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Cite this: DOI: 10.1039/c4an01963f

Label-free aptasensor for adenosine deaminase sensing based on fluorescence turn-on

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Received 28th October 2014,
Accepted 2nd December 2014

DOI: 10.1039/c4an01963f

www.rsc.org/analyst

A label-free and fluorescence turn-on aptamer biosensor has been developed for the detection of adenosine deaminase (ADA) activity with simplicity and selectivity. Adenosine aptamer will form a tight stem-loop structure upon binding with adenosine. In the absence of ADA, only a small quantity of picagreen intercalates into the stem section of aptamer, resulting in a low fluorescence of picagreen when excited at 490 nm. Interestingly, after the addition of ADA, adenosine is hydrolyzed to inosine, and the released aptamer forms double-stranded DNA (dsDNA) with its complementary single-stranded DNAc, followed by the intercalation of picagreen to dsDNA. When the solution is excited, picagreen emits strong green fluorescence. The increased fluorescence intensity of picagreen is dependent on the concentration of ADA. The detection limit of the ADA is determined to be 2 U L^{-1}, which is lower than ADA cutoff value (4 U L^{-1}) in the clinical requirement and more sensitive than most of the reported methods. Compared to other previous ADA sensors, the assay is not only label-free but also a turn-on signal, and possesses properties of lower cost and simpler detection system. Furthermore, this label-free strategy is also applicable to the assay of other enzymes and screening of corresponding inhibitors.

Introduction

Adenosine deaminase (ADA) can catalyze the conversion of adenosine to inosine by removing an amino group, existing in all human tissues, plants and bacteria.[1–3] It plays a critical part in the function, maturation and maintenance of immunological responses.[4] Both genetic ADA deficiency and ADA overexpression may trigger diseases, as it is well known that inherited ADA deficiency is a main cause of severe combined immunodeficiency disease (SCID), accounting for a high rate of SCID.[5] Conversely, an excess of ADA is closely connected with hemolytic anemia, liver cancer and breast cancer.[6] Thus, the sensitive assay of ADA activity may have potential diagnostic applications.

Aptamers selected from nucleic acids through the vitro evolution process called SELEX (systematic evolution of ligands by exponential enrichment) are able to bind various targets with desirable selectivity, specificity and affinity, such as small molecules, proteins and even cells and organism.[7–13] It has been noted that aptamers have advantageous merits, such as outstanding specificity, easy labeling, excellent stability and applicability.[14–20] In recent years, considerable attention has been paid to aptamer-based sensors. Several techniques based on aptamers have been employed to detect ADA activity, including fluorescent aptasensors, and electrochemical aptasensors.[6,21–26] However, some of these reported techniques are complicated, cumbersome and time-consuming,[21,25] and some of these methods are based on the strategy of labeling.[26–28] While these labeled fluorescent sensors have many interesting applications, label-free fluorescent sensors may offer certain better advantages, as they are cost-efficient and possess easy operation due to the absence of fluorescent labeling.[29–36]

Picagreen, a type of cyanine dye, is a double-strand-chelating dye that can intercalate into double-stranded DNA (dsDNA), which conducts the same way as that of picogreen. Picagreen has a maximum emission at 525 nm when it is excited at 490 nm after intercalating into dsDNA. It is worth mentioning that picagreen is even more sensitive than ethidium bromide as a dsDNA intercalator.[37] When picagreen is bound to dsDNA, the fluorescence of picagreen greatly increases. At the same time, little background occurs because the free picagreen virtually has no fluorescence. Moreover, picagreen is stable to photobleaching, allowing longer exposure time and assay flexibility.[38–40]

In this work, we developed a novel strategy to detect the enzymatic activity of ADA based on the label-free aptasensor. The aptamer can specifically bind with the target adenosine to form the adenosine-aptamer complex. In the presence of ADA, the stem-loop structure is damaged because of ADA hydrolysis,

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and thus the flexible aptamer hybridizes with its complementary single-stranded DNAc to form dsDNA, followed by the intercalation of picagreen to dsDNA. When the solution is excited, the fluorescence intensity of picagreen is exceptionally enhanced. Therefore, the ADA activity can be simply and effectively detected by measuring the fluorescence intensity of picagreen. Compared with other labeled strategies, the outstanding advantage of our strategy is label-free, possessing lower cost and simpler properties.

Experimental

Reagents and instruments

The oligonucleotides, adenosine deaminase, adenosine, and bovine serum albumin (BSA), were obtained from Shanghai Sangon Biological Engineering Technology & Service Co. Ltd. *erythro*-9-(2-Hydoxy-3-nonyl) adenine hydrochloride (EHNA), α-glucosidase, β-glucosidase and thrombin were purchased from Sigma. Picagreen was obtained from Beijing Fanbo Biochemicals Co. Ltd. The oligonucleotide sequences used in our experiments are as follows: aptamer, 5′-GCA CC**T GGG GGA GTA TTG CGG AGG AA**G GTG C-3′; DNAc, 5′-TCC TCC GCA ATA CTC CCC C-3′. The sequence shown in bold is an adenosine aptamer. DNAc is complementary to the aptamer. The concentrations of all oligonucleotides were determined by measuring the absorption at 260 nm in a quartz cuvette. UV-vis absorption spectra were taken on a Perkin Elmer Lambda 35 spectrophotometer. The fluorescence spectra were recorded on a Hitachi F-7000 spectrophotometer equipped with a xenon lamp excitation source. All solutions were prepared with ultrapure water purified using a Millipore filtration system.

Preparation of hairpin

The solution containing a hairpin structure of aptamer in the presence of adenosine was prepared according to the procedure reported previously.[26]

Assay of enzyme

In 2.0 mL 53.3 mM KH_2PO_4 buffer solution (PH 7.5), the hairpin (1.0×10^{-8} M) prepared above was treated with successive concentrations of ADA (0.002–0.1 U mL^{-1}) for 25 min at 25 °C. Then, the DNAc (1.0×10^{-8} M and, picagreen (dsDNA Kit 2000 assay, about 1.3×10^{-7} M) were added. The fluorescence spectra were measured at 25 °C with the excitation wavelength of 490 nm.

Assay for ADA activity as a function of incubating time

In 2.0 mL 53.3 mM KH_2PO_4 buffer solution, the solution of the aptamer-adenosine complex (1.0×10^{-8} M) was treated with ADA at 25 °C (0.1 U mL^{-1}) for 0, 5, 10, 15, 20, 25, and 30 min, respectively. After incubation, single-stranded DNAc (1.0×10^{-8} M) and picagreen (1.3×10^{-7} M) were added. The fluorescence spectra were measured at 25 °C with the excitation wavelength of 490 nm.

Inhibition assay by EHNA

In the experiment of detecting inhibitor EHNA, the solution of the aptamer-adenosine complex was fixed at 0.1 U mL^{-1} ADA and successive concentrations of inhibitor EHNA (0–500 nM) were added at 25 °C for 25 min in 2.0 mL 53.3 mM KH_2PO_4 buffer. Then, the DNAc (1.0×10^{-8} M) and picagreen (1.3×10^{-7} M) were added. The fluorescence spectra were measured at 25 °C with the excitation wavelength of 490 nm.

Specificity assay of ADA

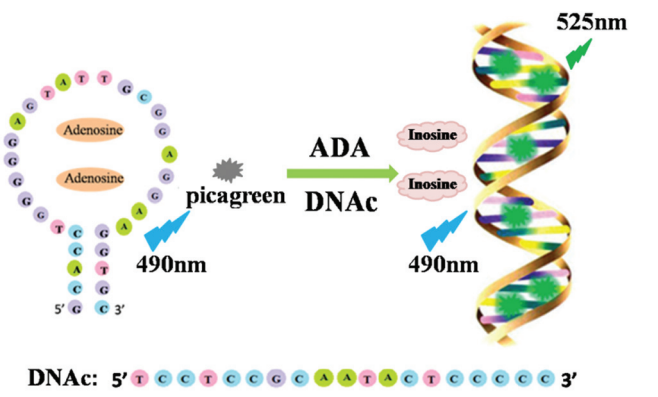
To detect the specificity of ADA to adenosine, we used α-glucosidase, β-glucosidase, bovine serum albumin (BSA), chymotrypsin and thrombin to replace ADA to react with DNA-adenosine complex. The experimental procedures were the same as those mentioned above, and the fluorescence spectra were measured at the same condition.

Assay of ADA activity in human serum

To investigate the practical application of our new approach in complicated conditions, the experiments in 10% human serum were carried out. The procedure is the same as the enzyme assay in buffer solution except for the use of 10% human serum instead of 53.3 mM KH_2PO_4 buffer.

Results and discussion

The proposed principle of label-free aptasensor for adenosine deaminase sensing is represented in Scheme 1. The adenosine aptamer will form a tight stem-loop structure once binding with the target of the adenosine. It is known that picagreen can intercalate within double-stranded DNA (dsDNA). Therefore, there is relatively low fluorescence of picagreen upon excitation at 490 nm because of the absence of dsDNA with valid length. Upon the addition of ADA, adenosine is converted to inosine by the catalysis of ADA, which makes the aptamer flexible, and the free ssDNA hybridizes with DNAc, followed by picagreen intercalating into dsDNA, resulting in the amplifica-



Scheme 1 Schematic presentation of the proposed principle of label-free aptasensor for adenosine deaminase sensing.

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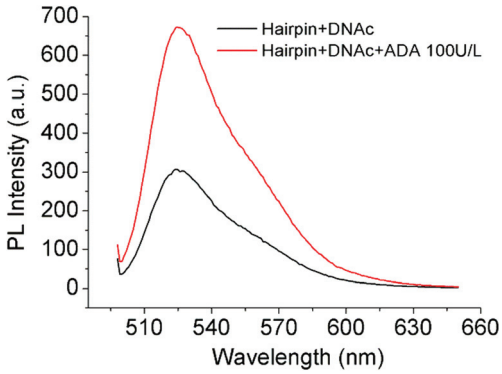

Fig. 1 Fluorescence emission spectra of aptamer/DNAc/picagreen and aptamer/DNAc/picagreen/ADA in KH_2PO_4 buffer solution (53.3 mM, pH = 7.5). [aptamer] = 1.0×10^{-8} mol L^{-1}, [adenosine] = 2.0×10^{-8} mol L^{-1}, [DNAc] = 1.0×10^{-8} mol L^{-1}, [picagreen] = 1.3×10^{-7} M. The excitation wavelength is 490 nm.

tion of fluorescence responses (Fig. 1). Accordingly, the change in fluorescent intensity can be used to detect ADA sensitivity.

In order to optimize the concentration of nucleic acids, we measured the fluorescence intensity of picagreen with the addition of dsDNA. In our experiments, the concentration of picagreen was fixed at 1.3×10^{-7} M in 2.0 mL 53.3 mM KH_2PO_4 buffer solution. Then, the solution of aptamer-adenosine complex was added, followed by the measurement of fluorescence intensity with an excitation wavelength at 490 nm. It was observed that the fluorescence intensity increases with the increasing concentration of the complex. When the concentration of the complex increases to 1.0×10^{-8} mol L^{-1}, the fluorescence intensity reaches the plateau (data not shown). Therefore, the optimized concentrations of aptamer and complementary single-stranded DNAc were both obtained for 1.0×10^{-8} mol L^{-1}.

First, to verify the enzymatic activity of ADA, we measured the fluorescence spectra in KH_2PO_4 buffer at pH 7.5 after the successive addition of ADA. As shown in Fig. 2a, the fluorescence intensity increases along with the increasing concentrations of ADA. There is no doubt that at a high concentration of ADA a large number of adenosine will be converted into inosine, which releases more flexible aptamer into the solution. Thus, in the presence of complementary sequence (DNAc), more hybridized dsDNAs form. In this case, more picagreen molecules could intercalate into dsDNA. Therefore, the fluorescence intensity enhances dramatically. These results present that the fluorescence intensity is closely related to the concentration of ADA. The Fig. 2b also demonstrates that the fluorescence ratio (I/I_0 at 525 nm) rises with the increasing ADA concentrations. As shown in the inset of Fig. 2b, the increased fluorescence ratio is linearly dependent on the concentrations of ADA. The fitting equation is y = 0.99868 + 0.02869× (R^2 = 0.998). Correspondingly, the detection limit of 2 U L^{-1} was obtained reasonably, which is lower than the ADA cutoff value (4 U L^{-1}) in the clinical requirement[28] and more sensitive than most of the reported methods (Table 1).[23,25,27,41] This homogeneous assay possesses the quality of being label-free, which makes ADA detection cost-effective, simple and rapid without any separation, further reaction and washing steps.[21,26] When the ADA concentration increases to 100 U L^{-1}, the ratio reaches the plateau and does not increase any more, which means the conversion of the adenosine is almost complete under this condition.

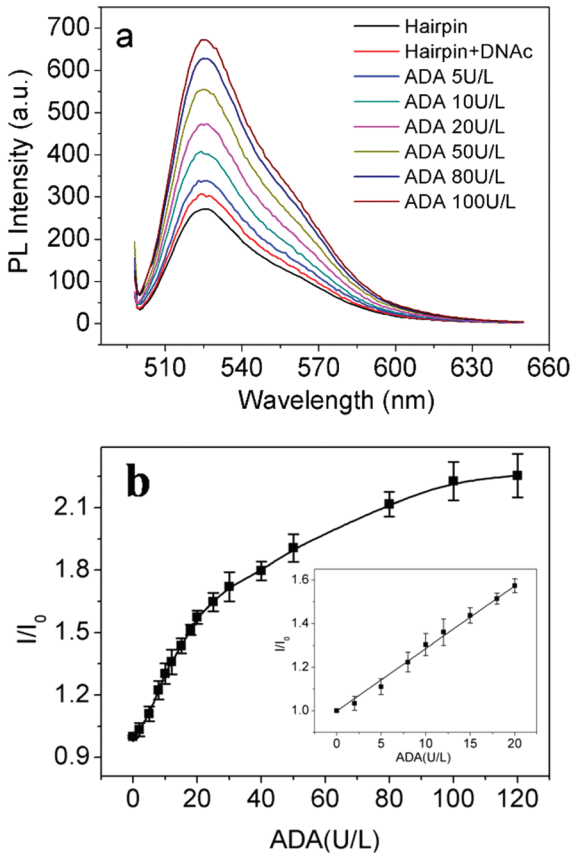

Fig. 2 (a) Fluorescence emission spectra of aptamer/DNAc/picagreen in KH_2PO_4 buffer solution (53.3 mM, pH 7.5) with the addition of aqueous ADA; (b) the ratio of fluorescence at 525 nm as a function of ADA concentrations (inset: linear relationship between the fluorescence ratio and low ADA concentrations). [aptamer] = 1.0×10^{-8} mol L^{-1}, [adenosine] = 2.0×10^{-8} mol L^{-1}, [DNAc] = 1.0×10^{-8} mol L^{-1}, [picagreen] = 1.3×10^{-7} M. The error bars illustrate the standard deviations of three independent parallel experiments. The excitation wavelength is 490 nm.

Table 1 Comparison of the limit of detection of measuring ADA from our work and a part of previous studies

Ref.	Methods	LOD (U L^{-1})
This work	Label-free aptasensor	2
27	Graphene oxide based fluorescent aptasensor	12.9
41	Fluorescence sensing	50
25	Electrochemical aptasensor	200
23	Colorimetric biosensors based on gold nanoparticles	400

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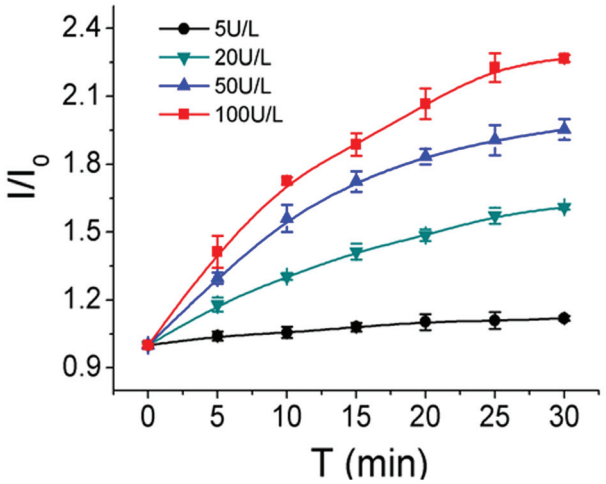

Fig. 3 Ratio of fluorescence at 525 nm as a function of ADA reaction time at different concentrations of ADA. [aptamer] = 1.0 × 10^{-8} mol L^{-1}, [adenosine] = 2.0 × 10^{-8} mol L^{-1}, [DNAc] = 1.0 × 10^{-8} mol L^{-1}, [picagreen] = 1.3 × 10^{-7} M. The error bars illustrate the standard deviations of three independent parallel experiments. The excitation wavelength is 490 nm.

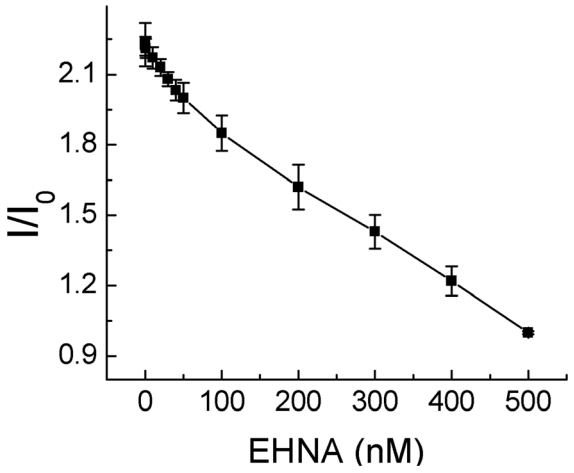

Fig. 4 Ratio of fluorescence at 525 nm as a function of EHNA concentrations. [aptamer] = 1.0 × 10^{-8} mol L^{-1}, [adenosine] = 2.0 × 10^{-8} mol L^{-1}, [DNAc] = 1.0 × 10^{-8} mol L^{-1}, [picagreen] = 1.3 × 10^{-7} M, [EHNA] = 0–500 nM. The error bars illustrate the standard deviations of three independent parallel experiments. The excitation wavelength is 490 nm.

Moreover, the ADA reaction time was optimized by measuring the fluorescence spectra of hairpin/DNAc/picagreen/ADA solution. In this case, various concentrations of ADA from 5.0 to 100 U L^{-1} are successively investigated. At first, the concentration of ADA is fixed at 100 U L^{-1} because the activity of ADA shows the maximum at this concentration, which is mentioned above. As shown in Fig. 3, the ratio quickly increases from around 1.0 to 1.3 when the hairpin incubates with ADA in the buffer for 5 min. After ADA hydrolysis for 10 min, the ratio rises to 1.7, which demonstrates the enzymatic activity of ADA is desirable. As the reaction time is further increased, the ratio still gradually enhances but the rate obviously slows down. When the reaction time is 25 min, the maximum fluorescence intensity is obtained and does not rise any more. Apparently, the same results could be obtained at other concentrations of ADA, such as 5, 20 and 50 U L^{-1}. As a result, the 25 min reaction time was selected for other analytical purposes. It should be noticed that the enzyme assay is homogeneously completed in a short time (25 min) without any time-consuming process. Therefore, our strategy presents a more simple and rapid assay with favorable sensitivity for ADA.

By taking advantage of the novel strategy of label-free aptamer biosensor, we investigated the inhibition of EHNA for ADA activity. As a well-known inhibitor of ADA, *erythro*-9-(2-hydroxy-3-nonyl) adenine hydrochloride was used to inhibit enzymatic activity of ADA.[27] Fig. 4 shows that the fluorescence ratio decreases when the concentration of EHNA increases in the range of 0–500 nM. It is also obvious that the activity of ADA can be totally inhibited when the concentration of EHNA is 500 nM. These results demonstrate that our approach has potential to detect the inhibitor of ADA efficiently and sensitively.

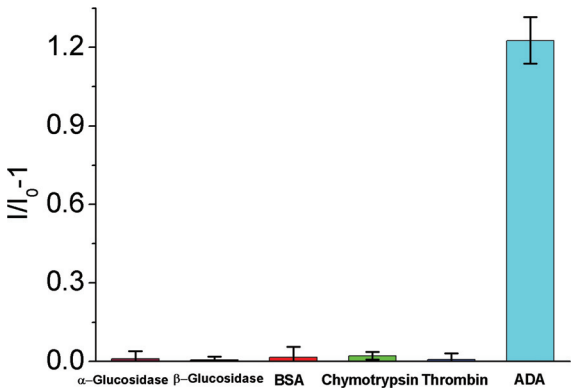

Fig. 5 Increased ratio of fluorescence at 525 nm in the presence of α-glucosidase, β-glucosidase, bovine serum albumin (BSA), chymotrypsin, thrombin and ADA. [aptamer] = 1.0 × 10^{-8} mol L^{-1}, [adenosine] = 2.0 × 10^{-8} mol L^{-1}, [DNAc] = 1.0 × 10^{-8} mol L^{-1}, [picagreen] = 1.3 × 10^{-7} M. The error bars illustrate the standard deviations of three independent parallel experiments. The excitation wavelength is 490 nm.

To investigate the specificity of this strategy, the fluorescence ratio was detected upon the addition of other enzymes. α-Glucosidase, β-glucosidase, bovine serum albumin (BSA), chymotrypsin and thrombin were used to replace ADA to react with hairpin/adenosine complex. The experimental procedures were the same as the ADA measurement. As shown in Fig. 5, compared with ADA, the increased fluorescence ratio resulting from other enzymes always keeps the same level as the control experiment (without any enzyme) which is close to 0. However, the ratio produced by ADA was much higher, reaching 1.2. These results indicate the assay demonstrates an outstanding specificity for ADA.

At last, we further studied the efficiency of this biosensor in complex biological samples such as in 10% human serum. As

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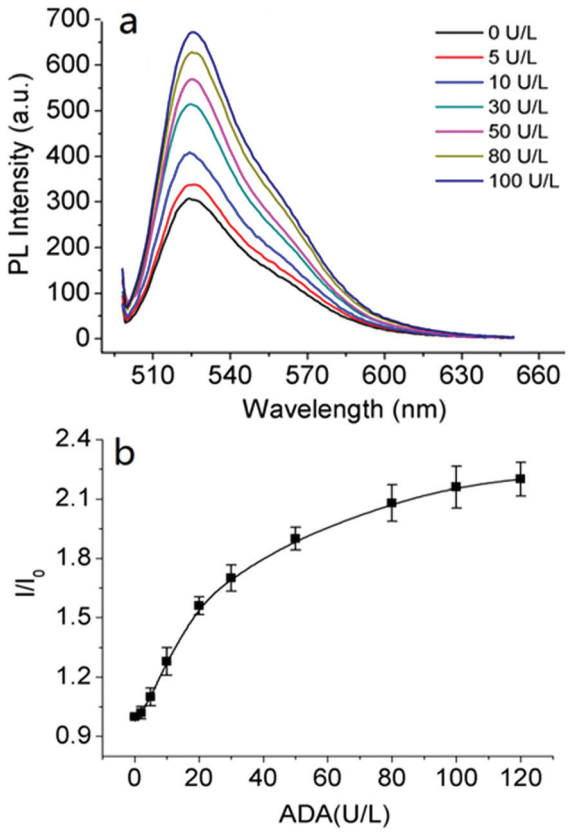

Fig. 6 (a) Fluorescence emission spectra of aptamer/DNAc/picagreen as a function of ADA concentration in 10% human serum; (b) the ratio of fluorescence at 525 nm as a function of ADA concentrations in 10% human serum. [aptamer] = 1.0×10^{-8} mol L^{-1}, [adenosine] = 2.0×10^{-8} mol L^{-1}, [DNAc] = 1.0×10^{-8} mol L^{-1}, [picagreen] = 1.3×10^{-7} M. The error bars illustrate the standard deviations of three independent parallel experiments. The excitation wavelength is 490 nm.

shown in Fig. 6, in the solution with 10% human serum the fluorescence intensity of picagreen increases along with the growth of ADA concentrations. The result is almost the same as what we measured before for the fluorescence intensity in KH_2PO_4 buffer at pH 7.5. However, when human serum content increased to 50%, the fluorescence intensity of picagreen did not increase significantly probably due to the effect of substances in serum on the interactions between picagreen and dsDNA. These results mean that our new strategy is applicable in relatively complex biological conditions.

Conclusions

To sum up, we have developed a novel strategy for the assay of the activity of ADA by taking advantage of label-free aptasensor. In the presence of ADA, picagreen can intercalate into hybridized dsDNA, allowing the detection of ADA by measuring the increased fluorescence signal. Our results indicate that the increased fluorescence intensity is consistent with the ADA activity. The detection limit of the assay is 2 U L^{-1}. which can meet the clinical requirement. Furthermore, the aptasensor possesses the label-free and signal turn-on properties and is cost-effective, simple and homogeneous without any complex treatments. The new strategy we proposed may provide a versatile platform for the detection of enzymatic activity and the screening of the inhibitor.

Acknowledgements

The authors are grateful for the financial support from the National Natural Science Foundation of China (grants 21222509, 21105061), Fundamental Research Funds for the Central Universities (GK201103002), Specialized Research Fund for the Doctoral Program of Higher Education of China (grant 20110202120006), Program for Changjiang Scholars and Innovative Research Team in University (IRT 14R33), and College Students' Innovative Projects of the State Ministry of Education of China (grant 201410718007).

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