RNA reporter based CRISPR/Cas12a biosensing platform for sensitive detection of circulating tumor DNA

Author:
Deng, Fei; Goldys, Ewa; Li, Yi; SANG, Rui; Hall, Tim; Zhang, Chengchen

Publication details:
2023 45th Annual International Conference of the IEEE Engineering in Medicine and Biology Society
v. 00
Medium: Print
pp. 1 - 4
9798350324471 (ISBN)
1557-170X (ISSN); 1558-4615 (ISSN)

Event details:
45th Annual International Conference of the IEEE Engineering in Medicine and Biology Society
Sydney
2023-07-24 - 2023-07-27

Publication Date:
2023-08-01

Publisher DOI:
https://doi.org/10.1109/EMBC40787.2023.10340759

DOI:
https://doi.org/10.26190/unsworks/28638

RNA reporter based CRISPR/Cas12a biosensing platform for sensitive detection of circulating tumor DNA

Fei Deng, *Member, IEEE*, Yi Li, *Member, IEEE*, Rui Sang, *Member, IEEE*, Chengchen Zhang, *Member, IEEE*, Tim Hall, *Member, IEEE*, Danting Yang, Ewa Goldys

***Abstract*—CRISPR/Cas biotechnology provides an exceptional platform for biosensor development. To date, the reported CRISPR/Cas biosensing systems have shown extraordinary performance for nucleic acids, small molecules, small proteins and microorganism detection. The CRISPR/Cas12a biosensing system, as a typical example, has been well established and applied for both nucleic acids and non-nucleic acids target detection. However, all established CRISPR/Cas12a biosensing systems are based on DNA reporters, which potentially limits further application.**

In this study, we established an RNA reporter based CRISPR/Cas12a biosensing system. A basic biosensing system was evaluated, and the limit of detection was found to be 1 nM. Afterwards, we optimized this biosensing system using both temperature and chemical enhancers. The final optimal biosensing system (with DTT & 37°C) shows fluorescence signal increased by a factor of ~10 compared with the basic system. The optimal biosensing system was further applied for the detection of circulating tumor DNA (ctDNA), which shows over 4 orders of magnitude detection range from 1pM to 25 nM, with the limit of detection of 1pM. This RNA reporter based CRISPR/Cas12a biosensing system provides an effective platform for nucleic acids quantification.

***Clinical Relevance*— This research provides a novel approach for ctDNA diagnostics, which is an attractive biomarker for noninvasive monitoring of tumor growth, response, and spread.**

I. Introduction

CRISPR/Cas biotechnology discovered through exploration of prokaryote biology has been widely utilized in the areas of gene editing[1, 2], gene imaging[3], and biosensing[4]. The CRISPR/Cas system typically consists of a restricted Cas protein and a programable guiding RNA (gRNA), with a specific region for the recognition of target nucleic acids [5, 6]. To date, two classes of Cas effectors have been discovered, including class one (multiple Cas effector) and class two (single Cas effector)[1]. Class two Cas effectors, which includes type II, V, and VI, have found a wide range of applications due to their simplicity[4]. Prior to the discovery of *trans*-cleavage activity, type II Cas effectors were first applied for biosensing of nucleic acids [5]. After the discovery of *trans*-cleavage activity in Type V and VI Cas effectors, further CRISPR/Cas biosensing systems have been developed for the quantification of nucleic acids [7], small proteins [8, 9], microorganisms [10] etc. The superb biosensing performance of CRISPR/Cas biotechnology originates from the exceptional recognition ability of gRNA and *trans*-cleavage ability of Type V&VI Cas effectors [1]. Among them, Cas12a has been widely utilized for biosensing development for the detection of DNA [7] and RNA [11] targets.

In a typical CRISPR/Cas12a biosensing system, a programable gRNA serves as a recognition moiety of target nucleic acids, and single strand DNA (ssDNA) reporters are added to produce signal amplification [12]. To date, diverse DNA reporters have been designed for CRISPR/Cas12a biosensing system, including 6C [13], G4 [14], G3 [15], and hairpin reporters [16] etc. However, all of them are DNA based reporters, which is due to the previously discovered DNA *trans*-cleavage ability of Cas12a[1]. Recently, researchers have found that Cas12a also shows *trans*-cleavage ability on RNA targets[11, 17], which paves a new way for RNA reporter development in CRISPR/Cas12a biosensing system.

In this study, we developed a novel RNA reporter based CRISPR/Cas12a biosensing system. A specific single strand RNA reporter was developed, which contains a fluorophore on the 5' end, and a quencher on 3' end. Before the recognition of target nucleic acids, Cas12a RNP was inactive, and unable to cleave RNA reporters. After the recognition of target nucleic acids, Cas12a RNP was activated, and RNA reporter was cleaved for signal readout. The established RNA reporter based CRISPR/Cas12a biosensing system was further optimized using temperature and diverse chemical enhancers. Afterwards, the optimal biosensing system was applied for circulating tumor DNA quantification.

II. Methodology

A. Materials and reagents

EnGen® Lba Cas12a (Cpf1) protein (New England Biolab), 10X NEB 2.1 buffer (New England Biolab), 1,4-dithiothreitol (DTT) (Sigma, DTT-RO), Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (Sigma, C4706), Polyvinyl alcohol (PVA) (Sigma, P8136), Phosphate

*This research was supported by Innovation Connections Grant (ICG001677 and ICG001676)

F. Deng, Y. Li, R. Sang, C. Zhang, T. Hall, & E. Goldys are with the Graduate School of Biomedical Engineering, UNSW, Sydney, Australia. D. Yang is with the School of Medicine, Ningbo University, China.

E-mail for correspondence: fei.deng@unsw.edu.au

Buffered Saline (PBS) (Sigma, 10 mM, pH=7.4), DNase/RNase free water (ThermoFisher).

All nucleotide oligos were synthesized by Sangon Co. Ltd. with designed terminal modifications. For the FAM-modified oligos, the excitation wavelength was 480 nm and emission wavelength was 520 nm.

TABLE I. SEQUENCES OLIGOS USED IN THIS WORK

Name	Oligo type	Sequence 5'-3'	Length (nt)	Modification
Guiding RNA (gRNA)	ssRNA	UAA UUU CUA CUA AGU GUA GAU GGG GGG GGU UGG UAG GGU GUC	42	N/A
Trigger ssDNA	ssDNA	GAC ACC CTA CCA ACC CCC CCC	21	N/A
ssRNA reporter	ssRNA	UUAUU	5	5'-FAM; 3'-BHQ1
ctDNA Guiding RNA (gRNA)	ssRNA	UAA UUU CUA CUA AGU GUA GAU CCU CUC UCU AAA AUC ACU GAG	42	N/A
ctDNA	ssDNA	CTC AGT GAT TTT AGA GAG AGG	21	N/A

B. Preparation and evaluation of standard CRISPR/Cas12a reaction mixture

10 μL 10 μM (100 pmol) Cas12a protein was gently mixed with 5 μL 20 μM (100 pmol) gRNA in 3.6 mL 1X NEB 2.1 buffer, which was diluted by Milli-Q water. Then, 6 μL 100 μM (0.6 nmol) ssRNA linked fluorescent reporter was added and well mixed to form the final reaction mixture. The prepared reaction mixture was kept on ice before use.

Subsequently, 10 μL of different concentrations of trigger DNA were added into 90 μL standard CRISPR/Cas12a and incubated for 120min. The final fluorescence signal was recorded using a SpectraMax i3x multi-Mode Microplate Reader (Molecular Devices).

C. Optimization of RNA reporter based CRISPR/Cas12a biosensing system.

The reaction temperature was first optimized under both room temperature (RT) and 37℃ using the standard CRISPR/Cas12a reaction mixture. Afterwards, 10 μL of 250 nM trigger DNA were added into 90 μL standard CRISPR/Cas12a and incubated for 120min. The final fluorescence signal was recorded using a SpectraMax i3x multi-Mode Microplate Reader (Molecular Devices).

The optimization of chemical enhancers was based on the standard NEB 2.1 buffer. Three different chemical enhancers were added in the standard CRISPR/Cas12a reaction mixture, and the final concentration of DTT was 10 mM, the concentration of TCEP was 0.5 mM, and the concentration of PVA was 10 mg/mL, which was originated from our previous research [7]. Afterwards, 10 μL of 250 nM trigger DNA was added into 90 μL standard CRISPR/Cas12a and incubated for 120min.The final fluorescence signal was recorded using a SpectraMax i3x multi-Mode Microplate Reader (Molecular Devices).

D. Application of RNA reporter based CRISPR/Cas12a biosensing platform for the detection of ctDNA

The chemical enhancer involved CRISPR/Cas12a reaction mixture was first prepared. Briefly, DTT was added into standard CRISPR/Cas12a reaction mixture, and the final concentration was 10 mM. Afterwards, 10 μL ctDNA sample with different concentrations was added into 90 μL DTT assisted CRISPR/Cas12a reaction mixture and incubated for 120min at 37℃. The final fluorescence signal was recorded using a SpectraMax i3x multi-Mode Microplate Reader (Molecular Devices).

III. RESULTS

A. The development and evaluation of RNA reporter based CRISPR/Cas12a biosensing platform.

As shown in Fig.1A, the standard CRISPR/Cas12a biosensing system contains three key components, including Cas12a protein, gRNA, and reporter. The Cas12a protein and gRNA were first mixed together, upon which spontaneously formed the Cas12a ribonucleoprotein (RNP). After the recognition of target DNA, the Cas12a RNP was activated to cleave surrounding RNA reporters for signal amplification. The fluorescent quenched RNA reporter was supplied to the reaction. After the Cas12a RNP cleaved the RNA, the fluorophore on the 5' end was separated from the quencher on 3' end, unquenching the fluorescent signals. In this study, FAM was selected as a fluorophore, and BHQ1 was selected as a quencher. Other selections of the fluorophore-quencher pairs are also possible for this biosensing system.

The *trans*-cleavage ability of Cas12a RNP on RNA reporter was first investigated (Fig. 1B). The fluorescence signal linearly increased with the increase of incubation time, indicating continuous *trans*-cleavage activity of activated Cas12a RNP on RNA targets [11]. After the demonstration of the *trans*-cleavage ability of Cas12a RNP on the RNA reporters, the corresponding biosensing platform was established. As shown in Fig. 1B, without the ssDNA trigger, there was no fluorescence increase, indicating that the Cas12a RNP could only be activated by its corresponding ssDNA trigger. After introducing different concentrations of the ssDNA trigger in our Cas12a biosensing system, the fluorescence signals continuously increased with the increase of incubation time (Fig. 1B). Furthermore, with higher concentrations of ssDNA trigger, higher fluorescence signals were observed, indicating that the fluorescence signals were positively correlated with the concentrations of ssDNA trigger at the same incubation time point, which is the

foundation for biosensing development. However, this basic RNA reporter based CRISPR/Cas12a biosensing system was only able to detect 1nM target ssDNA, which is not sufficient for practical biosensing applications [18]. Therefore, further optimization was carried out to improve sensitivity.

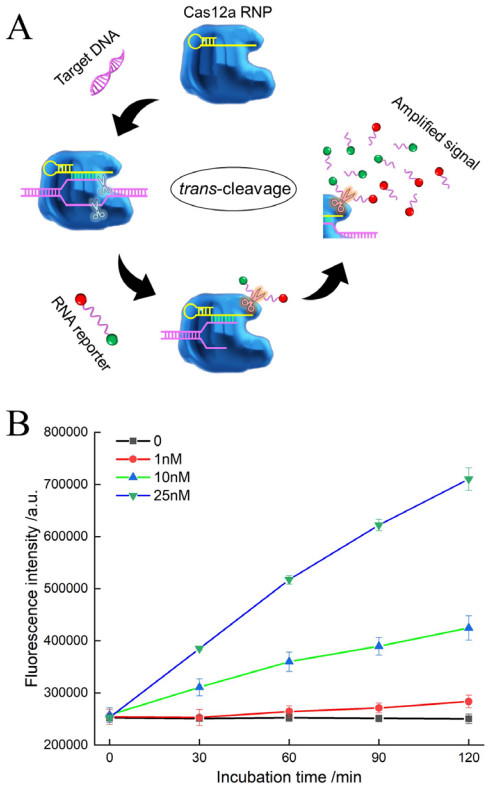

Figure 1. The development and evaluation of RNA reporter based CRISPR/Cas12a biosensing platform. (A) The schematic of RNA reporter based CRISPR/Cas12a biosensing platform; (B) The biosensing application of RNA reporter based CRISPR/Cas12a biosensing platform for the detection of different concentrations of trigger ssDNA (1, 10, 25 nM).

B. The optimization of RNA reporter based CRISPR/Cas12a biosensing platform.

In order to improve the sensitivity of RNA reporter based CRISPR/Cas12a biosensing system, we proposed two possible approaches, incubation temperature and chemical enhancers.

The effect of incubation temperature was first investigated as shown in Fig. 2A. Two different temperature points were investigated, room temperature (RT) and 37°C. The signals increased linearly with reaction time at both temperatures. Following the temperature increase from RT to 37°C, the fluorescence signal increased over 3 times.

To further improve the sensitivity of RNA reporter based CRISPR/Cas12a biosensing system, we introduced chemical enhancers to our basic biosensing system, including PVA, DTT, and TCEP. As shown in Fig. 2B, the fluorescence signals of all chemical enhancers assisted CRISPR/Cas12a biosensing systems were increased with the increase of incubation time. After introducing the non-ionic surfactant PVA into the biosensing system, the fluorescence signal increased around twice after two hours incubation. After introducing sulfhydryl reductants DTT or TCEP into the biosensing system, respectively, the fluorescence signals increased around 4 times and 3 times, respectively, after two hours incubation. Therefore, among the three chemical enhancers, DTT showed the optimum enhancement effect. Furthermore, after combining DTT with 37°C, the fluorescence signal increased around 10 times compared with the basic biosensing system (Fig. 2A). Therefore, DTT and 37°C was utilized for further biosensing application.

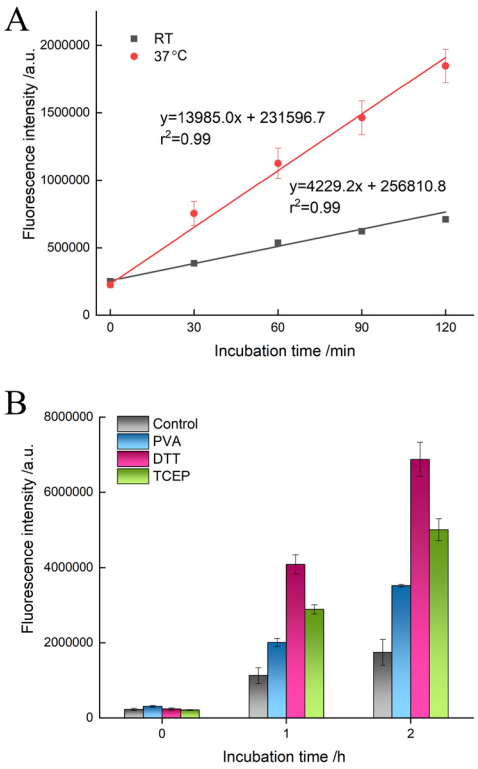

Figure 2. The optimization of RNA reporter based CRISPR/Cas12a biosensing platform (25 nM trigger ssDNA). (A) The optimization of reaction temperature; (B) The application of chemical enhancers in RNA reporter based CRISPR/Cas12a biosensing platform (37 °C).

C. The application of RNA reporter based CRISPR/Cas12a biosensing platform for the sensitive detection of ctDNA

After optimization of the RNA reporter based CRISPR/Cas12a biosensing platform, it was applied for detection of circulating tumour DNA (ctDNA), which is a key biomarker for cancer diagnostics. As shown in Fig. 3, different concentrations of ctDNA were applied in the optimized biosensing system. After two hours incubation, the final fluorescence signals were collected. Each testing contains three repeats, and *t*-test was applied to analyse the differentiations. As shown in Fig. 3, with the increase of ctDNA concentration, the corresponding fluorescence signals were all positively increased, indicating that the optimum biosensing system has the capability of quantification of ctDNA concentration. The limit of detection was 1pM, and the detection range was over 4 orders of magnitude from 1pM to 25 nM.

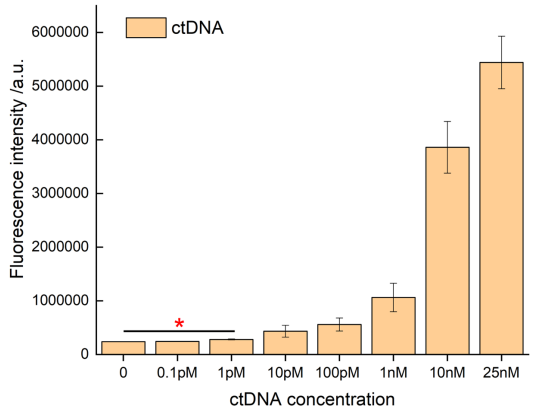

Figure 3. The application of RNA reporter based CRISPR/Cas12a biosensing platform for the sensitive detection of ctDNA (10 mM DTT, 37°C). * means $p < 0.05$. Student's *t*-test.

IV. Discussion and Conclusion

CRISPR/Cas12a biosensing system was established on its exceptional *trans*-cleavage ability for signal amplification [1]. To date, all the established CRISPR/Cas12a biosensing systems are using ssDNA-based reporters. Thus, our research is the first to use ssRNA-based reporters for CRISPR/Cas12a biosensing systems development. However, the basic RNA reporter based CRISPR/Cas12a biosensing systems shows limited sensitivity (1 nM), which was not sufficient for practical applications (fM to pM) [18].

In this research, we used two effective approaches to improve the sensitivity. Incubation temperature was first investigated, and 37°C was demonstrated to be the optimum temperature [11], which increased the fluorescence signal over 3 times. Afterwards, we introduced chemical enhancers to improve the sensitivity, which has been demonstrated to be effective for Cas12a RNP for the cleavage of ssDNA substrate in our previous research [7]. In this research, three different chemical enhancers were introduced, including PVA, DTT, and TCEP. After two hours incubation, comparing with the control group, the fluorescence signals of PVA, DTT, and TCEP involved groups increased 2, 4, 3 times, respectively. Thus, DTT was the optimum chemical enhancer. After combination of DTT with 37 °C, the fluorescence signal increased 10 times comparing with the basic biosensing system (Fig. 2A).

The optimized RNA reporter based biosensing system was further applied for the quantification of ctDNA since ctDNA analysis is an attractive biomarker for noninvasive monitoring of tumor growth, response, and spread [19]. The biosensing system shows over 4 orders of magnitude detection range with limit of detection (LOD) of 1pM (Fig. 3). Compared with the basic biosensing system, whose LOD is 1 nM (Fig. 1B), the optimized biosensing system increased the sensitivity up to 3 orders of magnitude, which is a significant improvement for biosensing application. Furthermore, comparing with DNA reporter based CRISPR/Cas12a biosensing system, whose LOD is 5 pM [11], better biosensing performance of our biosensing system was shown.

Therefore, in this research, we developed an RNA reporter based CRISPR/Cas12a biosensing system. It not only provides an alternative option for the development of CRISPR/Cas12a biosensing system, but also provides a sensitive biosensing platform for nucleic acids quantification.

References

[1] J. S. Chen *et al.*, "CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity," *Science,* vol. 360, no. 6387, pp. 436-439, 2018.

[2] M. Adli, "The CRISPR tool kit for genome editing and beyond," *Nature communications,* vol. 9, no. 1, pp. 1-13, 2018.

[3] C. Van Tricht, T. Voet, J. Lammertyn, and D. Spasic, "Imaging the unimaginable: leveraging signal generation of CRISPR-Cas for sensitive genome imaging," *Trends in Biotechnology,* 2022.

[4] Y. Li, S. Li, J. Wang, and G. Liu, "CRISPR/Cas systems towards next-generation biosensing," *Trends in biotechnology,* vol. 37, no. 7, pp. 730-743, 2019.

[5] M. Wang, R. Zhang, and J. Li, "CRISPR/cas systems redefine nucleic acid detection: principles and methods," *Biosensors and Bioelectronics,* vol. 165, p. 112430, 2020.

[6] D. C. Swarts, J. van der Oost, and M. Jinek, "Structural basis for guide RNA processing and seed-dependent DNA targeting by CRISPR-Cas12a," *Molecular cell,* vol. 66, no. 2, pp. 221-233. e4, 2017.

[7] F. Deng, Y. Li, B. Li, and E. M. Goldys, "Increasing trans-cleavage catalytic efficiency of Cas12a and Cas13a with chemical enhancers: Application to amplified nucleic acid detection," *Sensors and Actuators B: Chemical,* vol. 373, p. 132767, 2022.

[8] F. Deng, Y. Li, L. Qiao, and E. Goldys, "A CRISPR/Cas12a-assisted on-fibre immunosensor for ultrasensitive small protein detection in complex biological samples," *Analytica Chimica Acta,* vol. 1192, p. 339351, 2022.

[9] Y. Li, F. Deng, and E. M. Goldys, "A versatile CRISPR/Cas12a-based sensitivity amplifier suitable for commercial HRP-based ELISA kits," *Sensors and Actuators B: Chemical,* vol. 347, p. 130533, 2021.

[10] Y. Li, F. Deng, T. Hall, G. Vesey, and E. M. Goldys, "CRISPR/Cas12a-powered immunosensor suitable for ultra-sensitive whole Cryptosporidium oocyst detection from water samples using a plate reader," *Water Research,* vol. 203, p. 117553, 2021.

[11] J. Li *et al.*, "Discovery of the Rnase activity of CRISPR–Cas12a and its distinguishing cleavage efficiency on various substrates," *Chemical Communications,* vol. 58, no. 15, pp. 2540-2543, 2022.

[12] Y. Li, F. Deng, and E. M. Goldys, "A simple and versatile CRISPR/Cas12a-based immunosensing platform: Towards attomolar level sensitivity for small protein diagnostics," *Talanta,* vol. 246, p. 123469, 2022.

[13] J. Gong *et al.*, "An enhanced method for nucleic acid detection with CRISPR-Cas12a using phosphorothioate modified primers and optimized gold-nanopaticle strip," *Bioactive materials,* vol. 6, no. 12, pp. 4580-4590, 2021.

[14] R. Zeng *et al.*, "CRISPR-Cas12a-Derived Photoelectrochemical Biosensor for Point-Of-Care Diagnosis of Nucleic Acid," *Analytical Chemistry,* 2022.

[15] T. Li *et al.*, "G-triplex: A new type of CRISPR-Cas12a reporter enabling highly sensitive nucleic acid detection," *Biosensors and Bioelectronics,* vol. 187, p. 113292, 2021.

[16] M. Rossetti *et al.*, "Enhancement of CRISPR/Cas12a trans-cleavage activity using hairpin DNA reporters," *Nucleic acids research,* vol. 50, no. 14, pp. 8377-8391, 2022.

[17] R. T. Fuchs, J. Curcuru, M. Mabuchi, P. Yourik, and G. B. Robb, "Cas12a trans-cleavage can be modulated in vitro and is active on ssDNA, dsDNA, and RNA," *BioRxiv,* p. 600890, 2019.

[18] J. Phallen *et al.*, "Direct detection of early-stage cancers using circulating tumor DNA," *Science translational medicine,* vol. 9, no. 403, p. eaan2415, 2017.

[19] U. McDermott, J. R. Downing, and M. R. Stratton, "Genomics and the continuum of cancer care," *New England Journal of Medicine,* vol. 364, no. 4, pp. 340-350, 2011.