Sensors & Actuators: B. Chemical 320 (2020) 128164

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

CRISPR-Cas12a based aptasensor for sensitive and selective ATP detection

Lei Peng[a], Jin Zhou[a], Guozhen Liu[b], Lijuan Yin[a], Siyu Ren[a], Shuli Man[a,*], Long Ma[a,*]

[a] State Key Laboratory of Food Nutrition and Safety, Key Laboratory of Industrial Fermentation Microbiology (Ministry of Education), Tianjin Key Laboratory of Industry, Microbiology, School of Biotechnology, Tianjin University of Science & Technology, Tianjin 300457, China
[b] Graduate School of Biomedical Engineering, Faculty of Engineering, University of New South Wales, Sydney, NSW, 2121, Australia

ARTICLE INFO

Keywords:
CRISPR-Cas12a
Trans-cleavage
Small molecule detection
ATP
Aptamer
Fluorescent biosensor

ABSTRACT

CRISPR-Cas based detection platform opens up a new avenue for biosensing owing to its programmability, sequence specificity and high base resolution upon nucleic acid targets. In current study, we utilised the target DNA-induced non-specific single-stranded DNA cutting activity (*trans*-cleavage) of CRISPR-Cas12a to fabricate an aptamer mediated fluorescent biosensor for sensitive and selective detection of adenosine triphosphate (ATP). In this sensor, we designed an ATP-binding aptamer as the target ssDNA for Cas12a, thereby in the presence of ATP, the ATP-binding aptamer would be occupied by ATP, and less target ssDNA can be processed by Cas12a, which gave rise to an ATP concentration dependent change in fluorescence resulted from *trans*-cleavage of doubly labeled ssDNA reporter. In this way, we successfully "translated" ATP signals to nucleic acid signals that can be amplified by Cas12a process. The dynamic detection range was from 1 μM to 200 μM and the limit of detection was 400 nM. The entire sample-to-answer time for this biosensor was around 40 min. Overall, this novel biosensor balanced sensitivity, specificity, detection time and ease of use. Our proposed biosensor provided a principle-of-proof for detecting small molecule by using CRISPR-Cas12a system, which would unlock its potential and further its futuristic applications in the field of diagnostics.

1. Introduction

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated proteins (Cas), together referred to as CRISPR-Cas systems, have been discovered and proved to act as adaptive immune systems in bacteria and archaea for host defense against viral infection. Beyond this, it is also noted that the programmable endonucleases of CRISPR-Cas can be powered for diagnostics [1–5]. Such kinds of nucleic acid detection platforms have been realised and attracted wide attention. Some of them, for instance, SHERLOCK (Cas13a) [6], DETECTR (Cas12a) [7], and Cas14-DETECTR [8], were developed with high sensitivity and specificity. With regard to CRISPR-Cas12a, it owns a RNA-guided DNase activity for target DNA cleavage (*cis*-cleavage). Moreover, Cas12a was reported to have indiscriminate (non-specific) single-stranded DNase activity (also termed as *trans*-cleavage) upon correct target DNA recognition by cognate crRNA, which can be repurposed for nucleic acid detection [9,10]. CRISPR-Cas12a has been developed for sensing virus [7,11,12], mycoplasma [13], single nucleotide polymorphism (SNP) genotyping [10], exosome [14] and crop disease diagnosis and genetically modified organisms (GMOs) [15] and even small molecules [16,17]. It was also reported that CRISPR-Cas12a could be coupled with nucleic acid amplification technology such as recombinase aided amplification (RAA) [18] and loop-mediated isothermal amplification (LAMP) [19] or some visual detection methods for naked eye detection [20,21], which has added further values to point-of-care testing (POCT).

Nucleic acid aptamers are a new type of recognition molecule composed of DNA or RNA that fold into 3-D structures for specifically binding to targets with high affinity [22–24]. Lots of efforts have been focusing on adopting or engineering aptamers as recognition elements for sensor fabrication [25–28]. Adenosine triphosphate (ATP) is the main energy carrier for living organisms, playing central roles in cellular metabolism and biochemical reactions [29,30]. ATP concentrations are tightly regulated under normal conditions. Besides, ATP can be also used as an indicator for food quality control, hygiene, cell viability and environmental analysis [31–33]. Therefore, the accurate detection and quantification of ATP is urgently needed for both biochemical and medical applications. Adenosine triphosphate (ATP) aptamer has been well studied for its sequence, structure and functions and it is arguably one of the most widely used aptamers [34–38].

In this study, we reported a facile and sensitive CRISPR-Cas12a based biosensor mediated by aptamer for detecting ATP. The principle of proposed biosensor was depicted in Fig. 1, we used an ATP ssDNA aptamer as a "target ssDNA", in the absence of ATP, it was expected to

* Corresponding authors.
E-mail addresses: msl@tust.edu.cn (S. Man), malong@tust.edu.cn (L. Ma).

https://doi.org/10.1016/j.snb.2020.128164
Received 21 February 2020; Received in revised form 6 April 2020; Accepted 20 April 2020
Available online 04 May 2020
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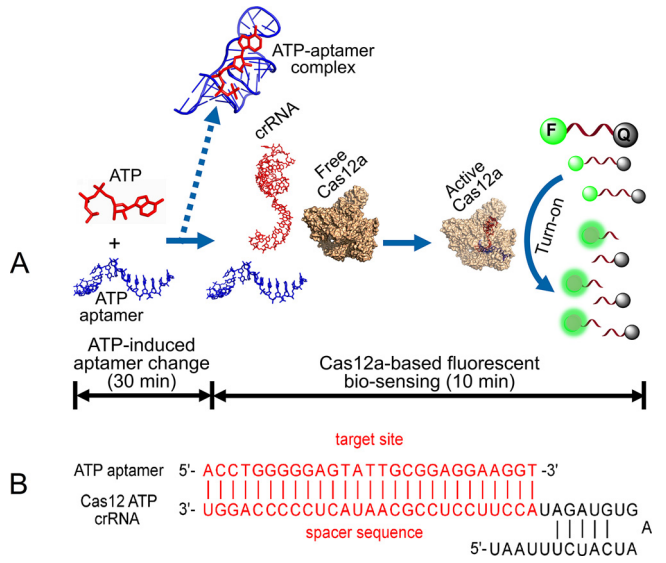


Fig. 1. Proposed CRISPR-Cas12a biosensor for ATP detection (A) and the schematic of target ssDNA as well as crRNA used (B). The target site is highlighted in red. The entire sample-to-answer time for this biosensor is appropriately 40 min. For the doubly labeled ssDNA reporter, F = FAM (carboxyfluorescein) dye and Q = BHQ1 (black hole quencher 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

bind crRNA and thus trigger the cleavage of a pre-added doubly labeled ssDNA, a "turn-on" fluorescence could be recorded. While in the presence of ATP, the aptamer would bind ATP and result in "ATP-induced aptamer changes" in terms of effective concentration for CRIPSPR-Cas12a analysis. Changes in fluorescence intensities corresponded to the varies of ATP concentrations, which was fully explored for ATP detection in this study. In this regard, we intended to utilised the striking features of CRISPR-Cas12a system, namely programmability, sequence specificity and *trans*-cleavage and combine them with aptamer to fabricate a biosensor, which would represent a novel type of sensing strategy and span the capability of CRISPR-Cas12a in small molecule detection.

2. Experimental section

2.1. Materials and reagents

ATP, UTP, CTP, GTP, AMP and ADP were purchased from Sigma-Aldrich (USA). The DNA were synthesized from Sangon Biotech Co. Ltd. (Shanghai, China). Common chemicals and organic solvents used in HPLC analysis were obtained from Sigma-Aldrich (USA) or Aladdin Biochemical Technology Co., Ltd. (China) unless otherwise specified. They were analytical grade or chromatographically pure where necessary and used directly without further purification. Fetal bovine serum (FBS) was obtained from Sijiqing Company Ltd., (Hangzhou, China). Ribonuclease (RNase) inhibitor (#M0314S) was purchased from New England Biolabs (USA). Distilled deionized water (ddH_2O) was used throughout the investigation. The DNA and RNA concentrations were quantified using 260 nm absorbance by a UV–vis spectrophotometer with the extinction coefficients calculated using online IDT OligoAnalyzer 3.1 (http://www.idtdna.com/calc/analyzer#).

2.2. LbCas12a protein purification

The protein expression vectors having *Lachnospiraceae bacterium* ND2006 LbCas12a protein (LbCas12a, GI number: 917059416) gene cloned were transformed into *Escherichia coli* BL21 (DE3) competent cells using the heat shock method. Specifically, a mixture of chemically competent bacteria and plasmid DNA was placed at 42 °C for 90 s and then placed back on ice. *Escherichia coli* BL21 (DE3) cells, transformed with the plasmids were grown overnight in Luria-Bertani (LB) medium supplemented with 100 μg/mL kanamycin, which was used to inoculate in LB for growth at 37 °C. Isopropylb-D-1-thiogalactopyranoside was added to a final concentration of 0.5 mM to initiate protein expression after the cell optical density (OD_{600}) reached 0.4 − 0.6. The cultures were then shaken for an additional 25 h at 16 °C, and harvested by centrifugation at 8000 rpm for 15 min at 4 °C. Cell pellets were harvested and stored at −80 °C for later purification. All subsequent steps of the protein purification were performed at 4 °C. The collected cells were crushed and resuspended in lysis buffer (50 mM Tris-HCl, 1.5 M NaCl, 1 mM DTT, pH 8.0) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) followed by sonication (XO-650D, Atpio, Nanjing, China). The lysate was centrifugated for 30 min at 12,000 rpm and the supernatant was filtered through a 0.22 μm filter. Filtered supernatant was applied to column containing Ni Sepharose™ 6 Fast Flow (GE) and incubated with rotation for 2 h followed by washing three times using lysis buffer. The target protein was eluted with a stepwise gradient of 50, 100, 200 mM imidazole. The collected eluted protein was dialysed against buffer (50 mM Tris-HCl, 200 mM NaCl, 1 mM DTT, 5% glycerol, pH 8.0) and then concentrated using Millipore concentrators. The protein concentration was measured based on Lambert-Beer law and frozen at −80 °C.

2.3. Preparation of crRNA

For preparation of crRNA, a single-stranded cognate DNA oligonucleotide with T7 promoter sequence was annealed with a primer sequence having a complementary T7 primer sequence (final concentrations 5 μM), which was then transcribed to crRNA by using the HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs). This transcription reaction was carried out at 37 °C overnight. Subsequently, the product crRNA was purified using Monarch® RNA Cleanup Kit (New England Biolabs).

2.4. The utility of this proposed CRISPR-LbCas12a biosensor for sensing ATP

Different concentrations of ATP were added in buffer composed of 20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ and 100 mM NaCl and incubated with 300 nM ATP aptamer at 37 °C for 30 min to allow the binding. The obtained mixtures were taken for the next step of detection. ATP detection was performed in a reaction containing 200 nM purified LbCas12a, 250 nM crRNA, 50 nM ssDNA reporter and pre-incubated ATP–ATP aptamer sampler buffered with 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 1 U/μL RNase inhibitor (reaction buffer). The assay was carried out at 37 °C and kinetic data were recorded in a Tecan Infinite® 200 Pro plate reader (Tecan, Switzerland) with excitation and emission wavelengths at 484 nm and 529 nm respectively and fluorescent data were measured every 30 s. The background fluorescence was subtracted accordingly and the obtained data were used subsequently for data plotting. The standard curve was made by plotting fluorescence intensity *vs.* ATP concentration for each point. The real aqueous ATP samples subjected to CRISPR-LbCas12a biosensor analysis were all done by using this standard curve. For real sample analysis, the reaction buffers with different concentrations of ATP were prepared by spiking different concentration of ATP into 5% fetal bovine serum (FBS).

2.5. ATP detection in aqueous solutions using HPLC (high performance liquid chromatography)

For standard curve preparation: Mobile phases for HPLC was composed of 95 % buffer A and 5% buffer B. Buffer A was 30 mM sodium phosphate buffer (pH 5.9) and buffer B was methanol. The HPLC was

operated at 30 °C with a flow rate of 1.0 mL/min. The wavelength of UV–vis detector was set at 254 nm. The aqueous stock solution of ATP was at 250 μM in water. Serial dilutions of stock solution were prepared whose final ATP concentrations were at 1, 5, 10, 20, 40, 80, 160 and 250 μM. The standard solution was subjected to HPLC (1260 Infinity, Agilent Technologies) analysis by using an analytical ZORBAX SB-C18 column (4.6 × 150 mm, 5 μm). The standard curve was made by plotting peak area *vs.* ATP concentration for individual point. The real aqueous ATP samples analysed by HPLC were all used this calculated standard curve. For real sample analysis, the reaction buffers with different concentrations of ATP were prepared by spiking different concentration of ATP into 5% fetal bovine serum (FBS).

2.6. Selectivity of proposed CRISPR-Cas12 biosensor for ATP detection

The selectivity for ATP detection was evaluated over some structurally related compounds including uridine 5′-triphosphate (UTP), cytidine 5′-triphosphate (CTP), guanosine 5′-triphosphate (GTP), Adenosine-5′-diphosphate (ADP) and Adenosine 5′-monophosphate (AMP). The concentrations of ATP and other compounds used were 200 μM and 2 mM respectively. The assaying condition was described as Section 2.4. ΔFL = FL in the absence of ATP - FL of samples with ATP or other tested compounds.

3. Results and discussion

To start with, we scrutinized the feasibility of the proposed CRISPR-Cas12a biosensor. As the core of this biosensor, the fidelity and specificity of LbCas12a were tested. After obtaining the cloned gene, its purification was also carried out (Figure S1). The crRNA was prepared using an *in vitro* T7 transcription reaction (Figure S2). As shown in Fig. 2A, in the absence of correct crRNA and target ssDNA, there was hardly any *trans*-cleavage of reporter ssDNA as evidenced by the fact that the readout stayed almost unchanged at the time of investigation; in contrast, only when LbCas12a met matched crRNA and target ssDNA, such *trans*-cleavage of reporter could be triggered. It was also noted that the doubly labeled reporter ssDNA was highly quenched by itself and only upon cleavage by LbCas12a, its fluorescence could be “turn-on”. We also confirmed that there was a target ssDNA concentration-dependent fluorescent signal increment for LbCas12a *trans*-cleavage of reporter (Fig. 2B). Fig. 2C showed the linear relationship between fluorescence intensities and target ssDNA, indicative of a huge possibility of LbCas12a for sensing of ssDNA.

Next, we intended to use this CRISPR-Cas12a biosensor for detecting ATP. In order to do so, some factors, which are in correlation with the performance of the fluorescence measurements, were investigated in detail. Based on previous studies, we found that a 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris) based buffer is the correct one to work with [6,8,9]. Besides, we optimised the pH values together with

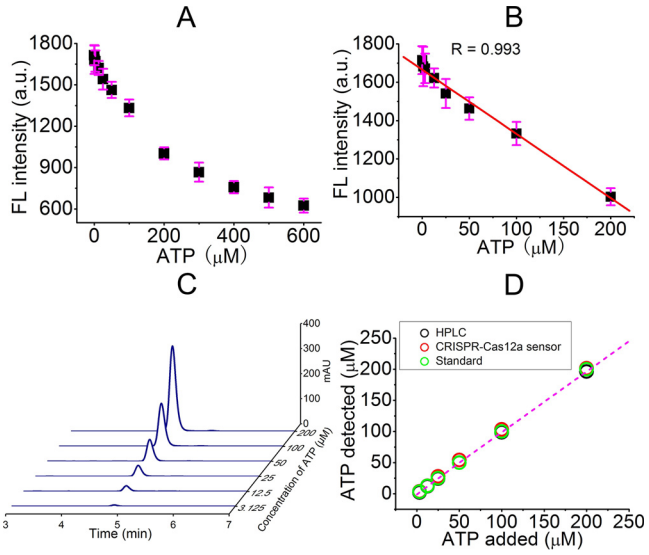


Fig. 3. The detection capability of the proposed CRISPR-Cas12a biosensor for ATP detection. A: The dependence of fluorescence intensities upon ATP concentrations. B: The linear relationship between fluorescence intensities and the concentrations of ATP. C: The HPLC profiles for ATP detection. D: The comparison of results between HPLC and CRISPR-Cas12a biosensor for ATP detection.

concentrations of Tris, Na^+, Mg^{2+} and BSA (Figure S3-S7), which might have effect on the performance of the biosensor. Changes of fluorescence signals in the presence and absence of correct target ssDNA were used as an indicator for the sensitivity of LbCas12a *trans*-cleavage. The nucleic acids used in the optimisation were listed in Table S1. We figured out that a 50 mM Tris-HCl buffer with a 8.0 pH value containing 100 mM NaCl and 10 mM $MgCl_2$ would be satisfactory. It was seemed that BSA was not necessary for the buffer. In addition, we worked out the appropriate LbCas12a/crRNA ratio for ATP sensing (Figure S8). The LbCas12a and crRNA were used at 200 nM and 250 nM respectively. It was assured that the addition of ATP did not inhibit the LbCas12a *trans*-cleavage activity (Figure S9). After that, the capability of the proposed biosensor for ATP detection was investigated. As shown in Fig. 3A, the relationship between fluorescence intensities and added ATP was illustrated. With incremental amounts of ATP, ATP–aptamer complex was expected to form, which resulted in less target ssDNA to be processed by Cas12a. This led to the gradual decline in fluorescence intensity (Fig. 3A). There was a linear relationship (R = 0.993) between fluorescence intensities and ATP concentrations over the range from 1.0 μM to 200 μM (Fig. 3B), and a 400 nM limit of detection (LOD) for ATP analysis was achieved, based on the 3σ/slope equation. To test the performance of our proposed CRISPR-Cas12a biosensor for detection of

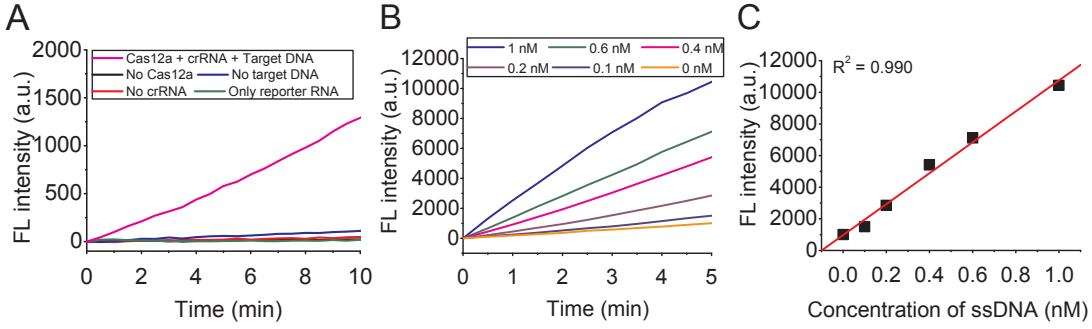


Fig. 2. Proof-of-principle of CRISPR-Cas12a biosensor. A: Feasibility assessment of CRISPR-Cas12a biosensor. B: The real time fluorescence recording for CRISPR-Cas12a *trans*-cleavage upon different concentrations of the target ssDNA. C: The linear relationship between fluorescence intensities of FAM-labeled reporter under different concentrations of input ssDNA (data extracted from Fig. 1B).

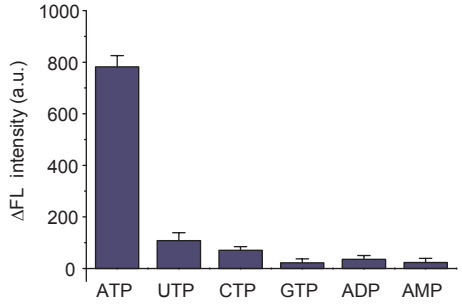


Fig. 4. The selectivity of the proposed method for ATP detection compared to five other ATP analogues. The ATP tested was at 200 μM while other five interfering compounds were at 2 mM.

ATP in real samples, we challenged our biosensor by detecting spiked ATP in serum, a complex and intractable matrix that would contain interfering molecules. We also applied conventional high performance liquid chromatography (HPLC) method to analyse the same spiked ATP standard solutions (Fig. 3C), which was considered as a highly reliable approach for ATP detection. The standard curves obtained from HPLC quantification was shown in Figure S10. Comparing with the results from both methods, we noticed that they were in a good correlation (Fig. 3D) and the results from both methods were highly close to the known concentrations of the standards. The above-mentioned data reinforced the possibility of CRISPR-Cas12a biosensor for qualitative ATP sensing with a superior detection limit and a wide dynamic range in comparison to the most reported fluorescent aptasensors (Table S2).

Lastly, the selectivity of our proposed biosensor for ATP detection was evaluated by examining the response of this biosensor to the analogues of ATP including AMP, ADP, UTP, CTP and GTP (Fig. 4). 200 μM ATP and 2 mM for the five interfering compounds were tested. It was observed that only ATP could markedly change the fluorescence and only minimal fluorescence changes were observed for AMP, ADP, CTP and GTP. The fluorescence response to UTP was less than 15 % that of ATP even if the concentration of the former was 10 folds compared with the latter. It was demonstrated that the biosensor displayed explicit selectivity for ATP over other nucleotide analogues. This selectivity was originated from the specificity of the ATP-binding aptamer. More, the high selectivity of CRISPR-Cas12a biosensor enabled its scope for futuristic use.

4. Conclusions

In summary, we have realised a prototype of aptamer mediated a CRISPR-Cas12a biosensor for detecting small molecule evidenced by sensitive and selective ATP detection. This biosensor harnessed the power of widely used small molecule binding DNA aptamer as a recognition element, and the recently discovered *trans*-cleavage of CRISPR-Cas12a for signal translation and amplification. In this case, it successfully translated ATP signals to nucleic acid signals. The fluorescence readout of proposed biosensor was fast, simple and sensitive [39–41], and could be immediately read out by a portable fluorescent sensor, making it more amenable to on-site detection. Overall, this strategy could be put to the “tool box” as a “module” for analysts, which could be further coupled with other well established methods in a “plugged in” manner. For example, our proposed biosensor could be assembled with some fast and/or visual detection techniques such as colorimetric, electrochemical signals, or lateral ow strip readouts [42,43]. Besides, if our proposed method can be established and recognised by other researchers, then some DNA amplification technologies (such as rolling cycle amplification, RCA or strand displacement amplification, SDA) can be subsequently wired or assembled with the current one to improve LOD in the futuristic work [44–46]. Though there is intensive research in nano-, aptamer-based biosensors [47–49], the pursuit of novel detection strategy has always been underway. CRISPR-Cas biology is expected to be developed as rapid, accurate, portable and multiplexed diagnostic tools of new generation. Our current work would enrich the reach of CRISPR-Cas biology based detection. Also, with proved reliability, sensitivity, specificity and simplicity, our proposed method could be extended and generalised for other analytes’ detection, promising to be developed to meet the need in a wide range of areas.

CRediT authorship contribution statement

Lei Peng: Methodology, Investigation, Data curation, Writing - original draft. **Jin Zhou:** Investigation, Methodology, Formal analysis, Validation. **Guozhen Liu:** Writing - review & editing. **Lijuan Yin:** Conceptualization, Writing - review & editing. **Siyu Ren:** Software. **Shuli Man:** Supervision, Project administration, Methodology. **Long Ma:** Conceptualization, Project administration, Supervision, Writing - original draft, Funding acquisition.

Declaration of Competing Interest

All of the authors would like to declare that there are no conflict of interest.

Acknowledgements

This work is supported by National Natural Science Foundation of China (No. 81503086, 21672161), Tianjin Municipal Science and Technology Committee (18PTSYJC00140, 19JCYBJC27800). Guozhen Liu acknowledges the Australian Research Council (ARC) for funding of the Future Fellowship (FT160100039), University of New South Wales (UNSW) for the Biomedical Engineering Seed Fund and Digital Grid Futures Institute Seed Fund.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2020.128164.

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