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Adenosine Deaminase Biosensor Combining Cationic Conjugated Polymer-Based FRET with Deoxyguanosine-Based Photoinduced Electron Transfer

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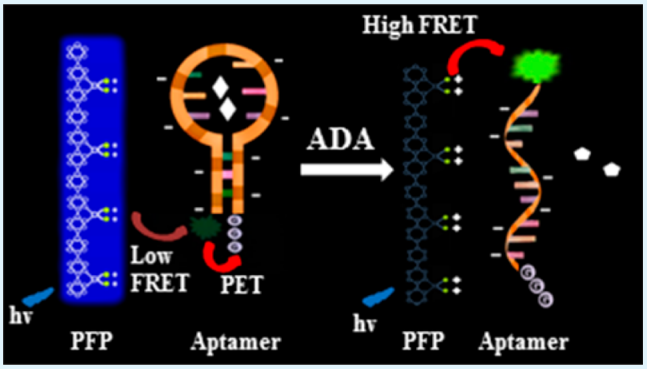
Supporting Information

ABSTRACT: We demonstrated a sensitive and selective adenosine deaminase (ADA) detection by modulating the fluorescence resonance energy transfer (FRET) between cationic conjugated poly(9,9-bis(6′-N,N,N-trimethylammonium) hexyl)fluorine phenylene) (PFP) and the deoxyguanosine-tailored hairpin aptamer. The hairpin aptamer was labeled with a fluorophore FAM at one end and three deoxyguanosines (Gs) at the other end as a quencher. In the absence of ADA, aptamer forms hairpin-like conformation with adenosines making close affinity of Gs and FAM, which results in the weak FRET from PFP to FAM because of FAM fluorescence being quenched by Gs via photoinduced electron transfer (PET). After addition of ADA, adenosine was hydrolyzed by ADA, followed by the release of free aptamer. In this case, FAM being far away from Gs, the strong FRET thus was obtained due to the quenching process being blocked. Therefore, the new strategy based on the FRET ratio enhancement is reasonably used to detect the ADA sensitively, combining the fluorescence signal amplification of conjugated polymers with the initiative signal decreasing by Gs. The detection limit of the ADA assay is 0.3 U/L in both buffer solution and human serum, which is more sensitive than most of those previously documented methods. Importantly, the assay is rapid, homogeneous, and simple without a complicated treating process. The ADA inhibitor, erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA), was also studied based on this assay, and the detection limit of EHNA is 10 pM. This strategy provides a new platform for the detection of other biomolecules and enzymes.



KEYWORDS: *fluorescence resonance energy transfer, photoinduced electron transfer, adenosine deaminase, conjugated polymers, deoxyguanosine*

INTRODUCTION

Adenosine deaminase (ADA) is an enzyme that can catalyze the reaction of adenosine and 2-deoxyadenosine converting to inosine and deoxyinosine, respectively. It plays an important role in the function, maturation, and maintenance of immunological responses.[1−4] Both inherited ADA deficiency and ADA excess may cause diseases.[5,6] For example, it was reported that an increase of ADA activity in serum has been found to be closely related to esophagus tumors, pancreatic cancer, liver cancer, breast cancer and colorectal cancer.[7] On the contrary, one of the main causes for severe combined immunodeficiency (SCID) is ADA deficiency.[6] Therefore, a sensitive detection of ADA is of great significance for clinical applications. So far, several techniques have been employed to detect ADA activity, including fluorescent aptasensor, electrochemical aptasensor based on magnetic beads, graphene oxide, or silver-nanoclusters, etc.[5,8−11] However, these reported techniques have inherent disadvantages, such as complexity and limited sensitivity,[11,12] or requiring a laborsome and time-consuming process.[9] Accordingly, it is still necessary to develop a simple and sensitive method to detect ADA.

Recently, much attention has been paid on aptamer because of its specific and high binding affinity toward the target molecules.[13−17] In addition, aptamer that consists of a fluorescent label at one end and a nonfluorescent molecule (e.g., nucleobase) at the other end is able to work as a molecular beacon. Importantly, this kind of aptamer avoids the limitations that molecular beacons suffer in overall yield and are expensive.[18] In the closed-state conformation, this kind of probe is able to work as an acceptor to quench the fluorescence of the fluorophore via photoinduced electron transfer (PET).[19−21] In this regard, a number of unique (rather than traditional) nonfluorescent quenchers, for example, guanosine, and deazaguanosine have already been successfully intro-

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duced.[22−25] The quenching efficiency of guanosine is probably due to its low oxidation potential, hydrophobic interaction between the dyes and DNA nucleotide, and formation of nonfluorescent ground state via photoinduced electron transfer because of the close proximity of the stacked arrangement of nucleobase G.[18−21,26−28] Interestingly, this type of aptamer provides a platform to design a novel biosensor with signal from turn-off to turn-on upon recognition of targets.

In recent years, cationic conjugated polymers (CCPs) have attracted more and more attention owning to their fluorescence signal amplification.[29,30] A variety of biosensors and chemosensors have been designed and developed by taking advantage of its particular optical properties.[31−37] These sensors have been successfully applied for detections of oligonucleotides, enzymes, protein fibrillation, inhibitors, as well as for biological applications, etc., with high sensitivity and specificity.[38−43] It is interesting to note that the fluorescence resonance energy transfer (FRET) technique plays an important role in most of these methods.[31−35,40,42,44] FRET is a popular technique in fluorescent biosensors, which is a well studied physical process whereby individual chromophore communicates its electronic states and provide the means for transferring excitations from a donor to an acceptor.[45] Therefore, the FRET technique based on fluorescent conjugated polymers is advantageous to enhance fluorescence of the receptors, and is attractive because of its simplicity of operation and use of standard optical equipment. We have reported a new biosensor to detect ADA based on two-step FRET from PFP to ethidium bromide (EB).[46] However, this method requires EB as energy receptor and the complementary nucleic acids to form double-stranded DNAs, which increased the complexity of the assay.[47]

Herein, we designed a hairpin-like adenosine aptamer with a fluorophore FAM at 5′-terminus and three deoxyguanosines (Gs) as a quencher at 3′-terminus because of three Gs producing the greatest quenching effect,[48] which undergoes a conformational change from a hairpin structure to open-chain conformation in the presence of ADA. In our strategy, aptamer and adenosine can form a hairpin-like adenosine-aptamer complex, which leads to the labeled FAM being quenched by Gs via PET due to its close proximity. Upon addition of PFP, the FRET from PFP to FAM is very low when PFP is excited at 380 nm. After addition of ADA, the FRET from PFP to FAM is highly efficient because the conformational change of aptamer from hairpin to open-chain results in the quencher, deoxyguanosines being away from FAM. This new biosensor can sensitively detect ADA simply through measuring FRET ratio from PFP to FAM, which utilizes the fluorescence signal amplification of CCPs and the initiative signal decreasing by Gs. Also, the method provides a new platform for other enzymes detection and has potential as a drug discovery screen.

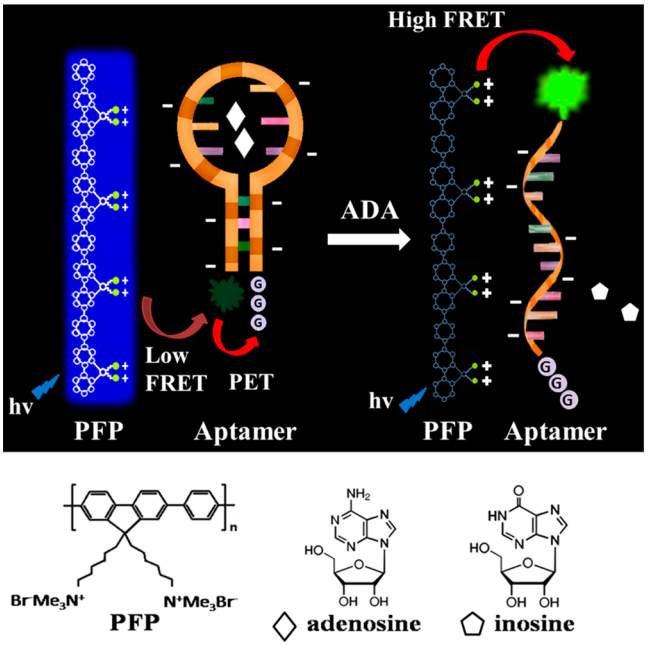
EXPERIMENTAL SECTION

Reagents and instruments. The oligonucleotides, adenosine deaminase, adenosine, and bovine serum albumin (BSA) were purchased from Shanghai Sangon Biological Engineering Technology & Service Co. Ltd. Erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA), α-glucosidase, α-chymotrysin, thrombin, human serum, and β-glucosidase were obtained from Sigma. Cationic poly(9,9-bis(6′-N,N,N-trimethylammonium)hexyl)fluorine phenylene) (PFP) was synthesized according to the literature.[44] The enzyme assay was carried out in KH_2PO_4 buffer (pH 7.5). The aptamer was hybridized in TE buffer containing 50 mmol/L Tris-HCl, 10 mmol/L KCl, 5 mol/L $MgCl_2$, and 100 mmol/L NaCl (pH 7.4). The oligonucleotide sequences used in our experiments are as follows: Ap-Gs: 5′-FAM-CAC TCT **GGG GGA GTA TTG CGG AGG AAG** AGT GGG G and C-Ap: 5′-FAM-GCA CCT **GGG GGA GTA TTG CGG AGG AAG** GTG C-3′. The sequence shown in bold is an adenosine aptamer. All solutions were prepared with ultrapure water purified by a Millipore filtration. The concentrations of all oligonucleotides were determined by measuring the absorbance at 260 nm in a 250 μL quartz cuvette. UV−vis absorption spectra were taken on a PerkinElmer Lambda 35 spectrophotometer. The fluorescence measurements were recorded on a Hitachi F-7000 spectrophotometer equipped with a xenon lamp excitation source.

RESULTS AND DISCUSSION

Scheme 1 shows the proposed principle of ADA sensing. The hairpin-like aptamer is labeled with fluorescein (FAM) at one

Scheme 1. Principle of Adenosine Deaminase Detection Using Conjugated Polymer-Based FRET Technique, Together with the Chemical Structures of PFP, Adenosine, and Inosine Used in the Detection



end and three Gs at the other end designed as a fluorescence quencher,[48] which can form stem-loop conformation with target adenosine. This probe is simple and low-cost compared with the molecular beacons. In this stem-loop conformation, the fluorescence of FAM is quenched efficiently by Gs *vis* photoinduced electron transfer due to the close affinity between FAM and Gs. Even after the addition of PFP, the fluorescence of FAM is weak and the FRET ratio from PFP to FAM is pretty low when PFP is excited. In the presence of ADA, adenosine is converted into inosine, which leads to the aptamer transferring into free conformation and results in the separation of FAM from Gs. Correspondingly, the FRET from PFP to FAM is distinctly high because of the electrostatic interactions between PFP and aptamer when PFP is excited at 380 nm. Notably, our previous research presented that the desirable spectral overlap between PFP emission and FAM absorption makes it possible for FRET from PFP to FAM.[46] Thus, the simple and sensitive detection of ADA is realized only by obtaining the FRET ratio of FAM to PFP. Importantly, this assay is simpler without any excess operation and less expensive than that reported previously.[9,11,46] Furthermore, the

conjugated polymers-based FRET technique and deoxyguanosine-based PET greatly enhances the sensitivity. Finally, the new method holds great potential for application in more complex conditions.

To optimize the concentrations of aptamer, we measured the fluorescence spectra after the addition of aptamer to PFP solution. As shown inFigure S1a in the Supporting Information, aqueous of aptamer without adenosine were added into the solution of PFP (2.0×10^{-6} M in RUs), and then the fluorescence emission spectra were obtained with excitation wavelength at 380 nm. In this case, the aptamer takes the free conformation in the absence of targets, which makes the fluorescence of FAM by seldomly quenched by Gs. Hence, the FRET from PFP to FAM is strong when free aptamer was added. Importantly, the FRET was improved dramatically with the increasing concentration of the aptamer. Finally, the optimized concentration of aptamer was obtained for 4.0×10^{-8} M. Furthermore, the ratios of FAM intensity to PFP fluorescence intensity at different concentrations of PFP and proportional aptamer were compared. As shown in Figure S1b in the Supporting Information, the ratio becomes the highest when PFP is 2.0×10^{-6} M and aptamer is 4.0×10^{-8} M. Hence, 2.0×10^{-6} M PFP and 4.0×10^{-8} M oligonucleotides were selected to obtain the best sensitivity.

Figure 1 shows that the FRET from PFP to FAM in the presence and absence of ADA. The hairpin structure aptamer

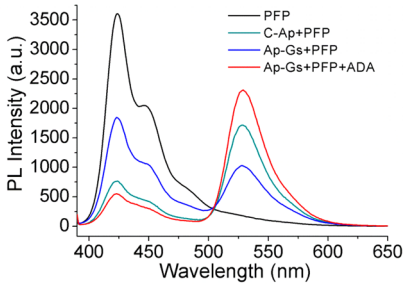


Figure 1. Fluorescence emission spectra of PFP, PFP/C-Ap (with adenosine), PFP/Ap-Gs (with adenosine) and PFP/Ap-Gs/ADA in KH_2PO_4 buffer solution (53.3 mM, PH = 7.5). [PFP] = 2.0×10^{-6} M in repeat units, [aptamer] = 4.0×10^{-8} M, [adenosine] = 8.0×10^{-8} mol/L, [ADA] = 150 U/L. The excitation wavelength is 380 nm.

was formed with adenosine by hybridization beforehand, and the concentration ratio between adenosine and aptamer is 2:1 as used previously.[9,46] PFP can emit strong blue fluorescence when PFP is excited at 380 nm. Upon the addition of aptamer with three Gs (Ap-Gs), in the absence of ADA, the FRET ratio (0.62) from PFP to FAM is pretty low because of the fluorescence of FAM is quenched by three deoxyguanosines, which indicates that the hairpin-like structure formed. In the presence of ADA, adenosine is converted into inosine, which makes the conformation change from hairpin to open-chain and then quencher, three deoxyguanosines is separated from FAM. The FRET ratio (4.2) from PFP to FAM is thus dramatically high due to strong electrostatic interactions between PFP and free aptamer when PFP is excited at 380 nm. To compare the FRET ratio with that without Gs, the fluorescence spectrum in the presence of PFP and control aptamer (C-Ap) that is labeled with FAM at the 5′ end was measured. As shown in Figure 1, the FRET ratio is about 2.2, which is much higher than that with Gs. The results indicate that this novel aptamer with three deoxyguanosines can greatly decrease the initiative signal and correspondingly is favorable for enhancing detection sensitivity. Accordingly, a new method is designed to detect ADA simply by measuring FRET ratio, which takes advantage of the fluorescence signal amplification of PFP and background signal decreasing from deoxyguanosine.

To test the ADA activity, we measured the fluorescence emission spectra of PFP/Ap-Gs (with adenosine) at different concentrations of ADA. As shown in Figure 2, in the absence of

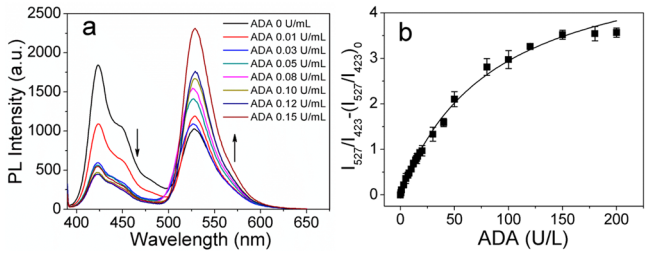


Figure 2. (a) Fluorescence emission spectra of PFP/aptamer (with adenosine) in KH_2PO_4 buffer solution (53.3 mM, pH 7.5) with addition of ADA. (b) Increase ratio of fluorescein intensity to PFP fluorescence intensity with the increasing addition of ADA. $(I_{527}/I_{423})_0$ means the ratio of fluorescein intensity in the absence of ADA. [PFP] = 2.0×10^{-6} mol/L in RUs, [aptamer] = 4.0×10^{-8} mol/L, [adenosine] = 8.0×10^{-8} mol/L. The error bars represent standard deviations three parallel measurements. The excitation wavelength is 380 nm.

ADA, aptamer forms stem-loop structure with adenosines. The fluorescence of FAM is weak when PFP is excited at 380 nm, because FAM is quenched by three guanosines via PET. Accordingly, the FRET ratio is pretty low because of the aptamer forming the nonfluorescence ground state. After ADA (from 0.3 to 150 U/L) was added into the solution and incubated for 25 min (The time has been optimized as followed) at 25 °C, the emission spectra were measured with the excitation wavelength at 380 nm. With the hydrolyzation of adenosine by ADA and the release of inosine, the aptamer changed from loop-stem structure to free single-stranded conformation. Then, PET process is inhibited because of FAM being far away from Gs. It is observed that the fluorescence of FAM increases dramatically, whereas the fluorescence of PFP decreases, indicating the efficient FRET occurring from PFP to FAM. The FRET ratio increases with the increasing concentration of ADA. When the concentration of ADA increased to 150 U/L, the ratio is almost no longer changed, which indicates that the hydrolysis reaction reaches the plateau. Figure 2b demonstrates the hyperbola relationship between the increased FRET ratio of FAM to fluorescein ($I_{527}/I_{423} - (I_{527}/I_{423})_0$) and the concentration of ADA, which is typical fitting mode for enzyme assay. The fitting equation is $y = 5.44663x/(84.82185 + x)$ ($R^2 = 0.998$). Notably, we can observe the distinct fluorescence intensity increases at a low ADA concentration of 0.3 U/L (The analyte's signal at the detection limit (S_{dl}) is given by $S_{dl} = S_{bl} + 3\sigma_{bl}$, where S_{bl} is the signal for the blank and σ_{bl} is the known standard deviation for the blank's signal.). These results represent that this method is sensitive to ADA and the limit of detection (LOD) is lower than most of previous biosensors reported by Wang et al.[46] (0.5 U/L, 2014), Zhang et al.[49] (0.5U/L, 2013), Feng et al.[50] (0.05 U/mL, 2013), and by Xing et al.[5] (12.9 U/L, 2012) because of CCPs-based FRET technique amplifying the fluorescence signal and deoxyguanosine-based PET decreasing background sig-

nal.[8,46,49,51] We also compared the sensitivity with that in the absence of PFP. The assays without PFP were carried out by simply measuring the fluorescence emission spectra of aptamer at different concentrations of ADA. As shown in Figure S2 in the Supporting Information, the detection limit of 2.0 U/L was obtained which is about 7-fold higher than that with PFP. These results further provide a proof of conjugated polymers amplifying the FRET ratio and improving greatly the detection sensitivity.

Besides, to optimize the ADA reaction time, we measured the fluorescence emission spectra of PFP/aptamer (with adenosine)/ADA at different reaction times. Figure S3 in the Supporting Information shows the FRET ratio of FAM to PFP as a function of reaction time. At 25 min, the maximum FRET ratio was obtained and reached the plateau no matter what the concentration of ADA was.[46] As a result, 25 min was selected as the optimized reaction time, which further demonstrates the detection of ADA is rapid and can be completed in a relatively short time. We also investigated the specificity of the proposed method by comparing the FRET ratio (I_{527}/I_{423}) after aptamer hydrolysis by ADA and a variety of other enzymes, such as alpha-glucosidase, beta-glucosidase, chymotrysin, and thrombin (see Figure S4 in the Supporting Information). The high selectivity of this method for ADA also was observed as reported previously.[9,46] More importantly, the detection of ADA is carried out in a homogeneous and very simple system, which includes only PFP and hairpin-like aptamer. These advantages make the method prior to other sensors that are time-consuming and/or acquire complex reaction process.

In order to study the efficiency of this biosensor in complex biological samples, we investigated the ADA activity in 10% human serum. As shown in Figure 3, the pretty similar

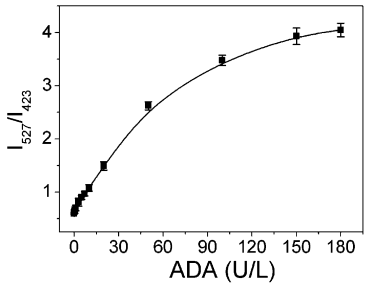


Figure 3. Ratio of FAM intensity to PFP fluorescence intensity as a function of ADA concentration in 10% human serum. [PFP] = 2.0 × 10^{-6} mol/L in RUs, [aptamer] = 4.0 × 10^{-8} mol/L, [adenosine] = 8.0 × 10^{-8} mol/L. The error bars represent standard deviations three parallel measurements. The excitation wavelength is 380 nm.

relationship to that in KH_2PO_4 buffer was observed between the increased FRET ratio and the concentration of ADA. The FRET ratio increases fast in the range of 0−50 U/L, and then the rate falls down. When the concentration of ADA is 150 U/L, the FRET ratio is about 3.9 and reaches the plateau. Furthermore, the obvious fluorescence intensity increase at a low ADA concentration of 0.3 U/L also was observed as in buffer solution. Therefore, this new strategy is applicable in complex biological conditions, which is superior to commonly used biosensors that are invalid to detect proteins in natural system.[52]

In addition, the inhibition of enzyme activity was studied by incubating ADA with different concentrations of erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA) which is a well-known inhibitor of ADA.[5] The concentration of ADA is fixed at 150 U/L because the activity of ADA gives the maximum response at this concentration and the incubation time is set for 25 min as mentioned above. Figure 4 shows that

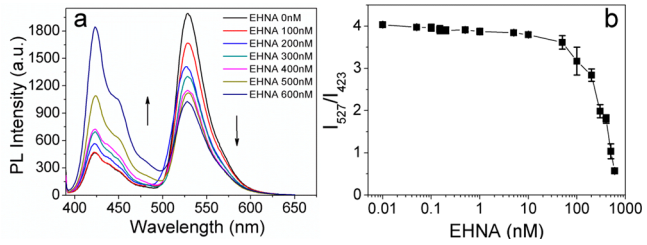


Figure 4. (a) Fluorescence emission spectra of PFP/aptamer (with adenosine) in KH_2PO_4 buffer solution (53.3 mM, pH 7.5) with addition of EHNA. (b) Ratio of fluorescein intensity to PFP fluorescence intensity with the increasing amount of EHNA. [PFP] = 2.0 × 10^{-6} mol/L in RUs, [aptamer] = 4.0 × 10^{-8} mol/L, [adenosine] = 8.0 × 10^{-8} mol/L, [ADA] = 150 U/L. The error bars represent standard deviations three parallel measurements. The excitation wavelength is 380 nm.

the fluorescence intensity of FAM decreases while the fluorescence intensity of PFP enhances with the increasing concentration of EHNA in the range of 0−600 nM. As a result, the FRET ratio of FAM to PFP decreases, which is dependent on the amount of EHNA. High concentrations of EHNA lead to FRET ratio dramatically decreasing. As shown in Figure 4b, it is observed that 600 nM EHNA can inhibit ADA completely and the detection limit of EHNA is 10 pM. These results confirm that our strategy has the potential for screening ADA inhibitors.

CONCLUSIONS

In summary, we designed a new method to sense ADA sensitively and specifically based on conjugated polymers and deoxyguanosine-modified aptamer. The detection limit of the ADA assay is determined to be 0.3 U/L not only in buffer solution but also in serum, which is lower than most of previously reported ones. Our method is pretty rapid, and the whole assay only takes about 25 min. Overall, our method possesses three advantages: First, conjugated polymer-based FRET technique amplifies the fluorescence signal; Second, the designed aptamer with three deoxyguanosines greatly decreases the initiative signal, which is helpful for increasing detection sensitivity; Third, the good water-solubility of CCPs makes the detection homogeneous and simple, avoiding complicated isolation and washing steps. Above all, it can be extended to the detection of other enzymes in complex conditions.

ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures and other figures as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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