Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Allosteric aptasensor-initiated target cycling and transcription amplification of light-up RNA aptamer for sensitive detection of protein

Danxia Song¹, Deyu Yuan¹, Xuemei Tan¹, Ling Li, Huan He, Liang Zhao, Gang Yang, Sirui Pan, Hongyuan Dai, Xu Song*, Yongyun Zhao*

Center for Functional Genomics and Bioinformatics, College of Life Science, Sichuan University, Chengdu, Sichuan 610064, PR China

ARTICLE INFO

Keywords:
Allosteric aptasensor
Target cycling signal amplification
Transcription amplification
Protein detection
Light-up RNA aptamer

ABSTRACT

The early detection of biomarker proteins in clinical samples is of great significance for the diagnosis of diseases. However, it is still a challenge to detect low-concentration protein. Herein, a label-free aptamer-based amplification assay, termed the ATC-TA system, that allows fluorescence detection of very low numbers of protein without time-consuming washing steps and pre-treatment was developed. The target induces a conformational change in the allosteric aptasensor, triggers the target cycling and transcription amplification, and ultimately converts the input of the target protein into the output of the light-up aptamer (R-Pepper). It exhibits ultrahigh sensitivity with a detection limit of 5.62 fM at 37 °C and the accuracy is comparable to conventional ELISA. ATC-TA has potential application for the detection of endogenous PDGF-BB in serum samples to distinguish tumor mice from healthy mice at an early stage. It also successfully detects exogenous SARS-CoV-2 spike proteins in human serum. Therefore, this high-sensitive, universality, easy-to-operate and cost-effective biosensing platform holds great clinical application potential in early clinical diagnosis.

1. Introduction

Proteins play a critical role in multifarious biological processes and are deemed to be important biomarkers for clinical diagnosis and prognosis. Early detection of serum biomarker proteins is of great significance for the early diagnosis and treatment of diseases, such as cancer and viral infection [1–3]. Protein can be measured by different methods including enzyme-linked immunosorbent assays (ELISA) [4], mass spectrometry-based proteomics [5,6], surface plasmon resonance (SPR) [7], electrochemiluminescence [8] and the lateral flow assay-based method [9,10]. Among them, ELISA has been the classical method for the quantitative assessment of water-soluble proteins. It is performed in the form of a commercial kit which has the advantage of convenience. Nevertheless, due to the low sensitivity, generally ranging from ng/ml to μg/ml [11], it can’t meet the growing demand for early diagnosis of diseases based on low-abundance biomarkers. To improve the sensitivity of ELISA, various signal amplification techniques, such as redox-cycling-based colorimetric ELISA [12], digital ELISA [13] and plasmonic ELISA [14], have been developed. However, the time-consuming washing steps in such assays not only make them labor-intensive but also prevent analysis in real-time, limiting further application. Although the lateral flow assay-based method is simple and suitable for rapid detection [15,16], it can only be used for semi-quantitative analysis and isn’t sensitive enough, which is not suitable for low-concentration protein quantification or early diagnosis [9,10].

Alternatively, aptamer-based amplification assays have been developed with great effort. Aptamers are short single-stranded DNA or RNA that can interact with corresponding targets with high affinity and specificity [17,18]. Based on the structure of nucleic acid, aptamers can be used for allosteric regulation to develop aptamer-based amplification assays [19], such as recombinase polymerase amplification (RPA) [20], rolling circle amplification (RCA) [21,22], hybridization chain reaction (HCR) [23,24], CRISPR-Cas13a/12a amplification reaction [25,26]. However, the sensitivity for most methods is at the picomolar level [27]. Therefore, it is still necessary to develop a highly sensitive technique for the detection of low concentrations of proteins.

In recent years, the light-up RNA aptamers, such as Spinach, Broccoli and Pepper, which can bind fluorogens (DFHBI/DFHBI-1 T, HBC, et) and enhance their fluorescence by several orders of magnitude upon

¹ The authors should be regarded as Joint First Authors

https://doi.org/10.1016/j.snb.2022.132526
Received 8 June 2022; Received in revised form 31 July 2022; Accepted 16 August 2022
Available online 18 August 2022
0925-4005/© 2022 Elsevier B.V. All rights reserved.
binding, have been applied as a promising label-free reporter for in biological detection [28]. Compared with light-up RNA aptamers, the non-specific binding of sequences, such as SYBR Gold and SYBR Green I, inevitably, leads to high background or nonspecific signals [29]. Although covalently labeled probes would allow specific labeling, however, compromised with complicated design and high cost [30]. Hence, the advantages of light-up RNA aptamers could be exploited as an ideal reporter for target-specific detection.

Herein, we designed a label-free allosteric aptasensor (Aa) to develop an aptamer-based cascade amplification assay for protein detection, termed the “allosteric aptasensor-initiated target cycling and transcription amplification” (ATC-TA) system, to overcome the limitation of sensitivity and cumbersome procedures in protein detection. As a proof of concept, platelet-derived growth factor-BB (PDGF-BB) was chosen as the model analyte, which is an important biomarker for tumor diagnosis [31,32]. The accuracy of this system is comparable to ELISA in complex samples, but exhibits ultra-high sensitivity. This system not only realizes fluorescent detection but also can detect protein in real-time and visual detection. Additionally, using the aptamer of SARS-CoV-2 spike protein previously reported by our group [33], an ATC-TA system for detecting spike protein was established, which showed that this system has universality, making it more applicable for protein detection.

## 2. Materials and methods

### 2.1. Materials

The PDGF-BB aptamer (ssDNA, 5′- CAGGCTACGGCACGTA′-GAGCATCACCATGATCCTG-3′), the aptamer of SARS-CoV-2 spike protein (ssDNA, 5′- GGGAGGGCGGTGATTGGATGCCGA – 3′) and all other oligonucleotides (Table S1) were purchased from Sangon Biotech (Shanghai, China) and purified by HPLC.

### 2.2. Binding assays of an allosteric aptasensor to PDGF-BB

PDGF-BB proteins (100 ng per well) were coated in the 96-well ELISA microplates with 100 μL coating buffer at 4 °C overnight. The PDGF-BB-protein-coated plates were washed twice with washing buffer before blocking with 200 μL of the solution (2% BSA in PBST) at RT for 1 h. Then, 200 nM final concentration biotin-labeled Aa at the 5′-end were added into the wells and incubated in binding buffer at RT for 30 min with gentle shaking. After washing two times, streptavidin-horseradish peroxidase (HRP) and its substrates were sequentially added to the reactions. Color development was carried out using TMB substrate and measured using a microplate reader at 450 nm absorbance.

### 2.3. ATC-TA for protein detection

The amplification was carried out in a 10 μL mixture containing 5 nM Aa, 5 nM Tp, 1 × Bst buffer (20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄ and 0.1% Triton X-100), 10 mM NaCl, 4 mM MgSO₄, 0.5 mM dNTPs, 0.12 units/μL of Bst DNA polymerase and protein at 37 °C for 1 h. After this extended reaction, the transcription was carried out. ATC products were transcribed in a 20 μL reaction containing 10 μL of ATC product, 0.75 mM of NTP, 1 × T7 RNA polymerase buffer (40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 10 mM DTT, 6 mM MgCl₂, 2 mM spermidine), and 0.75 units/μL of T7 RNA polymerase at 37 °C for 2 h. Afterward, fluorescence was measured at respective wavelengths.

### 2.4. Spectroscopic measurements

A solution of the ATC-TA products and HBC (10 μM) were incubated with the target in a buffer containing 40 mM HEPES (pH=7.4), 100 mM KCl and 10 mM MgCl₂ at 25 °C for 10 min. Fluorescence spectra were measured using Bio-Tek Synergy H1 equipped with 25 °C. The emission wavelength window, 495–600 nm; slit widths, 5 nm. The excitation wavelength was 460 nm; and the emission wavelength was 514 nm.

### 3. Results and discussion

#### 3.1. The principle of the ATC-TA system

The principle of the designed strategy of ATC-TA is illustrated in Fig. 1. Two label-free DNA probes, namely Aa and Tp are ingeniously designed. Allosteric aptasensor (Aa) without sticky end contained four functional modules: (1) aptamer modules (carmine) for specific recognition of target protein (purple); (2) T7 promoter modules (orange); (3) 6 successive adenine bases (black) between the aptamer and T7 promoter to reduce any possible steric hindrance effect, which has no interaction with other DNA sequences; (4) extend modules (blue) which is complementary with part of Tp on the 3′ terminals to initiate the downstream reaction.

In the absence of a target, the Aa is in an inactive conformation (stem-loop conformation). The extended modules form a stem with the 5′-end of aptamer, blocking the downstream reaction. Once the protein binds to the aptamer domain, the protein would mediate the conformational switch of the aptamer module, restoring the active structure and releasing the extended modules to hybridize to the probe Tp, which consists of two parts from the 3′- to 5′-end: the antisense sequence of the extend modules (blue) and the antisense sequence of light-up aptamer (green). Once the extended modules were captured by the probe Tp, DNA polymerase would extend on the Aa-Tp duplex to yield double-stranded DNA, followed by the displacement of the target, which will continue to bind with another Aa, initiating the target cycle. Meanwhile, T7 RNA polymerase recognized the T7 promoter sequence in double-stranded DNA and conducted RNA transcription to generate a large amount of RNA product, the light-up RNA aptamer with a tail on the 5′-end. Finally, those multiple light-up RNA aptamers bind to HBC to generate fluorescence signals as a reporter. The target cycle and transcription amplification are repeated continuously and enrichment of multiple light-up RNA aptamers, resulting in a cascade of reactions and amplified fluorescence signals.

#### 3.2. Feasibility studies of ATC-TA

To analyze the feasibility of this strategy, Aa with 11 bp stem (Aa11) and Tp with 8 bases (Tp8) complementary to Aa were designed. Firstly, we analyze whether the PDGF-BB aptamer (Kₐ = 0.1 nM) domain of Aa11 could bind to PDGF-BB protein by ELISA. The result revealed that Aa11 can successfully bind with PDGF-BB (Fig. 2A-B). Secondly, to select the light-up RNA aptamers, we compare the fluorescence of Peppers, which have superior properties, with that of Broccoli, which is one of the widely used light-up RNA aptamers [28,34,35]. Pepper exhibits a higher signal-to-noise ratio (S/N) (205.2-fold, Fig. 2C) and is used as the fluorescent reporter in this work.

Because the light-up RNA corresponding to Aa11 carries an 11-base tail at the 5′-end (Pepper-11), we asked whether the tail would interfere with the fluorescence signals. The result showed that the tail did interfere with Pepper’s fluorescence (Fig. 2D-E). To reduce this interference, we tried to reverse the Pepper sequence and obtained the R-Pepper (Reversed Pepper). Although the R-Pepper’s fluorescence is weaker than that of Pepper, the fluorescence of R-Pepper-11 (with the tail at R-Pepper) is stronger than that of Pepper-11 and is consistent with that of R-Pepper (Fig. 2D-E), indicating that R-Pepper can tolerate more unrelated sequences than Pepper, which broadened the application range of the light-up aptamer. Therefore, R-Pepper was chosen for the subsequent experiments.

Subsequently, to verify the proposed scheme step by step, native polyacrylamide gel (PAGE) was performed. Firstly, it is necessary to investigate whether the Aa11 can be opened by Tp8 and then extended by Bst DNA polymerase. The result shows that the product band was not...
observed (Fig. 2F, Lane 4; Fig. 2I). When PDGF-BB was added to the reaction, the Aa11 is allosteric, hybridizing with Tp8, and an extended product with predicted length was observed in PAGE analysis (Fig. 2G, Lane 5; Fig. 2I). Afterward, the T7 RNA polymerase is bound to the T7 promoter sequence in double-stranded DNA and transcript RNA (Fig. 2H, Lane 7; Fig. 2I). The bands in Lane 7 and Lane 9 (in vitro transcribed R-Pepper-11, as the marker) were consistent with each other, suggesting that in the presence of PDGF-BB, those two probes can work well and result in amplified fluorescence signals. It is worth noting that there is one extra band in the middle of lane 7. It was proved to be the hybridization product of R-Pepper-11 and Tp8 (Fig. S1A-B). Secondly, the possible false-positive results were also analyzed using native PAGE (Fig. S1C), indicating that the reaction could only be triggered by PDGF-BB. Moreover, to further confirm those results, we also measured the fluorescence intensity of each step (Fig. 2J) and the results were consistent with the native PAGE. All those results indicated that the strong fluorescence signal could be detected only in the presence of the PDGF-BB.

3.3. Optimization of reaction parameters

To acquire optimal performance for PDGF-BB detection, several important aspects were systematically investigated. The stem region of Aa was crucial for the binding efficiency of PDGF-BB. We designed different Aa variants for screening. Among them, when we designed the Aa10, aptamer and T7 promoter are just complementary, resulting in a different Aa variants for screening. Among them, when we designed the Aa was crucial for the binding efficiency of PDGF-BB. We designed important aspects were systematically investigated. The stem region of PDGF-BB.

3.4. Analytical performance of the ATC-TA system

To investigate the sensitivity of ATC-TA, we investigated various concentrations of PDGF-BB under optimal conditions. The fluorescence intensity increased with the elevated PDGF-BB concentration, showing a positive correlation (Fig. 4A). At low concentrations, especially in the range of 250 fM–250 pM, an equation is obtained by linear regression analysis of the calibration curve (Fig. 4B). The limit of detection (LOD) was calculated as our previously reported [36]. The LOD is defined as 3σ/S (in which S is the slope of the calibration curve in the range of 250 fM–750 pM, and σ is the standard deviation of the blank, n = 3) and the calculated LOD is 5.62 fM. Additionally, the detection limit and linear range of previously reported methods were compared in Table S2. Those results demonstrate that this ATC-TA system has ultrahigh sensitivity.

Furthermore, to avoid interference from other proteins, several kinds of proteins were investigated to assess the selectivity of ATC-TA. The result showed that the fluorescence was significantly increased only with the addition of PDGF-BB. Conversely, the addition of the other protein, such as IgG, TNF-a, BSA and the two isomers (PDGF-AA and PDGF-AB) presented a weak fluorescence signal (Fig. 4C), demonstrating that ATC-TA is highly specific. The reaction tube was illuminated with a blue gel imager (excitation with 488 nm), and PDGF-BB–positive correlation (Fig. 4A). At low concentrations, especially in the range of 250 fM–250 pM, an equation is obtained by linear regression analysis of the calibration curve (Fig. 4B). The limit of detection (LOD) was calculated as our previously reported [36]. The LOD is defined as 3σ/S (in which S is the slope of the calibration curve in the range of 250 fM–750 pM, and σ is the standard deviation of the blank, n = 3) and the calculated LOD is 5.62 fM. Additionally, the detection limit and linear range of previously reported methods were compared in Table S2. Those results demonstrate that this ATC-TA system has ultrahigh sensitivity.

Furthermore, to avoid interference from other proteins, several kinds of proteins were investigated to assess the selectivity of ATC-TA. The result showed that the fluorescence was significantly increased only with the addition of PDGF-BB. Conversely, the addition of the other protein, such as IgG, TNF-a, BSA and the two isomers (PDGF-AA and PDGF-AB) presented a weak fluorescence signal (Fig. 4C), demonstrating that ATC-TA is highly specific. The reaction tube was illuminated with a blue gel imager (excitation with 488 nm), and PDGF-BB could even be visualized in high concentrations with the naked eye (Fig. 4D), which provides a variety of optional analysis methods for rapid on-site detection.

Because of the laborious washing steps required in ELISA, it is unusable for real-time analysis. Nevertheless, to achieve a more accurate quantitative detection of targets, it is better to monitor the progress of amplification reaction on a real-time basis. To detect PDGF-BB in real-time, real-time ATC-TA was carried out and performed at 37 °C. The result exhibits the typical fluorescence intensity curves in the presence of varying PDGF-BB concentrations (Fig. 4E-F). Those results indicated that the ATC-TA system was convenient and effectively detected PDGF-BB in real-time.
3.5. Detection of endogenous PDGF-BB protein in complex biological samples

To evaluate the applicability of ATC-TA system in real samples, the fluorescence of PDGF-BB was measured in human serum solutions diluted in different proportions, from 10-fold to 10000-fold dilutions (Fig. 5A). Their concentrations were then calculated from the calibration curve. We also compared the ATC-TA with the commercialized ELISA (Fig. S4A–B) and the accuracy was consistent with the ELISA (Table S3), demonstrating that this ATC-TA system could accurately detect the target in complex biological samples without interference from the matrix. Notably, as expected, when the serum is diluted to 10000-fold, the concentration is undetectable by ELISA. Nevertheless, the ATC-TA still successfully detected PDGF-BB, indicating that ATC-TA can be used as a sensitive platform for complex biological samples.

To ask whether the ATC-TA system can be used for the early diagnosis and prognosis of the tumor, we first demonstrated that Aa10 was also capable of binding to mouse PDGF-BB (Fig. 5B). Subsequently, we established the mouse MC38 colon tumor model to evaluate its ability for early diagnosis of tumor (Fig. 5C). The mouse PDGF-BB abundance in serum showed an upward trend with tumor growth. Compared with healthy mice, ATC-TA could effectively distinguish tumor mice from healthy mice in the early stage (3-day after subcutaneous inoculation of MC38 cells to 9-day) (Fig. 5D–E), demonstrating that the ATC-TA system has great application potential in complex substrate samples, especially in the early clinical diagnosis of a biomarker.

3.6. Detection of the exogenous SARS-CoV-2 spike protein in human serum

Having demonstrated the ability of ATC-TA to detect endogenous proteins in serum, we wondered whether it could detect exogenous proteins in serum for the detection of viral infection. We designed an allosteric aptasensor (S-Aa) based on the aptamer ST-6–2 (27 nt, \( K_d = 80 \text{ nM} \)) [33] targeting the SARS-CoV-2 spike protein and analyzed its feasibility (Fig. S5A–B). The optimized stem length of S-Aa was 12 bp and the concentration was 10 nM (Fig. S5C–E). The fluorescence exhibited significantly increased in the presence of spike protein (Fig. 6A) and there was a good linear relationship (Fig. 6B). It also has ideal selectivity to distinguish SARS-CoV-2 from other spike proteins of viruses, reducing the risk of contamination from other coronaviruses (Fig. 6C). Additionally, this system exerts ideal universality for SARS-CoV-2 variants, including the Delta, Lambda and Omicron, whose fluorescence is essentially identical to that of the wild type (Fig. 6D).
Those demonstrat that this system has the potential to detect SARS-CoV-2 and its variants.

Afterward, a series of contrived samples were prepared by adding known concentrations of spike protein to human serum and the final concentrations were 0.25, 0.5, 1 and 1.5 nM, respectively. Then, we use the ATC-TA system to measure the fluorescence signal of these samples (Fig. 6 E-F) and calculate those concentrations. The recoveries ranged from 91% to 117% (Table S4) and the matrix did not affect the detection of exogenous proteins (Fig. S5F), indicating that the ATC-TA system can be used as an alternative for the screening of virus infections, having potential utility in clinical settings.

4. Conclusion

In summary, a highly sensitive and specific ATC-TA system for fluorescence detection of protein was developed. It exhibits several advantages: first, compared with the current common method, it has high sensitivity, providing a simple method for low-concentration protein quantification or early diagnosis of biomarker proteins. Second, it does not require time-consuming washing steps, pre-treatment and other
cumbersome operations, making the process simple and easy to practical application. Third, all probes used in this system require no modification, which is important for retaining its high target-binding activity and making the system more convenient and cost-effective. Fourth, it not only realizes fluorescent detection but also can detect protein in real-time and visual detection, providing a variety of optional analysis methods. Additionally, this system is technically variable, offering great potential for simple, low-cost detection of many other targets by designing corresponding allosteric aptasensor, including proteins, metabolic molecules and pathogenic microorganisms. Taken together, this ATC-TA system provides a simple and sensitive biosensing platform with practical utility in the clinical setting, particularly in the early stages of disease diagnosis.

CRediT authorship contribution statement

Danxia Song: Methodology, Formal analysis. Deyu Yuan: Methodology, Formal analysis. Xuemei Tan: Formal analysis, Validation. Ling Li: Formal analysis. Huan He: Software. Liang Zhao: Investigation. Gang Yang: Investigation. Sirui Pan: Resources. Hongyuan Dai: Resources. Xu Song: Supervision, Methodology, Writing – review & editing. Yongyun Zhao: Conceptualization, Methodology, Writing – review & editing.

Declaration of Competing Interest

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

Data will be made available on request.

Acknowledgments

This study was supported by the National Key Research and Development Project (2017YFA0504300), Chengdu Science and Technology Program (2021-YF05-01681-SN).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2022.132526.

References

[1] Z. Zhang, R.C. Bart, Y.H. Yu, J.N. Li, L.J. Sokoll, A.J. Rai, et al., Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer, Cancer Res 64 (2004) 5882–5890.

[2] J.F. Rusling, C.V. Kumar, J.S. Gutkind, V. Patel, Measurement of biomarker proteins for point-of-care early detection and monitoring of cancer, Analyst 135 (2010) 2496–2511.

Fig. 6. Detection of the SARS-CoV-2 spike protein in human serum. A. Fluorescence spectra of ATC-TA in response to different concentrations of the SARS-CoV-2 spike protein. Blank: without spike protein. B. The calibrated curve was made according to the fluorescence spectra. C. Selectivity study of ATC-TA system against the spike protein of coronaviruses. D. Fluorescence peak intensity to assess the performance of the ATC-TA system for the spike protein of wild-type SARS-CoV-2 to detect the spike protein from SARS-CoV-2 variants. E. The general workflow to detect contrived samples using ATC-TA. F. Fluorescence spectra were made by adding spike protein of SARS-CoV-2 to human serum. All the P values were determined using paired t-test. * P < 0.05. Error bars were shown as means ± S.D., n = 3, triplicate.
