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Water-Soluble Conjugated Polymer as a Platform for Adenosine Deaminase Sensing Based on Fluorescence Resonance Energy Transfer Technique

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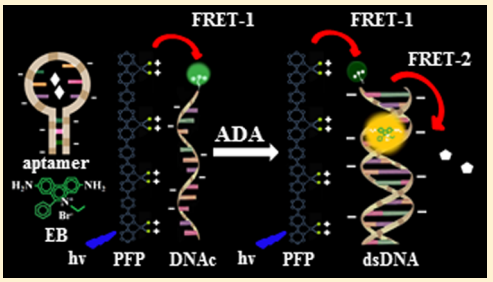
Supporting Information

ABSTRACT: We report a new biosensor for adenosine deaminase (ADA) sensing based on water-soluble conjugated poly(9,9-bis(6′-*N*,*N*,*N*-trimethylammonium)hexyl)fluorine phenylene (PFP) and fluorescence resonance energy transfer technique. In this biosensor, PFP, DNAc-FI labeled with fluorescein (FAM), and ethidium bromide (EB) were used as the fluorescence energy donor, resonance gate, and the final fluorescence energy acceptor, respectively. In the absence of ADA, the adenosine aptamer forms a hairpin-like conformation with adenosine, which is far from its complementary single-stranded DNA (DNAc-FI). When PFP is excited at 380 nm, fluorescein emits strong green fluorescence via one-step FRET while EB has no fluorescence. After addition of ADA, adenosine is hydrolyzed to inosine and then double-stranded DNA (dsDNA) is formed between the aptamer and DNAc-FI, followed by EB intercalating into dsDNA. Once PFP is excited, EB will emit strong yellow fluorescence after two-step FRET from PFP to fluorescein and from fluorescein to EB. The sensitive ADA detection then is realized with a low detection limit of 0.5 U/L by measuring the FRET ratio of EB to fluorescein. Most importantly, the assay is accomplished homogeneously in 25 min without further treatments, which is much more simple and rapid than that reported in literature. Hence, this method demonstrates the sensitive, cost-effective, and rapid detection of ADA activity. It also opens an opportunity for designing promising sensors for other enzymes.



Adenosine deaminase (ADA) plays an important role in the function, maturation, and maintenance of immunological responses.[1] As a deaminating enzyme, ADA can catalyze either adenosine or 2-deoxyadenosine, converting to inosine or 2-deoxyadenosine, respectively. It is noted that ADA is found in a wide variety of microorganisms, plants, bacteria, invertebrates, and mammals including all human tissues.[2−4] Both inherited ADA deficiency and ADA plethora may cause diseases. It is reported that one main cause for severe combined immune deficiency disease (SCID) is genetic ADA deficiency, accounting for a high incidence of SCID.[5] On the contrary, it is well-known that overexpression of ADA is related closely to hemolytic anemia, liver cancer, breast cancer, etc.[6] Therefore, sensitive detection of ADA and development of inhibitors may have potential clinical applications.

Aptamers are artificial nucleic acids that have been engineered through repeated rounds of in vitro selection or equivalently, SELEX (systematic evolution of ligands by exponential enrichment) to bind to various molecular targets with high affinity and specificity, such as small molecules, proteins, nucleic acids, and even cells, tissues, and organisms.[7] It has been reported that aptamers possess several advantages such as outstanding specificity, easy labeling, excellent stability, and convenient availability.[8−11] Therefore, much attention has been put on aptamers for developing new methods to detect targets from small organic molecules to complex multimeric structure.[12−14] So far, several techniques have been employed to detect ADA activity based on the adenosine aptamer, including fluorescent aptasensors, electrochemical aptasensors based on magnetic beads, graphene oxide or silver nanoclusters, etc.[6,15−20] However, some of these reported techniques are complicated and lowly sensitive,[15,20] or some techniques require a laborsome and time-consuming process.[17] Accordingly, it is still necessary to develop a simple and sensitive method to detect ADA.

In recent years, much attention has been put to water-soluble conjugated polymers (WSCPs) owing to their unique photo- and electrocharacteristics.[21,22] In comparison to small molecule counterparts, WSCPs are composed of a large number of absorbing units. After excited by UV light, the excitation energy along the whole backbone of the conjugated polymer transferring to an energy or electron acceptor quickly accounts for the amplification of fluorescent signals.[23] Recently, the present and some other authors have designed and developed a variety of biosensors and chemosensors by taking advantage of its fluorescence signal amplification.[24−32] These sensors

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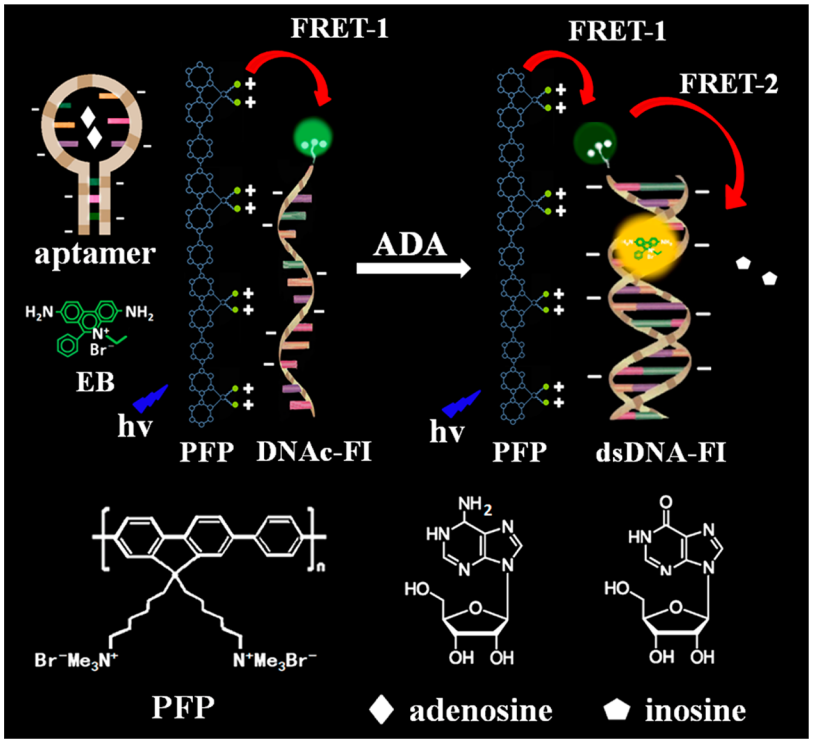
Scheme 1. Schematic Presentation of the Proposed Principle of Adenosine Deaminase Detection and the Chemical Structure of PFP, Adenosine, and Inosine



provided a number of sensitive and specific platforms for detections of oligonucleotides, enzymes, protein fibrillation, inhibitors, toxic metal ions, as well as for biological applications, etc.[33−41] It is interesting to note that most of these methods were based on the fluorescence resonance energy transfer (FRET) technique.[20,25−28,31,32,36,39,42−46] FRET is a well-studied physical process whereby individual chromophores communicate their electronic states and provide the means for transferring excitations from a donor to an acceptor.[42] Therefore, the fluorescent conjugated polymers-based FRET technique is advantageous to the enhancement of detection sensitivity, and is attractive because of its simple of operation and use of standard optical equipment.

In this work, we designed a new strategy to sense the enzymatic activity of ADA based on water-soluble conjugated polymers via fluorescence resonance energy transfer. In our strategy, an aptamer can be assembled into the adenosine−aptamer complex in the presence of adenosine, resulting in ethidium bromide (EB), the final energy acceptor, emitting no fluorescence. WSCPs were used as the energy donor, which can remarkably enhance the fluorescence of EB through two-step FRET upon adenosine converting to inosine by ADA hydrolysis. Thus, the ADA activity can be detected sensitively and simply by measuring the FRET ratio of EB to fluorescein. Compared to other techniques, the arrays are very easy without any excess operation, such as separation, denaturation, further reaction, washing steps, etc.[17,47] Therefore, this novel strategy would allow greatly improved detection sensitivity of ADA through a rapid method.

EXPERIMENTAL SECTION

Materials and Measurements. 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-chymotrypsin, thrombin, 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.[48] 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-FI, 5′-FAM-CAC CTT CCT CCG CAA TAC TCC CCC AGG TG-3′. The sequence shown in bold is an adenosine aptamer. DNAc-FI is complementary to the aptamer, which is two bases longer than the DNAc-FI. 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 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.

RESULTS AND DISCUSSION

The proposed principle of ADA sensing is illustrated in Scheme 1. Water-soluble PFP was used as the optical transducer in the biosensor. As shown in Figure S1 (Supporting Information), FRET from PFP to carboxyfluorescein (FAM) is possible because of the desirable spectral overlap between PFP emission and FAM absorption. Similarly, FRET from FAM to EB is also possible because of the favorable spectral overlap between FAM emission and EB absorption. However, despite the excellent spectral overlap between PFP emission and EB absorption, the direct FRET from PFP to EB is inefficient because of the unfavorable transition-dipole orientations of PFP and EB within the grooves of double-stranded DNA (dsDNA) (Figure S1b,

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Supporting Information). It was investigated by other groups that FAM with the free conformation thus provides the possibility of FRET from PFP to the intercalated EB, while FAM itself serves as a resonance gate for two-step FRET.[43,49] As shown in Scheme 1, the aptamer with the adenosine aptamer sequence can form a tight stem−loop structure once binding with adenosine, which is far from with its complementary single-stranded DNA (ssDNA) (DNAc-FI) with fluorescein (FAM) appended to the 5′-terminus. As is well-known that ethidium bromide (EB) can intercalate within the grooves of double-stranded DNA (dsDNA). Thus, in the presence of PFP and EB, FRET occurs from PFP to FAM upon excitation of PFP at 380 nm (FRET-1), where EB will not emit fluorescence because of the absence of dsDNA. Upon addition of ADA in the test solution, adenosine is converted to inosine by the catalysis of ADA. The release of inosine makes aptamer flexible and then the free ssDNA hybridizes with DNAc-FI. EB therefore intercalates into dsDNA-FI, which results in a two-step fluorescence resonance energy transfer: energy transfer from the PFP to the FAM (FRET-1), followed by FRET from the FAM to EB (FRET-2).[50] The stepwise energy-transfer process provides signal amplification of EB emission by the light-harvesting properties of WSCPs. Consequently, the energy transfer efficiency from FAM to EB can be used to detect ADA sensitively.

First, the concentrations of DNA and EB were optimized through measuring the fluorescence emission spectra. The aptamer and DNAc-FI were hybridized to form a dsDNA-FI (1.0×10^{-5} M) stock solution in advance. Then aqueous dsDNA-FI solution was added into the solution of PFP (2.0×10^{-6} M in RUs), followed by the measurement of fluorescence emission with an excitation wavelength at 380 nm. As shown in Figure S2a (Supporting Information), one-step FRET from PFP to FAM enhances with the increasing concentration of dsDNA-FI. When the concentration of dsDNA-FI is added to 4.0×10^{-8} M, the FRET efficiency reaches the plateau. Furthermore, to optimize the concentration of EB, the concentrations of PFP and dsDNA-FI were fixed to 2.0×10^{-6} and 4.0×10^{-8} M, respectively. Figure S2b (Supporting Information) shows that two-step FRET from PFP to FAM then from FAM to EB increases dramatically with the increase in the concentration of EB. The optimized concentration of EB was obtained for 7.0×10^{-6} M. Figure S2c (Supporting Information) distinctly presents the green fluorescence emission (one-step FRET) and yellow fluorescence emission (two-step FRET) under UV lamp excitation. Accordingly, 2.0×10^{-6} M PFP, 4.0×10^{-8} M oligonucleotide, and 7.0×10^{-6} M EB were selected for the following analytical purposes.

Figure 1 shows the fluorescence characterization of one-step FRET and two-step FRET in the solution of PFP, aptamer, DNAc-FI, and EB in the presence or absence of ADA. First, the aptamer (4.0×10^{-8} M) was converted to tight hairpin structure with its target adenosine. The binding molar ratio between adenosine and aptamer is 2:1.[17,51] Upon addition of complementary DNAc-FI (4.0×10^{-8} M), EB (7.0×10^{-6} M), and PFP (2.0×10^{-6} M), the fluorescence energy transfers efficiently from PFP to FAM (one-step FRET) once exciting PFP at 380 nm. The strong green fluorescence of FAM is observed while no obvious emission of EB can be obtained (Figure 1b), because the aptamer keeps the stem−loop structure in the presence of adenosine and the DNAc-FI is flexible without ADA. In the presence of adenosine deaminase, adenosine is converted into inosine and then the released

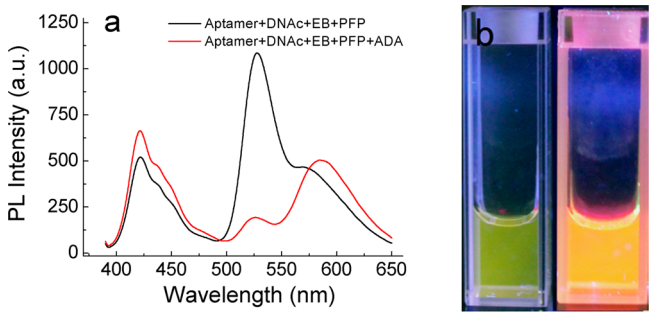

Figure 1. (a) Fluorescence spectra of PFP/DNAc-FI/aptamer (with adenosine)/EB in the absence and presence of ADA (incubating for 25 min). (b) Photographs of PFP/aptamer (with adenosine)/DNAc-FI/EB, and PFP/aptamer (with adenosine)/DNAc-FI/EB/ADA. [PFP] = 2.0×10^{-6} mol/L in repeat units, [DNAc-FI] = 4.0×10^{-8} mol/L, [aptamer] = 4.0×10^{-8} mol/L, [adenosine] = 8.0×10^{-8} mol/L, [EB] = 7.0×10^{-6} mol/L, [ADA] = 200 units/L. The excitation wavelength is 380 nm.

aptamer forms dsDNA-FI with DNAc-FI, followed by intercalation of EB to dsDNA-FI. Once PFP is excited, the EB emit strong yellow fluorescence via two-step FRET (Figure 1b). In this case, the addition of PFP to the solution leads to efficient FRET from PFP to fluorescein then from fluorescein to EB. The emission of PFP increases to some extent after addition of the ADA. As a biomacromolecule, ADA may reduce the potential intermolecular π-stacking of conjugated polymers, which leads to fluorescence enhancement due to a minimization of self-quenching of PFP. Thus, in view of the ratio of EB fluorescence intensity to the fluorescein intensity, we can monitor the adenosine deaminase activity by measuring fluorescence spectra in aqueous solutions.

To investigate the enzymatic activity of ADA, we measured the fluorescence spectra in KH_2PO_4 buffer at pH 7.5 ([PFP] = 2.0×10^{-6} mol/L in repeat units, [DNAc-FI] = 4.0×10^{-8} mol/L, [aptamer] = 4.0×10^{-8} mol/L, [adenosine] = 8.0×10^{-8} mol/L, [EB] = 7.0×10^{-6} mol/L) at various concentrations of ADA. ADA (from 0.5 to 250 U/L) was respectively added into the solutions of aptamer−adenosine complex that were formed in advance. After incubation at 25 °C for 25 min (the time was optimized in the following section), the EB and PFP were added, and the emission spectra were measured with the excitation wavelength of 380 nm. As shown in Figure 2a, the emission of fluorescein decreases while the emission of EB increases along with the increasing concentration of ADA. There is no question that a large number of adenosine will be converted into inosine at a high concentration of ADA, which releases more abundant flexible aptamer into the solution. Correspondingly, more hybridized dsDNA form in the presence of complementary sequence (DNAc-FI). In this case, a great deal of regions is provided for EB to intercalate. Therefore, the fluorescence emission of EB is enhanced after the two-step FRET process once adding more and more ADA. It is certainly reasonable that the fluorescence of fluorescein decrease gradually, because the energy of fluorescein transfers to EB via FRET-2. Obviously, the FRET ratio increases gradually with the increasing concentration of the ADA. These results present that the concomitant increase of the energy transfer efficiency from fluorescein to EB is a benefit from the addition of ADA. Figure 2b demonstrates the hyperbola relationship that is a typical fitting mode for enzyme assay between the increased FRET ratio of EB to fluorescein

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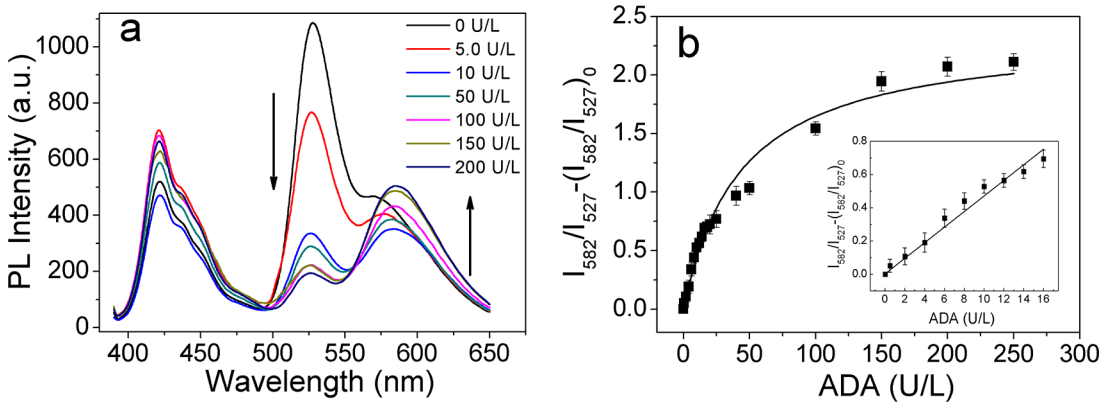

Figure 2. (a) Fluorescence emission spectra of PFP/DNAc-FI/aptamer (with adenosine)/EB in KH_2PO_4 buffer solution (53.3 mM, pH = 7.5) with addition of aqueous of ADA; (b) increased ratio of EB fluorescence intensity to the fluorescein intensity as a function of ADA concentration (inset: linear relationship between the increased ratio of EB fluorescence intensity to the fluorescein intensity and low ADA concentration). $(I_{582}/I_{527})_0$ means the ratio of EB to fluorescein intensity before addition of ADA. [PFP] = 2.0×10^{-6} mol/L in RUs, [DNAc-FI] = 4.0×10^{-8} mol/L, [aptamer] = 4.0×10^{-8} mol/L, [adenosine] = 8.0×10^{-8} mol/L, [EB] = 7.0×10^{-6} mol/L. The error bars represent the standard deviations of three parallel measurements. The excitation wavelength is 380 nm.

$(I_{582}/I_{527} - (I_{582}/I_{527})_0)$ and the concentration of ADA.[18] The fitting equation shown in Figure 2b is $y = 2.360\,29x/(43.321\,82 + x)$ ($R^2 = 0.988$). As shown in the inset of Figure 2b, in a range from 0 to 16 U/L, the increased FRET ratio is linearly dependent on the concentration of ADA. The fitting equation is $y = 0.002\,61 + 0.046\,83x$ ($R^2 = 0.989$). Thus, the detection limit of 0.5 U/L was obtained reasonably, which is lower than most of that reported previously by Zhang et al.[18] (2U/L) and by Xing et al.[6] (12.9 U/L). When the ADA concentration rises to 200 U/L, the ratio reaches the plateau and does not increase any more. That means the adenosine conversion nearly complete under this condition.

To compare the sensitivity in the absence of PFP, the assays without PFP were carried out by simple direct excitation of the fluorescein in the dsDNA-FI/EB at different concentrations of ADA. As shown in Figure S3 (Supporting Information), the FRET ratio only increases 1.5, even at the concentration of 200 U/L ADA. The detection limit of 2.0 U/L was obtained, which is 4-fold higher than that with PFP, which indicates that conjugated polymers amplify the FRET ratio and improve greatly the detection sensitivity. Thus, the FRET ratio of fluorescein to EB in the presence of PFP can be used to detect ADA sensitively and simply.

Furthermore, the ADA reaction time was optimized via measuring the fluorescence emission spectra of PFP/aptamer (with adenosine)/DNAc-FI/EB/ADA solution, and FRET from FAM to EB was studied as a function of the reaction time. In this case, different concentrations of ADA from 1.0 to 200 U/L are investigated, respectively. First, the concentration of ADA is fixed at 200 U/L, because the activity of ADA gives the maximum response at this concentration, which is above-mentioned. As shown in Figure 3, the ratio increases from around 0.4 to 1.2 quickly for the aptamer–adenosine complex incubating with ADA in the buffer for 2 min. After ADA reaction for 5 min, the ratio increases to 1.6, which demonstrates the enzymatic activity of ADA is desirable. Also, the higher initial reaction rate of ADA is accordant with one of the general enzymatic characteristics. As the reaction time is further increased, the ratio still enhances gradually but the rate slows down apparently. When the reaction time is 25 min, the maximum FRET ratio is obtained and does not change any more. Obviously, the same results were obtained at other

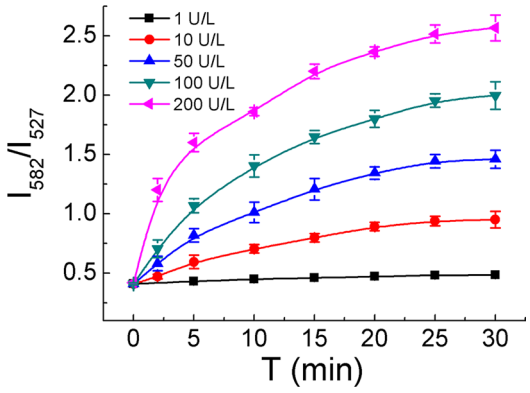

Figure 3. Ratio of EB fluorescence intensity to the fluorescein intensity as a function of ADA reaction time at different concentrations of ADA. [PFP] = 2.0×10^{-6} mol/L in RUs, [DNAc-FI] = 4.0×10^{-8} mol/L, [aptamer] = 4.0×10^{-8} mol/L, [adenosine] = 8.0×10^{-8} mol/L, [EB] = 7.0×10^{-6} mol/L, [ADA] = 1.0–200 U/L. The error bars represent the standard deviations of three parallel measurements. The excitation wavelength is 380 nm.

concentrations of ADA, such as 1, 50, 100, and 150 U/L. As a result, the 25 min reaction time was selected for the other analytical purposes. It should be noted that the enzyme assay is finished homogeneously in a short time (25 min) without any time-consuming process. Although some methods have equal (0.5 U/L; Zhang et al.)[47] or lower detection limits (0.05 U/L; Zhang et al.),[17] they are laborsome, and require multiply washing and/or a complicated process with a long reaction time. Thus, our method presents a more rapid and simple assay with high sensitivity for ADA.

To study the specificity of this method, the FRET ratio of fluorescein to EB (I_{582}/I_{527}) was examined in the presence of other enzymes. α-Glucosidase, β-glucosidase, BSA, chymotrypsin, and thrombin were chosen to be tested under the same experimental conditions as the ADA measurement. As shown in Figure S4 (Supporting Information), the FRET ratio always keeps the same low level as the control experiment (without any enzyme), no matter if in the presence of α-glucosidase, β-glucosidase, BSA, chymotrypsin, or thrombin. These results show the assay demonstrates an advantageous specificity for ADA.

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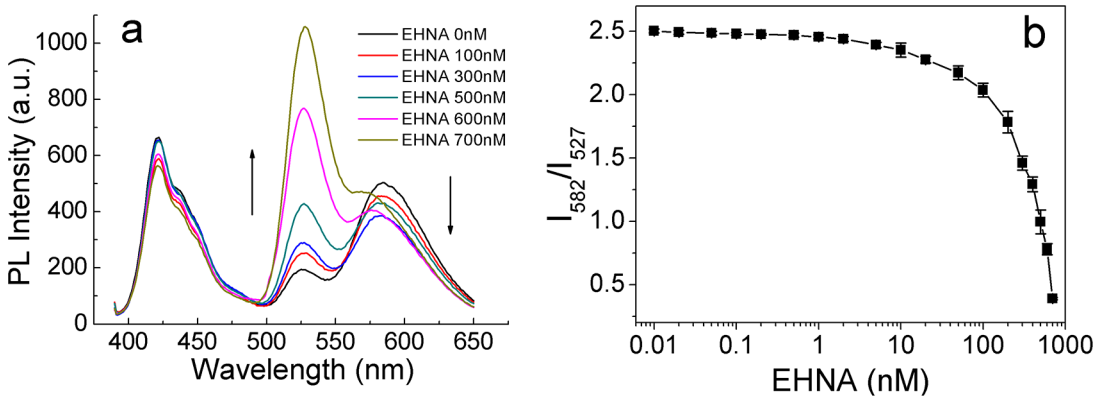

Figure 4. (a) Fluorescence emission spectra of PFP/DNAc-FI/aptamer (with adenosine)/EB/ADA in KH_2PO_4 buffer solution (53.3 mM, pH = 7.5) with successive addition of EHNA; (b) ratio of EB fluorescence intensity to the fluorescein intensity as a function of EHNA concentration. [PFP] = 2.0×10^{-6} mol/L in RUs, [DNAc-FI] = 4.0×10^{-8} mol/L, [aptamer] = 4.0×10^{-8} mol/L, [adenosine] = 8.0×10^{-8} mol/L, [EB] = 7.0×10^{-6} mol/L, [ADA] = 200 U/L. The error bars represent the standard deviations of three parallel measurements. The excitation wavelength is 380 nm.

Finally, we further investigated the inhibition of enzymatic activity of ADA by taking advantage of the new strategy of water-soluble conjugated polymers-based FRET. As a well-known inhibitor of ADA, erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA) was used to inhibit enzymatic activity of ADA. As mentioned above, the concentration of ADA is fixed at 200 U/L and the incubation time is set for 25 min. By incubating ADA with different concentrations of EHNA, the fluorescence spectra were then measured with the excitation wavelength of 380 nm. As shown in Figure 4a, the emission intensity of FAM increases while the emission intensity of EB decreases gradually along with the increasing amount of EHNA. Figure 4b shows that the FRET ratio of FAM to EB decreases when the concentration of EHNA increases in the range of 0−700 nM. Also, it is found that 700 nM EHNA can inhibit the activity of ADA fully. Correspondingly, the detection limit of EHNA obtained is 50 pM and the half-maximal inhibition concentration (IC_{50}) can also be obtained as 300 nM on the basis of the measurement of the FRET ratio (I_{582}/I_{527}). These results demonstrate that our strategy has potential to detect the inhibitor of ADA sensitively.

CONCLUSIONS

In conclusion, we have designed a new strategy to detect ADA activity by taking advantage of water-soluble conjugated polymers-based FRET. First, this technique combines the light-harvesting properties of WSCPs with fluorescence enhancement of the acceptor by FRET. The detection limit of ADA is 0.5 U/L, which is 4-fold lower than that of exciting directly fluorescein and also far lower than most of those reported in literature. Second, the detection of ADA is simple, rapid, and homogeneous, avoiding complicated procedures such as isolation and washing steps, multiple reactions, etc. Furthermore, this method provides a new platform for aptamer-based biosensors. In other words, this strategy has the advantages as a simple, rapid, and detection sensitive method, and provides new insight in the area of biosensors for enzyme detection and corresponding inhibitor screening.

ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures and other figures as noted in the 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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