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Novel strategy to improve the sensing performances of split ATP aptamer based fluorescent indicator displacement assay through enhanced molecular recognition

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ABSTRACT

Split aptamer strategy was often used to improve the sensitivity of aptasensor. However, traditional split aptamer strategy can not be directly used to improve the label-free aptamer based Thioflavin T (ThT) displacement assay for ATP because the split ATP aptamer display much lower enhancement effects on the fluorescence of ThT than intact aptamer. In order to address this issue, this is the first report using G-rich DNA sequence to enhance the affinity of the two split ATP aptamer halves to ThT and offer lower limit of detection (LOD), wider linear range and higher selectivity through the enhanced molecular recognition. Compared to the intact aptamer/ThT complex, the ensemble of two G-rich split ATP aptamer fragments/ThT are higher fluorescent. Consequently, G-rich sequences would improve the fluorescent signal and thus the sensing performance of the proposed assay. In the optimized conditions, the LOD of the proposed fluorescent ATP aptasensor is 2 nM, which is lower than the reported ThT/ATP aptamer based methods. Additionally, our aptasensor has a wider dynamic linear range (0.1 μM - 120 μM) and higher selectivity. The proposed aptasensor has been successfully applied to detect ATP in 15% human serum. More importantly, the current study not only provides a novel method for ATP assay but also presents a way to construct a label-free split aptamer based fluorescent sensor for other species where aptamer can be generated.

1. Introduction

Aptamers are single-stranded deoxyribonucleic acids (DNAs) or ribonucleic acids (RNAs) which were selected *in vitro* via Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Tuerk and Gold, 1990) to selectively recognize its targets with high affinity. Aptamers offer a practical alternative for the detection of biomacromolecules such as proteins and nucleic acids, small molecules, and even whole cells (Roncancio et al., 2014). Compared to antibodies, aptamers have many advantages, such as simple synthesis, high specificity, relatively small size, relatively easy labeling, good stability, non-immunogenic nature, cheap to produce, relatively fast, be chemically synthesized with extreme accuracy and reproducibility (Tan and Fang, 2015). Meanwhile, the target induced structure-switch of aptamers offers high flexibility in constructing novel aptasensors with good sensing performances, for example sensitivity and selectivity (Iliuk et al., 2011).

In the research field of fluorescent chemo/biosensor, non-covalent interactions maybe preferred when a quick response and a fast recovery rate are required, yet the weakness of the supramolecular forces can be disadvantageous when biomolecules need to be immobilized on the surface and to be stable during the assay, thus making covalent linkages more suitable (Anichini et al., 2018). The advantage of label free method is that it does not damage the affinity of the fitness to the target (Geng et al., 2018). Other than this, it is flexible in design and cheap in price, but its biggest challenge is low sensitivity. One reason for the low sensitivity is that the binding of aptamers with its target is essentially the binding of bases and the target sites, which leads to steric hindrance

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of excessive bases. To meet this challenge and improve the sensing performance especially the sensitivity, split aptamers, split DNA enzymes and split proteins have recently drawn extensive attention (Kolpashchikov, 2008; Zhang et al., 2018; Zhu et al., 2016). After a single aptamer is split into two fragments, the two separated parts also bind specifically to the target of the original aptamer and form a folded, associated complex (Zuo et al., 2009). It's worth noting that the target-binding ability of aptamer is almost undisturbed, although it is split into two or more parts (Liu et al., 2014b). It was reported that a 76-mer aptamer of 17β-estradiol was split into two short parts to design an Au NP-based colorimetric assay, and the limit of detection (LOD) was increased by 10-folds (Liu et al., 2014a). In addition to increasing sensitivity with split aptamer, other strategies such as indicator displacement have also been suggested to improve the detection sensitivity of optical aptasensor (Roncancio et al., 2014; Stojanovic and Landry, 2002). To improve the sensing performances of the fluorescent ATP aptsensor, the two above strategies of split aptamer and indicator displacement assay were used in this study.

ATP aptamer is isolated by Huizenga and Szostak with the advantage of high affinity for ATP while not for its analogues, such as guanosine triphosphate (GTP) and uridine triphosphate (UTP) (Huizenga and Szostak, 1995). ATP aptamers are widely used as sensor blocks in electrochemiluminscence (Bu et al., 2013; Liu et al., 2010; Wang et al., 2010b), electrochemical (Liu et al., 2013; Wen et al., 2014), phosphorescence (Xiong et al., 2018), colorimetric (Huo et al., 2016; Wang et al., 2010a), fluorometric (Hai et al., 2018; Li et al., 2016; Peng et al., 2018), surface plasmon resonance (SPR) (Ding et al., 2017), surface-enhanced raman scattering (SERS) (Ye et al., 2013) and field-effect transistors (Goda and Miyahara, 2012) biosensors because of their simplicity and specificity. Compared to other analytical technology, fluorescence-based ATP assays are more attractive, due to their high sensitivity, fast response, ease of visibility, operationally simple, cost-effective and the possibility for stand-off detection. Very recently, three research groups independently reported the novel ATP assay using ATP intact aptamer as recognition unit and Thioflavin T (ThT) as signal reporting using the indicator displacement assay (Ji et al., 2017; Liu et al., 2017; Wang et al., 2016). Cationic dye ThT does not emit in aqueous solution. As an effective G-quadruplex binder, the assembly of G-quadruplex/ThT emits strong fluorescence (Mohanty et al., 2013; Xu et al., 2018). However, the method of improving the sensitivity by using split ATP aptamer and ThT displacement assay has not been reported mainly due to the weak binding between the two split ATP aptamer fragments and ThT, which results in no significant increase in ThT fluorescence (Wang et al., 2016). To meet this challenge, here, a sensitive split aptamer based ThT displacement assay for ATP was successfully constructed utilizing ThT dyes as indicator and mediator, a label-free split ATP binding aptamer as the recognition element and G-rich DNA sequence which tagged at the end of the two split aptamer fragments as the signal amplifier. For the first time, G-rich DNA sequences were modified at one end of each of the two split ATP aptamer parts to enhance the molecular recognition between split ATP aptamer and ThT. G-rich sequences would improve the fluorescence and thus the performances of the split aptamer based ThT displacement ATP assay. The advantages of the method are as follows. First, the proposed method is more sensitive than the intact ATP aptamer/ThT complex based methods because the proposed G-rich split ATP aptamer/ThT assemblies were much brighter than the intact ATP aptamer/ThT complex because ThT works as the G-quadruplex binder. And the significant signal amplification and thus improved sensing performance can be achieved by G-rich sequence. Second, our proposed strategy shows an improved selectivity toward its analogues such as AMP and ADP. Third, the proposed approach has a wider linear range than the reported methods. Fourth, the current study not only provides a novel method for ATP assay but also presents a way to construct label-free split aptamer based fluorescent sensor for other species which a suitable aptamer can be generated. Finally, the split aptamer based ThT displacement ATP assay was successfully constructed and applied to detect ATP in 15% human serum.

2. Materials and methods

2.1. Materials and apparatus

Tris(hydroxymethyl)aminomethane (Tris) and ThT were purchased from Sigma-Aldrich. DNAs used in this work were provided from Sangon Biological Technology & Services Co., Ltd. (Shanghai, China) and the sequences were given in Table S1 in Supporting Information. $MgCl_2$ and KCl were provided from J&K Chemical Co., Ltd. (Beijing, China). The serum of healthy people comes from the first people's hospital of Shangqiu, Henan province.

The fluorescence spectra were recorded on an F-7000 spectrometer (Hitachi, Tokyo, Japan). The pH was given using a pHS-3C pH meter (Leici, Shanghai, China). Centrifugation was completed using a McAry (SQ) Eq-001 centrifuge.

2.2. Fluorescence measurements

For fluorometric detection of ATP, different amounts of ATP were introduced into 10 mM Tris-HCl buffer solution (30 mM Mg^{2+}, pH 6.0) containing 0.1 μM P1, 0.2 μM P2, 5 μM ThT and the solution was stirred well at ambient temperature. Then the fluorescence emission spectra were recorded immediately. The slits of excitation and emission were all set as 5 nm and scanning speed was medium (240 nm/min). The fluorescence emission spectra of the proposed sensing system were monitored from 460 to 600 nm with a excitation wavelength of 450 nm.

2.3. Colorimetric analysis

The blue-green fluorescence (486 nm) of ThT (5 μM) in the presence of 0.1 μM P1 and 0.2 μM P2 was visually observed by protected eyes. Pictures were taken using iphone 7 Plus under UV irradiation at 254 nm.

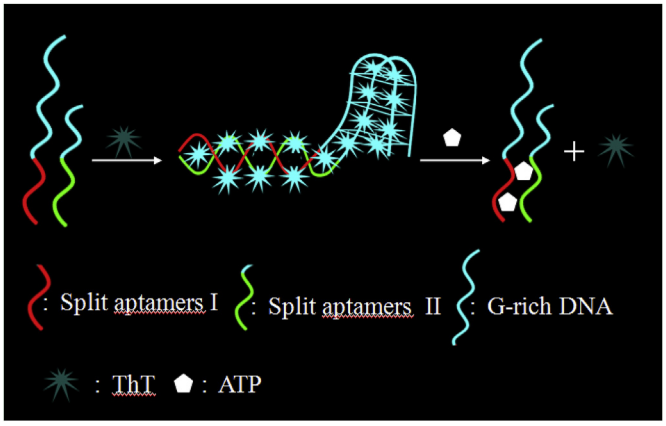
2.4. Real sample assay

Human serum was treated according to a previous report with a minor modification (Zhang et al., 2015). An aliquot of ethanol was added to human serum and then the mixture was treated by centrifugation at 15,000 rpm for 10 min. The supernatant was collected and passed through an Amicon Ultra-0.5 mL, 3 kDa Centrifugal Filter Unit at 13,000 rpm, 4 °C for 20 min. Then four identical 15% human serum (v/v, diluted with Tris-HCl buffer) samples were spiked with four different amount of ATP (0, 5, 100, and 500 μM in final concentrations, respectively) in order to mimic the real clinical samples. Finally, the fluorescence emission spectra were recorded immediately.

3. Results and discussion

3.1. Mechanism of the proposed split aptamer based ThT displacement assay for ATP

Scheme 1 illustrates the sensing strategy of the suggest ATP assay. The key point of the proposed split aptamer based indicator displacement assay for ATP is that the binding affinity between indicator and split aptamer should be moderate. The binding is strong enough to allow the formation of a stable complex with strong fluorescence, while still weak enough to allow quick indicator displacement when the target molecule exists. ATP binding aptamer has a higher affinity with ATP (K_d = 6 μM) than ThT (K_d = 89 μM). So ThT was chosen as the indicator and mediator. We began by splitting the intact 27-base ATP aptamer sequence (Apt 27, DNA7 in Table S1 in the supporting information) into two parts according to recently reported work



Scheme 1. Illustration of G-rich split aptamer-ThT based fluorescent ATP sensor.

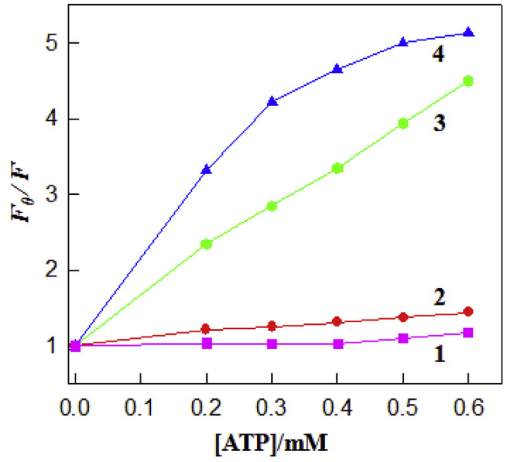


Fig. 1. The responses of fluorescent ATP sensors constructed by different DNA sequences to ATP. Curve 1: Split ATP aptamer; Curve 2: T-rich split ATP aptamer; Curve 3: Intact ATP aptamer; Curve 4: G-rich split ATP aptamer.

(Fedotova and Kolpashchikov, 2017). As we all know, free ThT in solution hardly fluoresces. However, once ThT is embedded in double-stranded DNA or G-quadruplex DNA, its fluorescence is greatly increased (Mohanty et al., 2013; Wang et al., 2011). The presence of the two split aptamer fragments (DNA5, DNA6 in Table S1) does not lead to a noteworthy increase in fluorescence of ThT (curve *a* in Fig. 1) which is consistent with reports in the literature (Wang et al., 2016). This observation clearly suggests that the split aptamer almost completely loses its ability to bind to ThT although the intact ATP aptamer has a strong affinity with ThT. Obviously, to make our design work, we need to find a novel way to increase the binding force between the split ATP aptamer and ThT. G-rich sequence can form the G-quadruplex in the presence of ThT (Mohanty et al., 2013; Xu et al., 2018). Therefore, we hypothesized that the G-rich sequence tagged the two ends of split ATP aptamer could improve its affinity to ThT. To test this idea, each half of the split aptamer was attached to a G-rich DNA sequence. Although the two split aptamer fragments cannot bind to ThT, the ensemble of the two split aptamer fragments tagged with G-rich sequences at both ends (P1, P2 in Table S1) and ThT generates higher fluorescence signal than the intact aptamer (DNA7 in Table S1) does (curve *4* and *3* in Fig. 1). In order to verify the G-rich sequence has the function of enhancing the binding, rather than the increase of base length, we synthesized two T-rich sequences split aptamer with the same number of bases (DNA3, DNA4 in Table S1), but it also did not bind to ThT. These data suggest that the G-rich sequences at both ends of split aptamer would improve the fluorescence intensity of ThT and thus the sensitivity of the method.

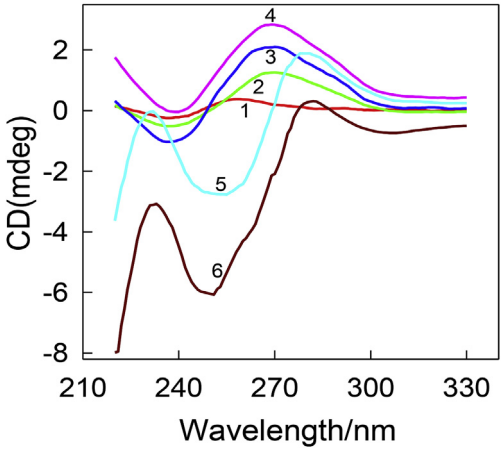


Fig. 2. CD spectra of the sensing system in the presence of different species in 10 mM Tris-HCl. Curve 1: P1, Curve 2: P2, Curve 3:P1 + P2, Curve 4:P1 + P2 + 25 μM ThT, Curve 5:P1 + P2 + 25 μM ThT + 0.8 mM ATP, Curve 6:P1 + P2 + ThT + 1.0 mM ATP.

As expected, in the presence of ATP, ATP can effectively displace ThT from the ThT/DNA complex, accordingly the fluorescence of ThT decreases considerably (curve *d* in Fig. S1). Thus, ThT/DNA complex is susceptible for the fluorescent detection of ATP.

3.2. CD and fluorescence spectra characterize of the formation and disappearance of G-quadruplex

CD spectrum is a powerful tool for studying the secondary structure of DNA. To confirm the proposed sensing mechanism, CD spectrum is used to characterize of the formation and disappearance of G-quadruplex in the proposed assay. A Characteristic CD spectral positive peak of ssDNA (P1) is located at 254 nm (Fig. 2 Curve 1). (Mohanty et al., 2013) This phenomenon is consistent with the fluorescence spectrum (Fig. S1 in the supporting information, curve 1). In the presence of single strand DNA, ThT fluorescence did not increase significantly. Under the experimental conditions, P2 also exhibits a certain extent characteristic peaks in the CD spectrum of G-quadruplex (the native peak at 240 nm and positive peak at 268 nm), which may be due to the formation of parallel G-quadruplex between its own molecules (Fig. 2 Curve 2) (Zhu et al., 2018) The effect of P2 on ThT fluorescence was further investigated by fluorescence spectra (Fig. S1, curve 2). Since P2 can form intramolecular G-quadruplex structure, ThT fluorescence is significantly increased. When there were both P1 and P2 in the system, the CD signal is significantly enhanced and the position remains unchanged, indicating that there was more G-quadruplex in the solution (Fig. 2 Curve 3). In the presence of ThT (25 μM), the CD signal was significantly enhanced again, indicating that more and more G-quadruplex was formed (Fig. 2 Curve 4). This is consistent with the report that ThT can induce the formation of G-quadruplex (Mohanty et al., 2013). This phenomenon can also be illustrated by the further fluorescence enhancement in the presence of P1and P2 (Fig. S1, curve 3). When the system contains different concentrations of ATP, the positive peak at 268 nm and the negative peak at 240 nm disappeared, which indicating that the G-quadruplex is destroyed (Fig. 2 Curve 5 and 6). It is also shown that ATP can effectively displace ThT from the ThT/DNA complex. Although the fluorescence enhancement of ThT is partly due to the formation of intramolecular G-quadruplex structure of P2, this has no affect on the determination of ATP. Because in the presence of ATP, ATP has a strong binding ability with the two split aptamer parts in P1 and P2, so there is no free P2 in the solution (Fig. S1, curve 4). Meanwhile, in the presence of ATP, the negative CD signal at 250 nm and positive peak at 280 nm gradually increases, which indicates the formation of duplex complex between ATP and the split aptamer

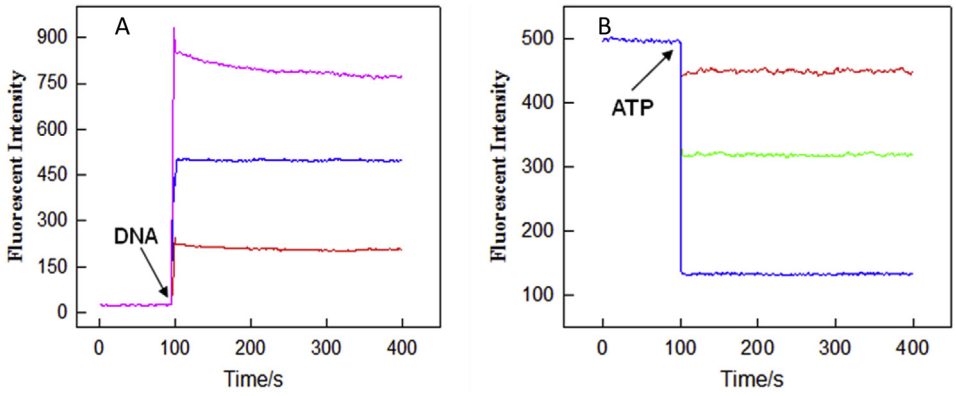


Fig. 3. Time-based curves of 5 μM ThT in the presence of split ATP aptamer (A) and the kinetic responses of the sensor to ATP (B).

(Heinen and Walther, 2017). The strong negative CD signal at 250 nm may be caused by ATP too. To confirm this, the CD spectrum using pure ATP as a control experiment was performed (Fig. S2). Fig. S2 clearly shows that the strong CD signal at 250 nm is caused by ATP itself indeed.

3.3. Kinetics of fluorescence enhancement of ThT caused by P1/P2 binding and the quenching of P1/P2/ThT ensemble induced by ATP

The proposed fluorescent biosensor was investigated to monitor ATP in real time. As shown in Fig. 3A, the quickly and significant fluorescence increase of ThT was observed upon the addition of different concentrations of P1/P2, most probably due to the formation of P1/P2/ThT ensemble. The slight decrease in fluorescence intensity of ThT in the presence of P1/P2 could be due to the photobleaching (Jokic et al., 2012). As shown in Fig. 3B, the dramatically and instantly decrease in fluorescence was seen upon further addition of ATP within half a minute. This observation clearly suggests that ATP binds effectively to the split aptamer and ThT is displaced and move away from the P1/P2/ThT ensemble.

3.4. Performance of the split aptamer based ThT displacement assay for ATP

The ability of the proposed assay for quantitative detection of ATP was then explored. Fig. 4 shows fluorescent spectral changes during titration of DNA/ThT ensemble with the increasing presence of ATP. As anticipated, there is a remarkable reduction in the fluorescence signal as the ATP concentration is increased within the range of 50 nM to 5 mM. This quenching effect is due to competition between ATP and thioflavin T for access to the split aptamer.

When the target concentration is further increased over 5 mM, no significant changes are seen in the emission spectra and a plateau is reached (Fig. 4A inset). The quenching efficiency is about 90% calculated by formula $(F_0\text{-}F)/F_0$, where F_0 and F are the fluorescent intensity at 486 nm in the absence and presence of ATP, respectively. A plot of F_0/F versus [ATP] shows a good linear relation from 0.1 to 120 μM under the experimental conditions employed here. The regression equation was $y = 2.59c + 1.06$ with correlation coefficient $R^2 = 0.996$ (Fig. 4B). The LOD was estimated to be 2 nM based on $3\sigma/k$ rule (where σ is the standard deviation of the blank solutions, $n = 11$, and k is the slop of the linear equation). Such a lower LOD and a wide dynamic range of 3 orders of magnitude were much better than the existing intact ATP aptamer-ThT based method.[30] Additionally, the changes in the fluorescence spectrum can also be seen in the fluorescence color. As shown in Fig. 4C, the fluorescence at 486 nm was changed from bright bluish green to dark with the increase of ATP concentration from 0 mM to 2 mM.

3.5. Parameters optimized

Parameters of this sensing system were optimized, including Mg^{2+} concentrations, K^+ concentrations, pH, levels of ThT and P1 and P2, ratio of P1 to P2 etc. The effect of Mg^{2+} ion was found to be an important factor affecting the stability of the aptamer/target complex (Jiang et al., 2004). As can be seen from Fig. S3, the optimal concentration of Mg^{2+} is 30 mM. Monovalent cation (such as K^+, Na^+) plays an important role in the stability of G-quadruplex (Aslanyan et al.,

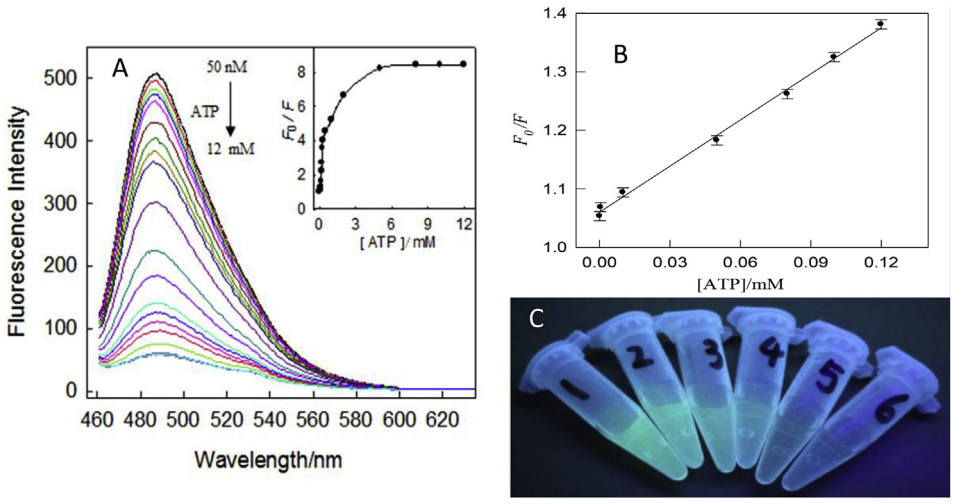


Fig. 4. (A)Fluorescence spectra of the suggested sensor to different amounts of ATP (from 50 nM to 12 mM). Inset: the plot of F_0/F to the ATP concentration. (B) Linear relationship between the F_0/F and the concentration of ATP (from 0.1 μM to 120 μM). F_0 and F are the fluorescent intensity at 486 nm in the absence and presence of ATP. And (C) Fluorescence images of the proposed sensor with different concentrations of ATP under ultraviolet light (254 nm) excitation. The concentrations of ATP were 0 μM, 100 μM, 200 μM, 500 μM, 1 mM and 2 mM from left (1) to right (6).

2017). Next, the effect of the K^+ on the fluorescence intensity was further investigated. As demonstrated in Fig. S4, the fluorescence quenching efficiency is the highest in the absence of K^+. There was no noteworthy decrease in fluorescence intensity of the aptasensor upon the addition of ATP in the presence of 50–250 mM K^+. These data strongly suggest that ThT cannot be displaced by ATP. The main reason for this may be due to the high stability of ThT/G-quadruplex in the presence of K^+. As the both indicator and mediator, the concentration of ThT has a dramatic impact on the sensing performance. As can be seen from Fig. S5, the fluorescence quenching efficiency is the highest in the presence of 5 μM ThT. When the concentration of ThT is lower than 5 μM, the initial fluorescence of the system is lower, while when the concentration of ThT is higher than 5 μM, ATP may not be able to replace all ThT. As the capturing probe, the concentration and ratio of the two split aptamers are also critical. Fig. S6 suggests the best ratio of P1 and P2 is 1:2. The concentration of P1 and P2 is 0.1 μM and 0.2 μM, respectively (Fig. S7). The effect of pH is also very pronounced. To further study the practical applicability of this sensor, the influence of the pH on the fluorescence response of the suggest aptasensor to ATP was investigated. The results show that the quenching efficiency is relatively high at pH 6.0 (Fig. S8).

3.6. Selectivity of the split aptamer based ThT displacement assay

A specific recognition to the target over other species is very necessary for biosensor with potential application in complex biological matrix. The proposed fluorescent aptasensor should theoretically display a good selectivity toward ATP because the ATP aptamer was used as the recognition unit. As shown in Fig. 5A, 100 μM ATP could induce an intensive decrease of the aptasensor, while the ATP analogues such as ADP, AMP etc did not induce considerable changes of the fluorescence. At the same time, the selectivity of the similar sensor based on the intact ATP aptamer was examined (Fig. S9). It is very clear that the proposed aptasensor is much more selective toward ADP and AMP than intact ATP aptamer-ThT based types. The results strongly indicate that the suggested aptasensor could meet the selective requirements for complex biological samples such as human serum. The good selectivity of the proposed fluorescent aptasensor was further confirmed by fluorescence color change. As shown in Fig. 5B, the fluorescence bright bluish green centered at 486 nm was intensively quenched only in the presence of ATP. There was no noteworthy change in fluorescence color in the presence of ATP analogues.

3.7. Detection of ATP in human serum samples

To evaluate the application feasibility of the developed aptasensor for detecting ATP in real samples, experiments were carried out in complex bio-samples such as 15% human serum (Fig. S10A). The recover experiments were performed. With the calibration curve in Fig. S10B, the recoveries and the relative standard deviations (RSDs) were obtained and were shown in Table 1. The recoveries for all the samples were in the range from 96 to 108% and RSDs were lower than 5% at three concentration levels (5, 100, 500 μM), signifying that the proposed system has high accuracy and good precision and is highly reproducible for detecting ATP in human serum. These results strongly suggest that the developed aptasensor could be considered as a robust and reliable approach for detection of ATP in real complex biological samples.

Table 1
Recovery studies of spiked ATP in 15% human serum with designed sensor.

Sample	Added/μM	Found[a]/μM mean[a] ± SD[b]	Recovery(%)	RSD(%)
1	5	5.46 ± 0.38	108	4.9
2	100	96.21 ± 3.20	96	2.3
3	500	526.01 ± 9.8	105	1.3

[a] Mean of three determinations.
[b] SD: standard deviation.

4. Conclusions

In summary, traditional split aptamer strategy can not directly used to improve the label-free aptamer based ThT displacement assay for ATP. To address this challenge, G-rich sequences were modified to the ends of the two split ATP aptamer fragments. ThT can interactions with the two G-rich split ATP aptamer fragments to form higher fluorescent complex than the intact aptamer/ThT complex. The high fluorescent G-rich split ATP aptamer fragments/ThT complex can be used as effective, sensitive and selective aptasensor for ATP assay. In the proposed assay, G-rich sequences would improve the fluorescence intensity and thus the sensitivity of the suggested ATP biosensor. The ATP concentration in human serum has been successfully determined, further demonstrating the potential utility of the novel system. The design offers a new opportunity for simple and sensitive detection of other targets that can cause structure switching of the corresponding target-specific aptamer. Further investigations on whether the platform can be applied to other object determination by changing aptamer are currently being carried out in our laboratory.

Declaration of interests

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.

Author Contribution

Fenghua Geng and Yongxiang Wang designed research; Yu Ma and Geng Fenghua performed research; Yongxiang Wang, Fenghua Geng, Maotian Xu, Congying Shao, Peng Qu, Yintang Zhang, and Baoxian Ye analyzed data; and Yongxiang Wang, Fenghua Geng and Congying Shao wrote the paper.

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

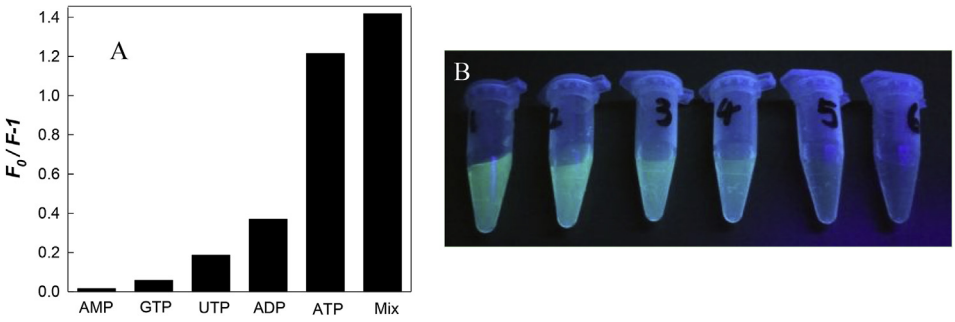


Figure 5. (A) Selectivity of the proposed sensor towards 100 μM ATP and its analogies at the concentrations of 100 μM. F_0 and F are the fluorescent intensity at 486 nm in the absence and presence of ATP. (B) Fluorescence images of the proposed sensor in the present of 100 μM ATP or/and its analogies at the concentrations of 100 μM under ultraviolet light (254 nm) excitation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2019.03.047.

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