts of targets. The oligonucleotide mixture was firstly denatured for 3 min at 80 °C and gradually annealed to room temperature. Then, 2 μL 1× CutSmart buffer containing 1 μL Exo I (1 U) was added

to conduct the digestion step of Exo I. The reaction mixture was incubated at 40 °C for 1 h and inactivated at 80 °C for 20 min. After that, 6 μL of digestion products were reacted with 1× T7 RNA polymerase reaction buffer (40 mM Tri-HCl, 6 mM $MgCl_2$, 2 mM spermidine, 1 mM DTT, pH 7.9), 10 nM T7 promoter, 50 U T7 RNA polymerase, 0.1 mM NTP mix, and 8 U Recombinant RNase Inhibitor in a final volume of 10 μL at 37 °C for 3 h. The transcription reaction was finalized by heating at 80 °C for 10 min.

For protein and exosome analysis, the Exo I cleavage reaction steps were the same as those for let-7a analysis except that the corresponding protein-specific or exosome-specific transcription templates were employed.

2.3 Cas12a-based trans-cleavage amplification and fluorescence measurement

The Cas12a-based fluorescent assay was carried out in a final volume of 10 μL containing 1× NEBuffer 2.1 (10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 100 μg/mL BSA, pH 7.9), 5 μL transcription products mentioned above, 10 nM ssDNA activators, 1 μM fluorophore/quencher-labeled reporter, and 100 nM Cas12a at 37 °C for 50 min, followed by incubation at 95 °C for 5 min to inactivate Cas12a. After that, the Cas12a cleavage reaction solution was diluted to 90 μL with 1× TE buffer (20 mM Tris-HCl, 1 mM EDTA, pH 7.9) and the fluorescence spectra were recorded by using an F-7000 fluorescence spectrophotometer (Hitachi, Japan) with the excitation wavelength of 488 nm.

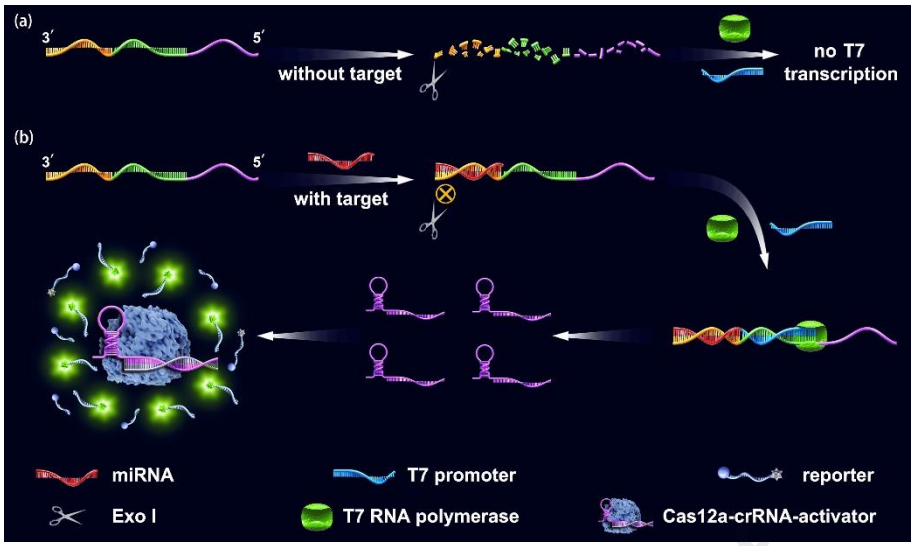
3. Results and discussion

3.1 Principle of the proposed TPCas assay for miRNA analysis

It is well known that under the guidance of crRNA, Cas12a recognizes DNA targets but not RNA targets. So conventional Cas12a is only suitable for DNA detection (Zetsche et al., 2015). To broaden the CRISPR/Cas12a platform's application to the detection of nucleic acid targets other than DNA,

in this study, we explore the application of the proposed TPCas system for miRNA analysis by using let-7a as a model target. As illustrated in Scheme 1, an ssDNA transcription template (referred to as 'let-7a template' in the text) consists of three parts: a target binding sequence that is complementary to let-7a (yellow color), a complementary sequence of T7 promoter (green color), and a transcription-based crRNA-producing region (purple color). In the absence of let-7a, the let-7a template will be completely digested into mononucleotide by Exo I along the 3' to 5' direction, so the subsequent transcription process as well as the Cas12a amplification processes will not occur. On the contrary, in the presence of let-7a, target let-7a can hybridize to the 3'-terminus of the let-7a template to form a complementary duplex structure, which can effectively protect the let-7a template from Exo I digesting since Exo I can only recognize the 3' end of ssDNA (Gao et al., 2018; Gao et al., 2020; Suea-Ngam et al., 2019). With the participation of T7 RNA polymerase, the intact let-7a template can initiate the transcription amplification process by adding additional T7 promoter sequences, producing a large amount of Cas12a crRNA. Upon recognizing the ssDNA activator, the ternary complex (Cas12a/crRNA/ssDNA) will be formed to activate the *trans*-cleavage activity of Cas12a. Then, the ssDNA reporter with a fluorophore (FAM) and a quencher (BHQ-1) modified at its terminals respectively was cleaved at a high turnover rate to release amplified fluorescence signals, which is proportional to the original concentration of target let-7a.

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Scheme 1. Schematic illustration of the TPCas strategy for let-7a miRNA analysis.

3.2 Feasibility of the TPCas assay for miRNA analysis

To verify the feasibility of the proposed method for miRNA detection, we firstly tested the Exo I digestion and the transcription products by denaturing polyacrylamide gel electrophoresis (dPAGE). As displayed in Figure 1A, the let-7a template exhibits a clear single band in lane 1. When the pure let-7a template was transcribed without adding let-7a and Exo I, a large number of ssRNA fragments can be generated (lane 1). In the absence of let-7a, if the let-7a template is premixed with Exo I, it can be observed from lane 2 that the band of transcription products become negligible, which indicates that most of the let-7a template was degraded to single nucleotides by Exo I and the transcription process cannot be carried out. However, as the concentration of let-7a increased, more and more let-7a template was protected and thus more transcripts were generated (lane 3 and 4). The dPAGE results clearly proved that the binding of let-7a with the template can successfully protect the let-7a template from digestion by Exo I, certifying the feasibility of the proposed TPCas assay for miRNA analysis.

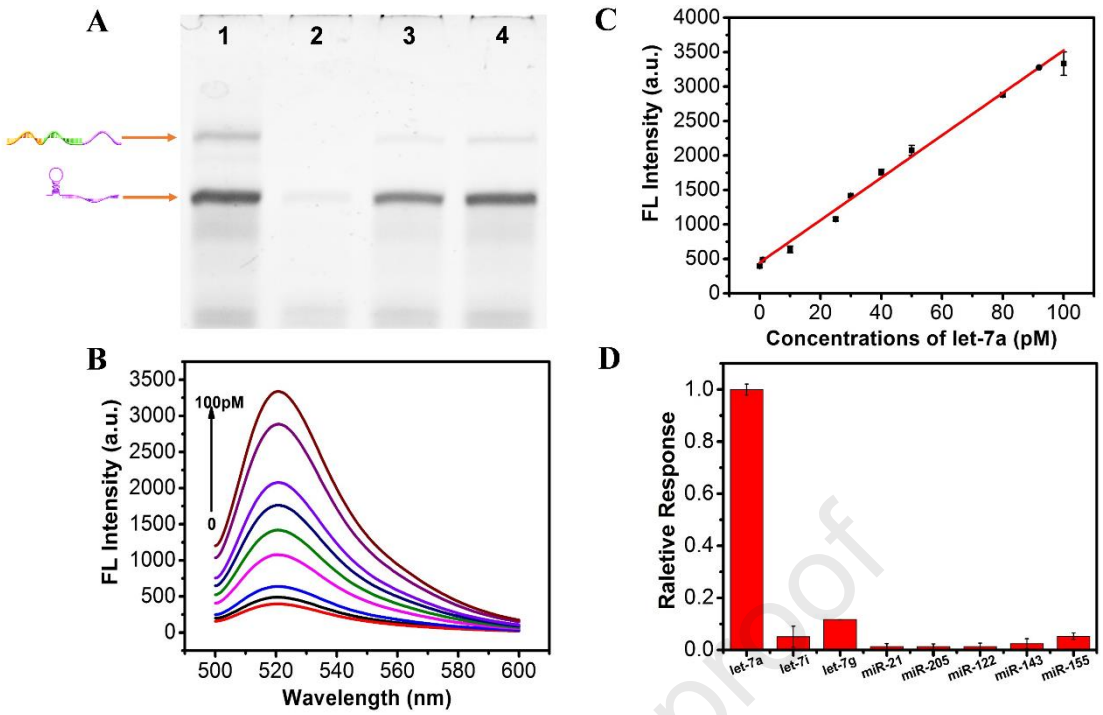


Figure 1. Results of let-7a analysis by using the TPCas assay. (A) Evaluating the feasibility of the TPCas assay for let-7a detection by 16% dPAGE analysis. Lane 1: transcription products of 50 nM let-7a template in the absence of let-7a and Exo I; Lane 2: transcription products of 50 nM let-7a template treated with Exo I (10 U) in the absence of let-7a (blank control); Lane 3: transcription products of 50 nM let-7a template treated with Exo I (10 U) in the presence of 10 nM let-7a; Lane 4: transcription products of 50 nM let-7a template treated with Exo I (10 U) in the presence of 20 nM let-7a. (B) Fluorescence spectra of the TPCas system upon the addition of different concentrations of let-7a (0~100 pM). (C) The linear plot between fluorescence intensity (at 520 nm) and let-7a concentration in the scope of 0 to 100 pM. (D) Fluorescence responses of other species of miRNA relative to that of let-7a. Relative response refers to the ratio of the fluorescence signal (background subtracted) produced by other miRNAs to that produced by let-7a. The concentrations of all miRNAs were 25 pM. Error bars are the standard deviation of three parallel experiments.

3.3 Analytical performance of the TPCas assay for let-7a analysis

Next, under the optimized experimental parameters (see Figure S1-S3 in the supporting information), the analytical performance of the proposed strategy is evaluated by detecting different

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concentrations of target let-7a. As displayed in Figure 1B, the fluorescence intensity increased gradually with the increasing concentration of let-7a from 0 to 100 pM. There was a good linear relationship between the fluorescence intensities and let-7a concentrations (Figure 1C), and the corresponding regression equation was $F = 446.37 + 30.77\ C_{let\text{-}7a}$ (F represented the fluorescence intensity at 520 nm and C represented the concentration of let-7a) with a correlation coefficient of $R^2 = 0.9937$. Based on the 3σ/S criterion, the detection limit of let-7a was estimated to be 0.75 pM. Furthermore, the specificity of the method was confirmed under the same conditions by using other random miRNA sequences, including homologous let-7 family members (let-7g and let-7i with two and four bases difference to let-7a, respectively) and nonhomologous miRNAs (miR-21, miR-205, miR-122, miR-143, and miR-155). As displayed in Figure 1D, only let-7a led to a remarkable fluorescence response while the other interferences resulted in only negligible fluorescence responses comparable to that of the blank sample, implying the proposed method has good specificity to discriminate different even similar miRNA sequences.

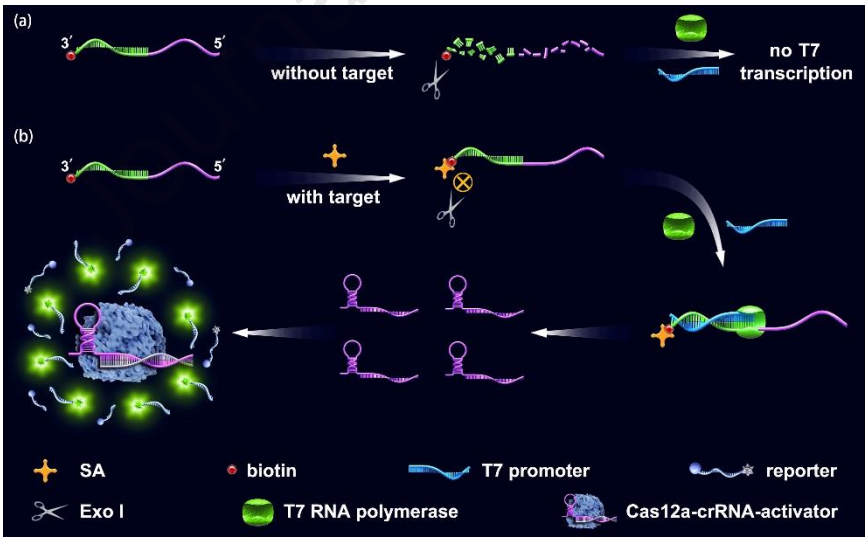
To validate the practicability of the TPCas assay in real complex samples, we have applied it to the determination of let-7a content in the total small RNA extracted from Hela cells, and the results are presented in Table S2. With a simultaneously constructed calibration curve by using synthetic let-7a standards, the amount of let-7a in 5 ng total small RNA samples is calculated to be 222.2 amol. To further verify the accuracy of the result, 250 amol of synthetic let-7a is spiked into 5 ng total small RNA samples, and the determined let-7a level in the spiked sample is 471.4 amol with a recovery of 99.7%, indicating that the proposed strategy works well in the complex biological samples.

3.4 Protein and exosome analysis by using the TPCas assay

Specific interactions between small molecules and corresponding protein receptors (or protein-protein interaction) play a key role in molecular diagnostics and drug development (Cao et al., 2012;

Journal Pre-proof

Wu et al., 2009; Wu et al., 2011). Given the good performance of the proposed strategy toward let-7a detection, we further extend the application scenarios of the TPCas assay to detecting other kinds of biospecies. Herein, the streptavidin (SA)-biotin system was used as a model system to verify the feasibility of the proposed strategy in protein analysis. As illustrated in Scheme 2, the 3'-biotinylated ssDNA template (referred to as the 'SA template' in the text) consists of three parts: biotin molecules at the 3'-end (red), complementary sequences of T7 promoter (green), and transcription-based crRNA-producing sequence (purple). In the absence of target SA, the biotin-linked SA template was hydrolyzed stepwise from the 3' end by Exo I to generate mononucleotides, leading to transcription termination. Upon binding to the target SA, the specific and strong interaction between SA and biotin protects the ssDNA template from enzymolysis by Exo I. Consequently, the intact SA template proceeds to perform subsequent transcription and Cas12a signal amplification processes, resulting in amplified fluorescent signals that can quantitatively reflect the SA dosage.



Scheme 2. Schematic illustration of the TPCas strategy for SA analysis.

The dPAGE results also validated the feasibility of the design for SA detection. It can be seen from Figure 2A, if without Exo I treatment, the pure SA template as well as the successfully transcribed ssRNA products can be clearly observed in lane 1. Upon the introduction of Exo I to the template but without the addition of SA, no obvious transcription band was observed (lane 2). When the SA

template was incubated with SA and then treated with Exo I, more and more SA templates were protected as the target SA concentration increased, and the bands of ssRNA products and SA template gradually recovered (lanes 3 and 4). These results clearly suggest that the specific interaction with SA renders the biotinylated SA template resistant to digestion by Exo I, providing a feasible way for SA quantification. Under the above optimized conditions, the fluorescence responses against different concentrations of SA were investigated. One can see from Figure 2B that the fluorescence intensities were gradually elevated with rising concentrations of SA. The specificity of the SA detection system was also investigated by comparing the fluorescence responses of SA with other potential interfering proteins including AFP, BSA, CEA, and PSA. As shown in Figure 2C, the fluorescence intensity of the sensing system was greatly increased only after the addition of SA, while the change in fluorescence intensity of other interfering proteins at the same concentration was negligible, indicating that non-specific proteins cannot bind to biotin molecules to prevent the Exo I digestion. Furthermore, other than the strong SA-biotin interaction, we have also verified that through an aptamer-based affinity interaction mode, the TPCas design is also applicable to the detection of exosomes. As depicted in Scheme S1, by simply changing the 3'-end of the ssDNA template (exosome template) into an aptamer sequence, the aptamer sequence at the 3' end of the exosome template can specifically bind to the membrane protein of HepG2 exosomes, effectively protecting the exosome template from Exo I cleavage based on the huge steric hindrance of exosomes. Then, the exosome-protected intact template can further proceed to a modified T7 polymerase transcription amplification and Cas12a-based fluorescence signal readout. As shown in Figure 2D, the fluorescence intensity of the proposed sensing system is positively correlated with the concentration of exosomes. It should be noted that although the presented SA and exosome analysis are only proof-of-concept demonstrations, we have undoubtedly verified that the target-protected Cas12a sensing strategy is very versatile,

which can broaden the Cas12a-based sensing toolbox from DNA target to miRNA, protein, as well as exosome analysis.

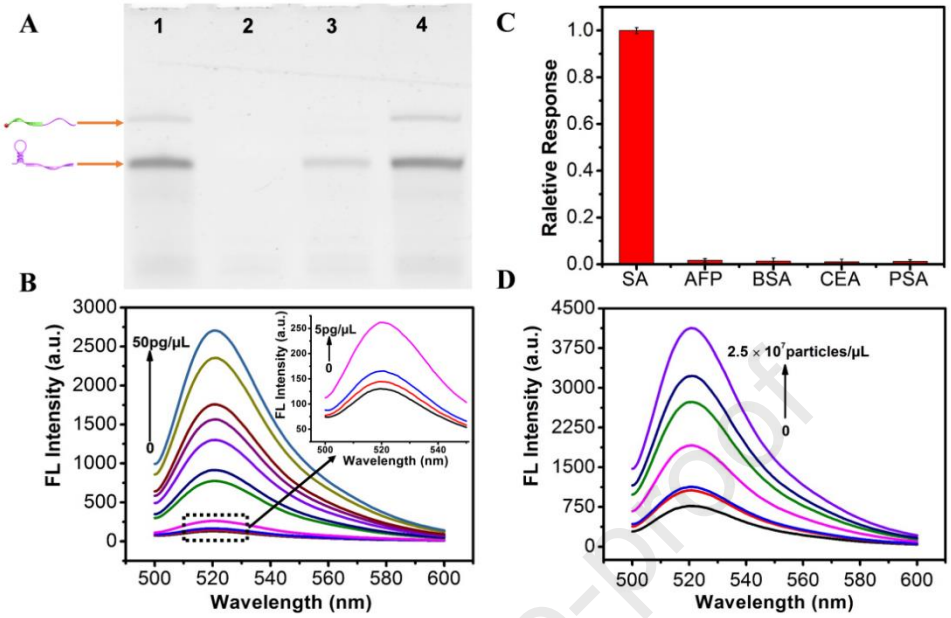


Figure 2. Results for SA and exosome analysis by using the TPCas assay. (A) Evaluating the feasibility of the TPCas assay for SA detection by dPAGE analysis. Lane 1: transcription products of 50 nM SA template in the absence of SA and Exo I; Lane 2: transcription products of 50 nM SA template treated with Exo I (10 U) in the absence of SA (blank control); Lane 3: transcription products of 50 nM SA template treated with Exo I (10 U) in the presence of 1 ng/μL SA; Lane 4: transcription products of 50 nM SA template treated with Exo I (10 U) in the presence of 10 ng/μL SA. (B) Fluorescence spectra of the TPCas system upon the addition of different concentrations of target SA (0~50 pg/μL). The inset shows the fluorescence spectra generated from 0~5 pg/μL target SA. (C) Fluorescence responses of other proteins relative to that of SA. Relative response refers to the ratio of the fluorescence signal (background subtracted) produced by other proteins to that produced by SA. The concentrations of all proteins were 10 pg/μL. Error bars are the standard deviation of three parallel experiments. (D) Fluorescence spectra of the TPCas-based exosome assay in the presence of different concentrations of target exosomes (0~2.5 $\times 10^7$ particles/μL).

4. Conclusions

In conclusion, we have proposed a target protection-based enzyme cleavage mechanism, which

combines transcription and CRISPR/Cas12a signal amplification routes to construct a multifunctional biosensor for sensitive detection of miRNA, protein, and exosomes. In the absence of the target, the ssDNA transcription template was gradually hydrolyzed by Exo I, thereby efficiently suppressing the inherent T7 RNA polymerase-associated non-specific transcription background. When different kinds of biomolecule targets are present, a wide range of bio-affinity systems in nature, including but not limited to the nucleic acid hybridization, small molecule-protein receptor interaction, and the aptamer-based affinity binding events mentioned in this work, can all efficiently prevent the transcription template from Exo I digestion. As such, the target dosage-correlated intact ssDNA template can perform the consequent transcription and CRISPR/Cas12a signal amplification processes and generate amplified fluorescence signals. By using Exo I to specifically hydrolyze the 3' end of ssDNA, this system can theoretically be used to detect target substances such as nucleic acids, proteins, and small molecules, as long as it can hybridize with the 3' end of the template sequence, or specifically bound to the receptors labeled at the 3' end of the template. Therefore, the platform provides a more general solution to the detection of numerous biological molecule targets and may pave a new way for the versatile and efficient CRISPR/Cas biosensing design.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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