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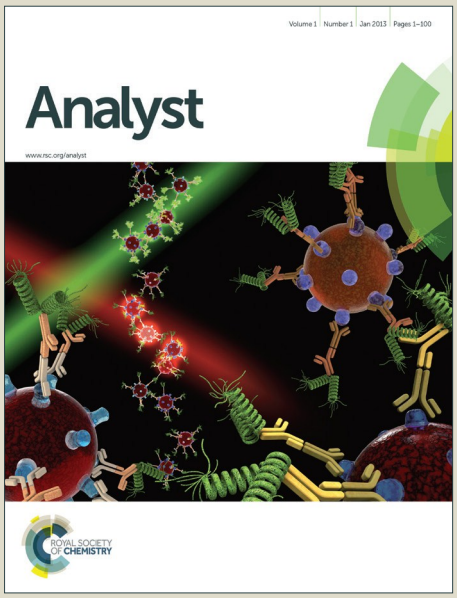

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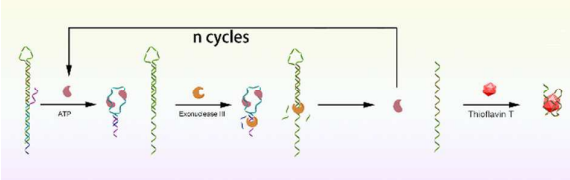

Textual abstract:

A label-free fluorescent adenosine triphosphate aptasensor is fabricated using overhanging aptamer that can trigger enzyme protection and target recycling amplification.

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A Label-free Fluorescent Adenosine Triphosphate Biosensor *via* Overhanging Aptamer-Triggered Enzyme Protection and Target Recycling Amplification

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Herein, a label-free fluorescent adenosine triphosphate (ATP) aptasensor is fabricated with DNA hairpin and overhanging aptamer. In the presence of ATP, overhanging sequences of aptamer may form prefered substrates of exo III, and thus trigger the enzyme-assisted amplification, which results in the release of G-rich sequences. Free G-rich sequences subsequently generate enhanced flourescent signal by binding with thioflavin T. However, if ATP is absent, overhanging sequence can induce steric hindrance and protect DNA hairpin against the digestion of exo III, significantly reducing noise of this biosensor. Accordingly, the signal-to-noise ratio of the sensing system is greatly improved, which ensures desirable analytical performance of the proposed aptasensor both in pure samples and real samples.

Besides its biological functions, nucleic acids have also been recognized as an engineering material that is widely used to fabricate various functional nanostructures,[1] such as logic gates,[2] drug delivery systems[3] and nanomachines.[4] Among diverse nucleic acid-based nano-architectures, DNA hairpins (HPs) attract great attentions for their applicability in monitoring living systems,[5] investigating protein-DNA interactions,[6] and in particular constructing biosensors.[7-9] Notably, nucleic acids can be precisely operated by base pairing and metal ion-mediated allostery,[10-12] thus provide different recognition strategies for these applications. Moreover, the discovery of aptamer further enriches the variety of nucleic acid-based recognition, broadening the realm of target molecules from small molecules to whole cells.[13,14] Therefore, HPs combined with aptamer are promising in designing advanced biosensors.[15]

Although aptamer-based HPs are of great help in fabricating biosensors, these analytical protocols are still afflicted with some inherent challenges. On one hand, conventional HPs are typically labelled with two non-native moieties, *i.e.*, quencher and fluorophore, leading to a high cost of running and more complex experimental processes. To overcome these limitations, lots of label-free fluorescent HPs mainly relying on a specific sequence or structure have been developed.[7,16] To be noted, G-quadruplex/Thioflavin T (ThT) complex, a newly discovered fluorescent system, is attractive to build label-free biosensors, because of its simple operation and low background signal.[17] On the other hand, the sensitivity of HPs is limited due to the incomplete quenching of quencher to fluorophore. In a great number of biosensors, enzyme-assisted amplification strategy is induced to improve their sensitivity.[18,19] However, the activities of most enzymes are not sole. For example, exonuclease III (exo III) has prominent activity to catalyze the hydrolyzation of blunt and recessed 3'-hydroxyl terminus of duplex DNA.[20] However, it is reported that exo III can also act at nicks in duplex DNA,[21] and has RNase H, 3'-phosphatase and AP-endonuclease activities.[22] As a result, occurrence of undesirable catalytic reaction may significantly influence the performance of biosensors.

Herein, taking adenosine triphosphate (ATP) as the model target molecule, a novel label-free fluorescent aptasensor has been developed by using two DNA, *i.e.*, G-rich HP (GHP) and overhanging aptamer. Besides the inherent function of target recognition, this overhanging aptamer has two other functions that are displayed in different cases. When ATP is absent, 5'-overhanging sequence can cover the 3'-recessed terminus of GHP, thus prevent the undesirable catalysis of exo III to GHP, which greatly reduce the noise of the analytical system. However, when ATP is present, 3' and 5'-overhanging of aptamer will hybridize to construct a 3'-recessed duplex. Therefore, overhanging aptamer and GHP can be simultaneously digested by exo III, releasing ATP and G-rich sequence. The released ATP further triggers target recycling amplification, producing more G-rich sequences. With the help of potassium ions, G-rich sequence can readily form G-

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Electronic Supplementary Information (ESI) available: Experimental details, optimal conditions for G-quadruplex/ThT complex, and comparison of different ATP aptasensors. See DOI: 10.1039/x0xx00000x

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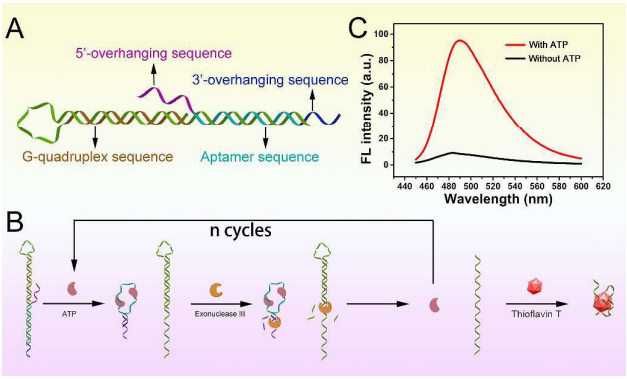

Fig. 1 (A) Schematic representation of the composition of an ATP aptasensor. (B) Fluorescence spectra of the proposed aptasensor with and without ATP. (C) Schematic illustration of the mechanism for fluorescent ATP quantification *via* overhanging aptamer-triggered enzyme protection and target recycling amplification.

quadruplex that can further form G-quadruplex/ThT complexes by binding with ThT.[23] G-quadruplex/ThT complexes are finally used to quantify ATP with amplified fluorescent signal. Taking advantage of ATP aptamer, various ATP aptasensors hase been built with different methods, such as photochemistry,[24] surface-enhanced Raman scattering,[25] quartz crystal microbalance[26] and fluorescence.[27] Among them, DNAzyme and G-quadruplex/N-methylmesoporphyrin complex is widely used in label-free and enzyme-assisted ATP aptasensors.[18,28] However, G-quadruplex/ThT complex is a better choice, because fluorescent signal of G-quadruplex/ThT complex can be easily obtained and greatly enhanced.[17] In addition, the biosensor proposed by us has improved signal to noise ratio by employing overhanging sequence and exo III-assisted system, which is desirable in related biosensors.

The proposed ATP biosensor consists of two DNA, *i.e.*, GHP and overhanging ATP aptamer. GHP is a hairpin DNA containing a G-rich sequence (Brown in Fig. 1A). The G-rich sequence hybridizes with 3'-terminal sequence of GHP, forming a 3'-recessed duplex. 5'-terminal sequence of GHP is used to hybridize with the ATP aptamer (Cyan in Fig. 1A). The ATP aptamer is prolonged with consecutive thymine (5'-overhanging sequence, Purple in Fig. 1A) and consecutive adenine (3'-overhanging sequence, Blue in Fig. 1A). Although 3'-recessed terminus is the preferred substrate of exo III, 3'-recessed terminus of GHP is covered by the 5'-overhanging sequence of aptamer, thus cannot be catalyzed by exo III. On the contrary, after binding with ATP, ATP aptamer can dehybridize with its complementary DNA. Therefore, in the presence of ATP, overhanging aptamer separates with GHP. Moreover, since there are complementary sequences in 3' and 5'-terminus of overhanging aptamer, the separated aptamer forms an additional 3'-recessed duplex. As a result, both GHP and overhanging aptamer are simultaneously hydrolyzed by exo III, releasing ATP and single-stranded G-rich sequence. ATP is further used to bind with another overhanging aptamer, triggering recycling amplification to release more G-rich sequences. In comparison with G-rich sequence in duplex,

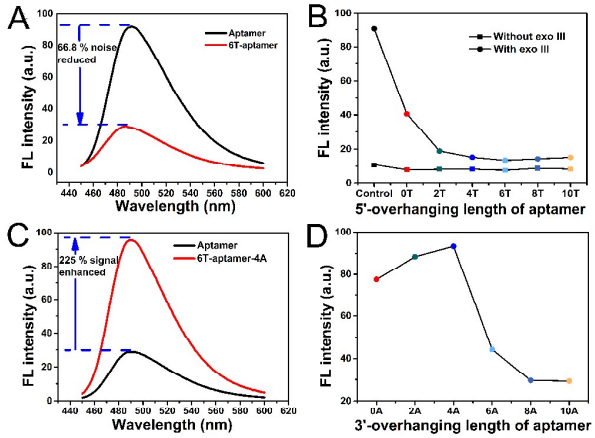

Fig. 2 Effects of (A) 5'-overhanging sequence and (B) its length on the fluorescent noise of the aptasensor. "Control" assay means that GHP was directly treated with exo III without the participation of overhanging aptamer. Effects of (C) 3'-overhanging sequence and (D) its length on the fluorescent signal of the aptasensor.

single-stranded G-rich sequences can readily form G-quadruplex/ThT complexes that can generate fluorescence emission at 485 nm by exciting at 415 nm (Fig. 1B). Taken the fluorescent behavior of G-quadruplex/ThT complex as characteristic signal, the aforementioned procedure is used to label-free ATP quantification (Fig. 1C).

Since many factors can affect the performance of the proposed sensing system, a number of experimental conditions are first optimized. High concentration of ThT can result in high fluorescence signal of G-quadruplex/ThT complex as well as high background signal of ThT. As shown in Fig. S1, 1/10 is chosen as the optimal ratio of $C_{(G3T3)}/C_{(ThT)}$ ratio that is used in the following assays. Effects of exo III on the fluorescent signal are further studied. According to the results in Fig. S2A and Fig. S2B, 20 U/mL and 30 min are the optimal concentration and incubation time of exo III, respectively. Considering that the biosensor is composed of two DNA, the ratio of $C_{(GHP)}/C_{(aptamer)}$ is also investigated. Equal amount of GHP and aptamer is proved to be the best ratio to build the biosensor (Fig. S3).

Having surveyed the above experimental results, it can be found that there is significant noise in the absence of ATP (background signal), even if the biosensor is constructed with the optimal conditions. In comparison with hairpin structure with a 3'-recessed terminus and a 5'-overhanging sequence, catalytic activity of exo III would be greatly affected, if the 5'-overhanging sequence hybridizes with its complementary sequence.[29] However the nick between 3'-terminus and 5'-terminus can be catalyzed by exo III, leading to a large noise. To solve this problem, the 5'-terminus of aptamer is lengthened with consecutive thymine (T). The overhanging sequence covers the nick and reduces the interaction between exo III and 3'-terminus of HP by steric hindrance.[30] Therefore, undesirable digestion of exo III is prevented, resulting in the decrease of noise (Fig. 2A). Furthermore, the effect of 5'-overhanging length on enzyme protection is carefully

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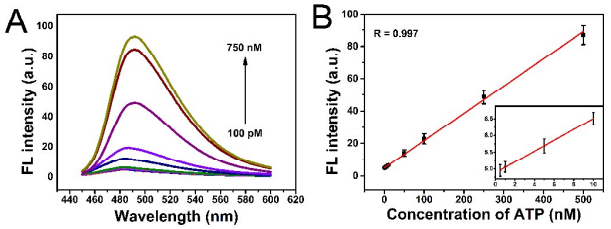

Fig. 3 (A) Fluorescence spectra of the aptasensor in different concentrations of ATP. (B) The linear relationship between the fluorescence intensity and the concentration of ATP. Inset: the first four points of the linear relationship.

investigated. As the 5'-overhanging sequence length increases from 0T to 6T, the noise reduces manifestly (Fig. 2B). By employing 6T-aptamer to construct the biosensor, 66.8 % of noise can be effectively reduced. On the other hand, the 5'-overhanging aptamer is further functionalized by lengthening its 3'-terminus to enlarge the signal of this biosensor. By introducing consecutive adenine (A) at 3'-terminus, overhanging aptamer can form 3'-recessed duplex after binding with ATP. As a result, overhanging aptamer and GHP can be simultaneously hydrolyzed by the same enzyme, exo III, causing the release of ATP and G-rich sequence. Released ATP triggered the recycling amplification, releasing more G-rich sequences to form G-quadruplex/ThT complexes. Therefore, the signal of sensing system is enhanced (Fig.2C). However, if consecutive A at 3'-terminus is longer than 5'-consecutive T, 3'-protruding terminal duplex is formed in the presence of ATP. Since 3'-protruding terminal duplex cannot be catalyzed by exo III, ATP-induced recycling amplification does not occur in this condition. As shown in Fig. 2D, 6T-aptamer-4A has the best effect on signal enhancement and is chosen to construct the proposed biosensor in the subsequent experiments. Benefited from both enzyme protection and recycling amplification caused by overhanging aptamer, it can be calculated that the signal-to-noise ratio of this sensing system is improved from 2.29 to 22.4.

Encouraged by the improvement of overhanging sequence on signal-to-noise ratio, feasibility of the proposed sensing system is evaluated by ATP quantification. Under the optimal conditions, ATP at different concentrations is measured with the aptasensor. As shown in Fig. 3A, the florescence intensity (FI) increases with the increase of ATP concentration ($C_{(ATP)}$) from 100 pM to 750 nM. In the range from 500 pM to 500 nM, a linear dependency between FI and $C_{(ATP)}$ is obtained (Fig. 3B). The regression equation is $FI = 4.86 + 0.17C_{(ATP)}$ (nM), R = 0.997. The first four points of the regression equation are depicted in inset of Fig. 3B. Calculated detection limit of this biosensor is 280 pM (S/N = 3). The linear range and detection limit are better than or at least comparable to other ATP aptasensors (Table S2). The improved sensitivity of the proposed method should be attributed to both the reduced background signal and the recycling signal amplification.

Selectivity of this ATP biosensor has been investigated by challenging it with three ATP analogues (CTP, GTP and UTP). Even if the concentrations of CTP, GTP and UTP are 1000 times

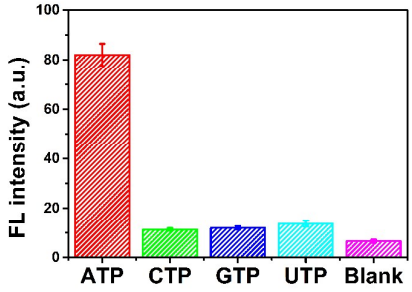

Fig. 4 Comparison of fluorescence intensity of ATP, CTP, GTP, UTP and blank assay.

Table 1. Detection of ATP in human serum samples with the proposed biosensor.

Sample	ATP added (nM)	ATP found (nM)	Recovery (%)	RSD (%, n=3)
Sample 1	100.0	113.3	97.7	2.62
Sample 2	10.0	25.25	96.5	4.54
Sample 3	1.0	16.55	95.0	5.02

higher than ATP, FI from ATP is at least 5-fold higher than those from ATP analogues (Fig. 4). The comparative results suggest satisfactory ability of this biosensor to identify ATP, which should be ascribed to the high selectivity of ATP aptamer.

It is reported that the ATP concentration in different human samples varies in a broad range from picomolar to sub-micromolar,[27,31,32] which is in the linear range of this biosensor. Abnormal levels of ATP are highly related to multiple physiological and pathological processes.[33-35] According to the results of our biosensor, concentration of ATP in human serum is 15.6 nM. Different concentrations of ATP are first spiked in human serum, and then are detected by the proposed method. Although there are complex components in serum, high recovery percentage and low relative standard deviation (RSD) demonstrated that no serious interferences have been found in human serum samples (Table 1), which indicates the feasibility of this sensor in practical applications.

In summary, an ATP aptasensor is designed based on label-free fluorescent G-quadruplex/ThT complex. In this biosensor, overhanging aptamer is employed to regulate the behaviour of enzyme-aided system from two aspects. First, 5'-overhanging sequence of aptamer can impede the catalysis of 3'-recessed G-rich DNA hairpin by exo III, reducing the fluorescent noise. Second, in the presence of ATP, 5' and 3'-overhanging sequence can induce an additional 3'-recessed duplex, thus triggering exo III-aided recycling amplification and enhancing the signal. Accordingly, relying on the multi-functional overhanging aptamer, ATP both in pure sample and serum sample is sensitively quantified with a high signal-to-noise ratio. To be noted, the inherent function of a certain aptamer is not affected by prolonging its terminal sequence. In addition, the protection of overhanging sequence is not

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limited to exo III. Therefore, the regulation strategy of overhanging sequence is versatile by simply coupling different aptamer with corresponding enzymes, which may significantly benefit vast enzyme-aided aptamer systems.

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