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A universal aptameric biosensor: Multiplexed detection of small analytes via aggregated perylene-based broad-spectrum quencher

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ABSTRACT

A universal aptameric system based on the taking advantage of double-stranded DNA/perylene diimide (dsDNA/PDI) as the signal probe was developed for multiplexed detection of small molecules. Aptamers are single-stranded DNA or RNA oligonucleotides which are selected *in vitro* by a process known as systematic evolution of ligands by exponential enrichment. In this work, we synthesized a new kind of PDI and reported this aggregated PDI could quench the double-stranded DNA (dsDNA)-labeled fluorophores with a high quenching efficiency. The quenching efficiencies on the fluorescence of FAM, TAMRA and Cy5 could reach to 98.3% ± 0.9%, 97.2% ± 0.6% and 98.1% ± 1.1%, respectively. This broad-spectrum quencher was then adopted to construct a multicolor biosensor via a label-free approach. A structure-switching-triggered enzymatic recycling amplification was employed for signal amplification. High quenching efficiency combined with autocatalytic target recycling amplification afforded the biosensor with high sensitivity towards small analytes. For other targets, changing the corresponding aptamer can achieve the goal. The quencher did not interfere with the catalytic activity of nuclease. The biosensor could be manipulated with similar sensitivity no matter in pre-addition or post-addition manner. Moreover, simultaneous and multiplexed analysis of several small molecules in homogeneous solution was achieved, demonstrating its potential application in the rapid screening of multiple biotargets.

1. Introduction

Biological multiplexing has recently attracted tremendous interest in disease diagnostics, drug screening, and biodefense applications, because it could extract the most information from the smallest amount of sample volume at low cost (MacBeath et al., 2000; Zheng and he (2009); Nicewarner-Pena et al., 2001). Therefore, simultaneous determination of multiplexed analytes with ultrahigh sensitivity is of great interest. In recent years, a number of planar microarrays (Stoeva et al., 2006; Pan et al., 2012; Liu et al., 2014a; Wu et al., 2013), suspended encoding particles (Liu et al., 2010; Wang et al., 2013; Hu et al., 2010), and electrochemical methods (Tang et al., 2011; Wu et al., 2008) for multiple analytes detection have been reported, which often require an extra labeling step for the targets or repeated cleaning process. Due to their simplicity, rapid analysis, high sensitivity, low cost and little proclivity to sample or cell damage, fluorescent methods have been widely employed for the detection of various biomolecules (Martinez-Manez et al., 2003; Borisov et al., 2008, Wang et al., 2017; Zuo et al., 2016). To date, small organic molecules-based quenchers, nanomaterial-based quenchers and fluorescent polymers (CPEs)-based quenchers were frequently used for multiplexed detection. The fluorescence of different kinds of dyes could be efficiently quenched via small organic molecules quencher. However, small organic molecules quenchers usually require conjugation on the probe through tedious modifying steps, and they suffer from low, or variant, quenching efficiency for fluorophores which emit at different wavelengths (Tyagi et al., 1998; Dubertret et al., 2001). Nanomaterials usually possess poor salt, thermal stability, and interfere with catalytic activity of nuclease, including gold nanoparticles (Song et al., 2010), carbon nano materials (Mann et al., 2013; De et al., 2011) and so on. While the synthesis of CPEs is rather complicated, time-consuming and expensive (Liu et al., 2003; Chen et al., 1999). The water solubility of CPEs is relatively poor. Therefore, the development of a novelty strategy with high sensitivity, as well as high biocompatibility and stability is highly

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desirable for multiplexed detection.

Aptamers are single-stranded DNA or RNA oligonucleotides which are selected *in vitro* by a process known as systematic evolution of ligands by exponential enrichment (SELEX) (Tuerk et al., 1990; Ellington et al., 1990; Nguyen et al., 2016). Aptamers possess the ability to form defined three-dimensional structure for specific target binding. Compared to antibody proteins, nucleic acids are more stable, adaptable and they can be easily produced using a commercial DNA synthesizer. Due to the advantages of high affinity and specificity, good stability and low cost, aptamers have been widely used for biomolecules detection. For example, Wang et al. reported a graphene-based aptamer logic gates for multiplex detection (Wang et al., 2012). Xu et al. developed a nanocomplex based on the fluorophore-labeled aptamer and the polydopamine nanospheres for the detection of ATP (Qiang et al., 2015). Liu et al. employed an aptamer beacon covalently linked to graphene oxide resisting nonspecific probe displacement for ATP detection (Liu et al., 2014b). However, most of these strategies generally require a fluorophore labeled aptamer. The covalent labeling may weaken the affinity of the aptamer to its target. Due to the perturbation of the conformational equilibrium or steric hindrance, the site-specific introduction of the fluorophore could led to a significant loss of binding energy (Jhaveri et al., 2000; Ho et al., 2004; Xing et al., 2016). Consequently, some label-free aptamer based detection methods have been successfully developed such as the employ of DNA-intercalating dyes. Xiao et al. demonstrated a rapid and specific aptamer-based method based on fluorescent molecule 2-amino-5, 6, 7-trimethyl-1, 8-naphthyridine (ATMND) for one-step cocaine detection (Roncancio et al., 2014). However, these strategies lack of multiplex detection capability. Accordingly, there is still an urgent need for the development of label-free, simple and universal aptamer based multiplexed biosensors.

Perylene diimide (PDI) derivatives are excellent fluorophores because of their high fluorescence quantum yield and photostability, high thermal stability and excellent chemical inertness (Wang et al., 2010). In our previous work, we reported that cationic aggregated and quenched perylene could act as a broad-spectrum and label-free quencher via strong electrostatic interactions (Hu et al., 2014). In this work, we synthesized a new kind of PDI compound (Fig. 1). Methyl iodide was used in our previous work. However, methyl iodide is highly toxic substances. The toxicity of ethyl iodide is much lower than that of methyl iodide. Moreover, we found this aggregated PDI not only could quench ssDNA-labeled fluorophores which emit over a wide wavelength range from the visible to NIR region, but also quench the dsDNA-labeled fluorophores with a high quenching efficiency. The quenching efficiencies on the dsDNA-fluorescence of FAM, TAMRA and Cy5 could reach to 98.3% ± 0.9%, 97.2% ± 0.6% and 98.1% ± 1.1%, respectively. The use of dsDNA/PDI complex as an indicator probe for multiplexed detection has rarely been performed. Therefore, it is interesting using this broad-spectrum quencher to develop a universal aptameric multicolor system. Aggregated PDI exhibits a very high quenching efficiency on all dsDNA-labeled fluorophores of the sensing system, allowing low background fluorescence and therefore low detection limits.

Herein, we report a universal aptameric system based on the taking advantage of dsDNA/PDI as the signal probe for multiplexed detection of small molecules. Adenosine and cocaine were used as the model analytes, because adenosine plays important roles in a number of physiological and pathological processes in the brain. Cocaine is a powerfully addictive stimulant drug. While abusing cocaine has a variety of adverse effects on the body. For other targets, changing the corresponding aptamer can achieve the goal. A structure-switching-triggered enzymatic recycling amplification was employed for signal amplification. High quenching efficiency combined with autocatalytic target recycling amplification afforded the biosensor with high sensitivity towards target. The detection limit of 400 nM for adenosine was obtained. Simultaneous and multicolor analysis of several small molecules in homogeneous solution was also achieved by the proposed sensing system, thus demonstrating its potential application in the rapid screening of multiple biotargets. The PDI quencher did not interfere with the catalytic activity of nuclease. No matter in pre-addition or post-addition manner, the biosensor could be manipulated with similar sensitivity. Finally, our designed strategy is universal and may be useful in the detection of other target analytes via changing the corresponding aptamers.

2. Experimental section

2.1. Reagents and apparatus

Perylenetetracarboxylicdianhydride was purchased from Alfa Aesar (Beijing, China). Adenosine, uridine, guanosine, cytidine and 3-dimethylaminopropylamine were purchased from Aladdin (Shanghai, China). Methyl iodide and ethyl iodide were obtained from J & K Scientific Ltd (Beijing, China). The nuclease exonuclease I was purchased from New England Biolabs. Other compounds were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). All chemicals were of analytical grade and used without further purification. All solutions were prepared in Milli-Q water (resistance > 18 MΩ cm) from a Millipore system. All fluorescence measurements were carried out on an F-4600 spectrofluorometer (Hitachi Company, Tokyo, Japan). All measurements were carried out at room temperature unless stated otherwise. DNA oligonucleotides used in this work were synthesized and purified by Sangon Biotechnology Co., Ltd (Shanghai, China), and their sequences are shown in Table S1.

2.2. Synthesis of PDI

PDI was synthesized according to the literature (Wang et al., 2010; Hu et al., 2014). 3-dimethylaminopropylamine (1.5 mL, 118 mmol) and perylene tetracarboxylic dianhydride (0.50 g, 12.1 mmol) were added to 20 mL of isobutanol. The reaction was heated at 90 °C for 24 h with stirring. Then, the obtained crude product was filtered, washed with ethanol and deionized water. Afterwards, the crude product was treated with aqueous NaOH solution at 80 °C for 30 min to remove the unreacted perylene tetracarboxylic dianhydride. This precipitation was washed with ethanol and water and then dried.

0.30 g of the above product and 0.15 mL of ethyl iodide were added to 15 mL of toluene, refluxed for 3 h with stirring under N_2 atmosphere. Then, the precipitate was filtered after the mixture was cooled to room temperature. Afterwards, the product was washed with ether and dried under vacuum to give a brown-red product.

2.3. Quenching efficiency investigation

10 μL of 1 μM aptamer (AD, and Coa) were incubated with 10 μL of 1 μM various fluorophore-labeled DNA (FAM-P1, TAMRA-P2, Cy5-P3) for 10 min. Then, different concentrations of PDI were added to solutions. In all cases, the total volume of the reaction solution was 100 μL. After incubation for 10 min, the fluorescence was measured by an F-4600 spectrofluorometer.

2.4. Procedures for small molecules detection (post-addition manner)

For small molecules detection, 10 μL of 1 μM aptamer (AD or Coa), 10 μL of 1 μM various fluorophore-labeled DNA (FAM-P1 or TAMRA-P2), 14 μL of 1 μM PDI, 10 μL of different concentrations of small molecules (adenosine or cocaine) were first incubated at room temperature in a buffer solution (20 mM Tris-HCl (pH 7.4), 50 mM NaCl). After 30 min, 10 U of Exo I was added to the sample solution. Then the mixture was incubated at 37 °C for 40 min. In all cases, the total volume of the reaction solution was 100 μL. The fluorescence was measured by an F-4600 spectrofluorometer.

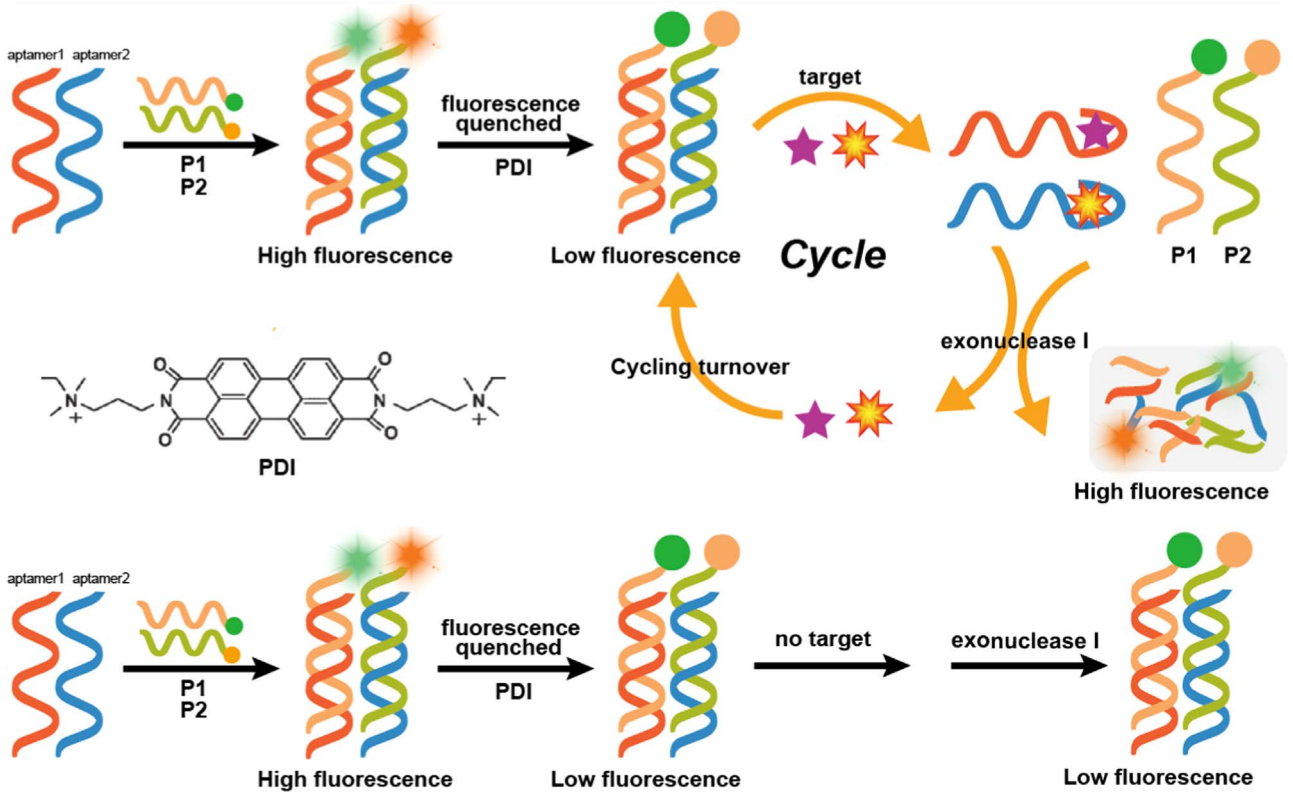

Fig. 1. Schematic illustration of a universal aptameric system based on the aggregated perylene-based broad-spectrum quencher with the Exo I-assisted enzymatic recycling amplification strategy for amplified multiplexed analysis of small molecules.

2.5. Procedures for small molecules detection (pre-addition manner)

10 μL of 1 μM aptamer (AD or Coa), 10 μL of 1 μM various fluorophore-labeled DNA (FAM-P1 or TAMRA-P2), 10 μL of different concentrations of small molecules (adenosine or cocaine) were first incubated at room temperature in a buffer solution (20 mM Tris-HCl (pH 7.4), 50 mM NaCl). After 30 min, 10 U of Exo I was added to the sample solution. Then, the mixture was incubated at 37 °C for 40 min. And then, 14 μL of 1 μM PDI was added to the above solution. In all cases, the total volume of the reaction solution was 100 μL. The fluorescence was measured by an F-4600 spectrofluorometer.

2.6. Multiplex small molecules simultaneous detection

10 μL of 1 μM aptamer (AD and Coa), 10 μL of 1 μM various fluorophore-labeled DNA (FAM-P1 and TAMRA-P2), 10 μL of different concentrations of small molecules (adenosine and cocaine) were first incubated at room temperature in a buffer solution (20 mM Tris-HCl (pH 7.4), 50 mM NaCl). After 30 min, 30 μL of 1 μM PDI and 20 U of Exo I were added to the sample solution. Then the mixture was incubated at 37 °C for 40 min. In all cases, the total volume of the reaction solution was 100 μL. The fluorescence was measured by an F-4600 spectrofluorometer.

3. Results and discussion

3.1. Design strategy

A multiplexed bioassay platform was developed for simple, sensitive and multiplex analysis of small molecules. The design scheme is shown in Fig. 1. The 5′-end of the signal probe DNA (P1 and P2) is labeled with fluorophores (FAM and TAMRA), which is complementary to aptamer AD, and Coa, respectively. In the absence of the target, the probe DNA hybridizes with the aptamer forming dsDNA, which prevent the digestion of the probe DNA by Exo I. The PDI associates with the probe and quenches the fluorescence of the fluorophores. Thus, the fluorophores-labeled dsDNA exhibit low fluorescence emission owing to their efficient quenching. Upon addition of the target, the aptamer prefers to form aptamer/target complex rather than the dsDNA as result of the stronger binding affinity of the aptamer towards its target. Therefore, the single DNA structure will become the substrate for Exo I cleavage. Then, Exo I catalyze the stepwise removal of mononucleotides from the blunt 3′ termini, resulting in the release of the target and fluorophore. The released fluorophores are no longer quenched by the aggregated perylene derivative, thus increasing the fluorescence intensity. Because the target is released and hybridizes with another aptamer, which could initiates a next round of cleavage. One target could initiate numerous probe digestions, leading to a highly sensitive small molecule detection method. By monitoring the increase in fluorescence intensity, the target could be detected with high sensitivity. Therefore, a multiplexed sensor for analysis of multiple molecules in homogeneous solution could be achieved when different aptamer are employed.

The fluorescence intensity of P1 under different conditions was detected to confirm the general feasibility of this approach. The results are shown in Fig. 2. In the absence of PDI, the fluorescence of P1/aptamer had very high fluorescence (curve a). In the presence of PDI, the initial fluorescence of P1/aptamer complex was almost quenched (curve b), indicating the high quenching ability of PDI because of the strong binding between dsDNA and PDI. When Exo I was added to the mixture containing aptamer and P1 in the absence of adenosine (curve c), only as light fluorescence signal change was detected after the addition of PDI, which implied that the P1 could hybridize with the aptamer forming dsDNA preventing the digestion of the P1 by Exo I. However, in the presence of adenosine, the fluorescence intensity of the assay system was increased greatly (curve d), suggesting that the aptamer prefered to form aptamer/target complex rather than the dsDNA as result of the stronger binding affinity of the aptamer. Exo I

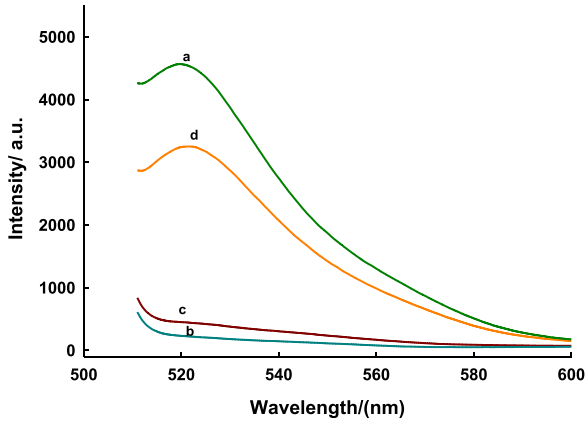

Fig. 2. Fluorescence spectra of P1 at different conditions: (a) P1+ aptamer; (b) P1+ aptamer+ PDI; (c) P1+ aptamer+PDI+Exo I; (d) P1+aptamer+PDI+Exo I+small molecule. [Exo I]=10 U; [PDI]=1.4 μM.

catalyze the stepwise removal of mononucleotides from the blunt 3′ termini, resulting in the release of the target and fluorophore. These above results confirm that this platform could be used for the assay of small molecule.

3.2. Quenching efficiency investigation

The fluorescence quenching ability of aggregated PDI was first investigated with the fluorophore- labeled dsDNA. As shown in Fig. 3a-c, upon addition of PDI to the solution of fluorescein FAM-, TAMRA- or Cy5-labeled dsDNA (P1, P2 or P3), and the fluorescence intensity greatly decreased. More than 98% of FAM's fluorescence was quenched with 1.4 μM of PDI in the solution. The quenching efficiencies on the fluorescence of FAM, TAMRA and Cy5 could reach to 98.3% ± 0.9%, 97.2% ± 0.6% and 98.1% ± 1.1%, respectively. This result reveals that aggregated PDI could efficiently quench the fluorescence of fluorophores labeled on dsDNA. Moreover, quenching time of PDI on the fluorophore- labeled ssDNA and fluorophore-labeled dsDNA (P1) was compared. As seen in Fig. 3d, the fluorescence intensity of fluorophore-labeled ssDNA reached a steady value within 2 min, and fluorescence of fluorophore-labeled dsDNA was completely quenched within 5 min. These results indicated the fast quenching kinetics of aggregated PDI.

Fluorescence quenching usually consists of static quenching and dynamic quenching. Static quenching can be described by the Lineweaver-Burk equation (Eq. (1)), while dynamic quenching can be described by Stern-Volmer's equation (Eq. (2)).

$$1/(F_0 - F) = 1/F_0 + K_{LB}/(F_0 cq) \quad (1)$$

$$F_0/F = 1 + Ksvcq, \quad (2)$$

where F and F_0 are the fluorescence intensities of the fluorophores in the presence of and in the absence a quencher (aggregated PDI), respectively, K_{LB} is the static quenching constant, cq is the concentration of the quencher and K_{SV} is the dynamic quenching constant (Jones et al., 2001; Murphy et al., 2004; Baptista et al., 1998; Lakowicz et al., 1999; He et al., 2008). When the concentration of aggregated PDI increased, the change of fluorescence intensity for fluorophore-labeled dsDNA showed a Stern-Volmer plot (Fig. 3e, f and S1, in the Supplementary Information), and the Lineweaver-Burk plot also showed a good correlation (Figs. S2, S3). Thus, the fluorescence quenching mechanism of PDI followed both dynamic and static processes (Chen et al., 1999; Russell et al., 1980). Since dsDNA is a polyanion, it could induce aggregation of the positively charged aggregated PDI via electrostatic interactions. Therefore, the quenching mechanism appears to be a function of the strong interaction between fluorophore-labeled dsDNA sequences and aggregated PDI by ionic interaction, strong intermolecular hydrophobic and π–π stacking interactions.

3.3. Optimization of the experimental condition

In order to achieve the best sensing performance, the concentration of Exo I was optimized. Experimental results showed that a concentra-

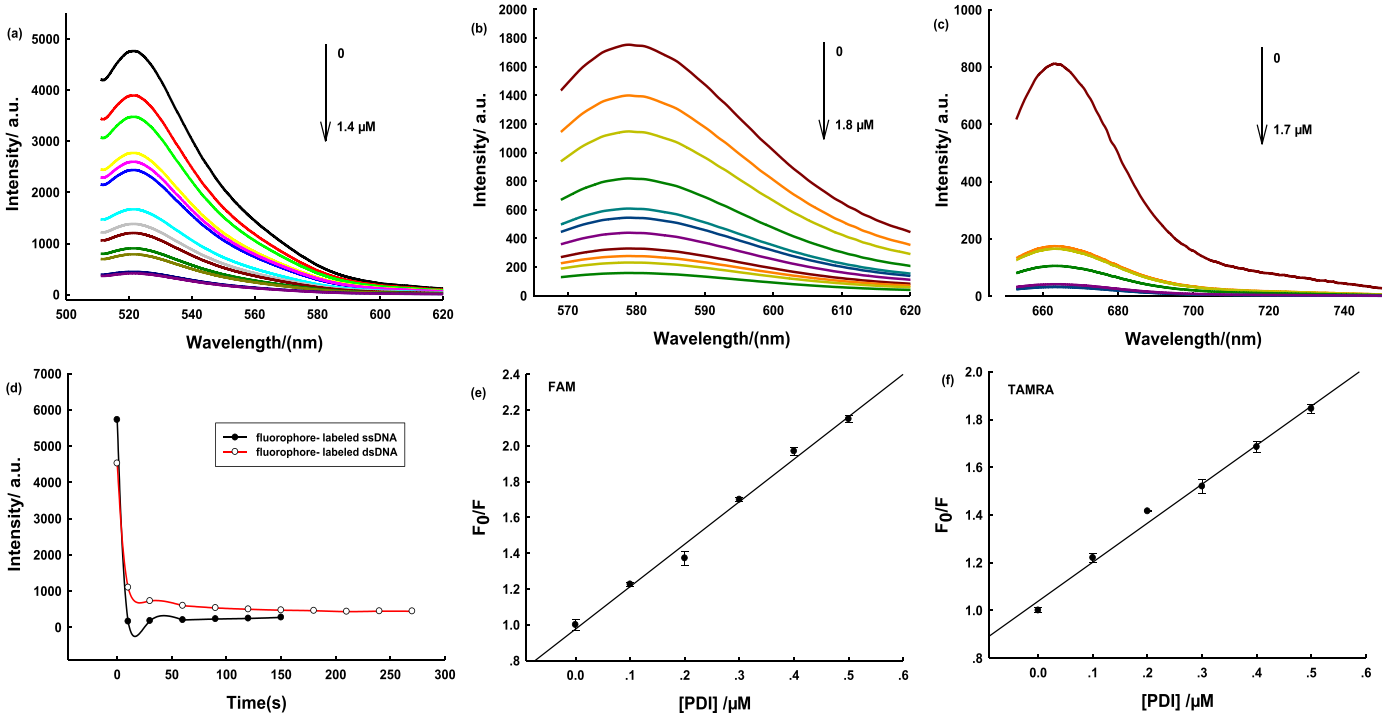

Fig. 3. Fluorescence spectra of 100 nM fluorophore-labeled P1/aptamer (a), fluorophore-labeled P2/aptamer (b) and P3/T3 (c) in the presence of different concentration of PDI; (d) Fluorescence kinetics curves of 100 nM fluorophore-labeled dsDNA and fluorophore-labeled ssDNA upon addition of 1.2 μM of PDI; (e, f) Stern-Volmer plots for 100 nM fluorophore-labeled dsDNA (e) P1 and (f) P2. F_0 and F are the fluorescence intensity of fluorophore in the absence and presence of PDI, respectively.

tion 10 U of Exo I could provide maximum signal-to background ratio (SBR) for the sensing system (Fig. S4), and the value was chosen as optimized conditions for further investigation.

3.4. Amplified multiplexed target detection

To assess the amplification function of endonuclease, the target-induced fluorescence enhancements in the presence a P1 probe with PDI. Upon the addition of the target adenosine, the exonuclease-assisted autocatalytic target recycling amplification could lead to a dramatic fluorescence increase. As shown in Fig. S5, in the absence of Exo I, only a 281 ± 10% signal increase was detected when 500 μM of target adenosine was added. While, under the same conditions, the introduction of Exo I for signal amplification provided a 930 ± 15% fluorescence enhancement. These results confirm that this assay could provide significant signal amplification for target detection. The low binding affinity of adenosine to its aptamer would be affluence this result. If an aptamer-target pair with strong affinity, the signal amplification with Exo I could be demonstrated more clearly.

To demonstrate that the aggregated PDI sensing system could be employed for multiplexed target detection in a homogeneous solution, the multiplexed detection ability was then studied. Two probes, P1 and P2, were labeled with FAM and TAMRA, respectively. Significant dye-to-dye energy transfer was avoided through choice of these two fluorophores, which were individually excited at 494 nm and 559 nm, emitting blue (520 nm) and orange (583 nm) colors, respectively (Hu et al., 2014). Upon addition of the target, the aptamer prefers to form aptamer/target complex rather than the dsDNA as result of the stronger binding affinity of the aptamer towards its target. The addition of Exo I will catalyze the stepwise removal of mononucleotides from the blunt 3′ termini, resulting in the release of the target and fluorophore (FAM or TAMRA). As shown in Fig. 4a, upon the addition of target adenosine, signal increase was observed only in the FAM channels, while weak signal change was observed in the TAMRA channels. The method could also be used for the specific detection of target adenosine or target cocaine (Figs. 4a, b). The simultaneous detection of the two small molecules was also carried out. When target adenosine and target

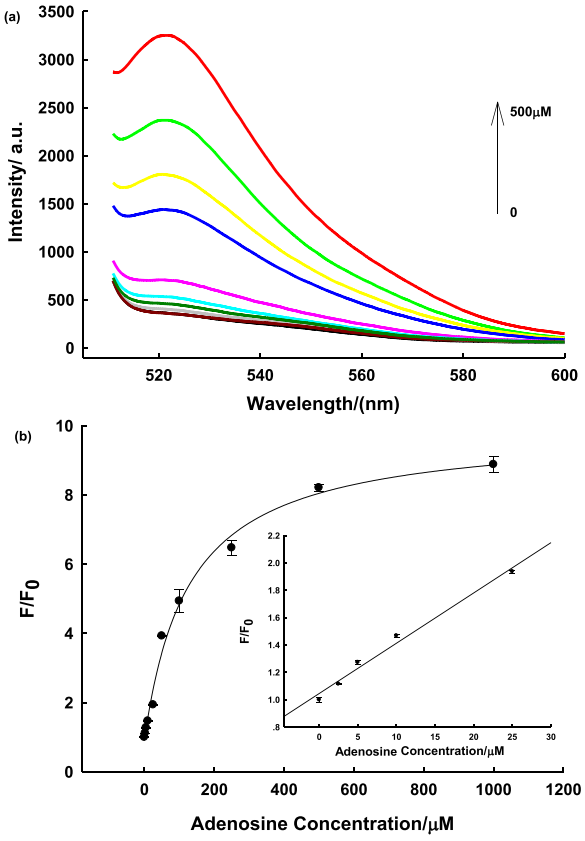

Fig. 5. (a) Fluorescence spectra of assay systems with "post-addition" strategy at various concentrations of target corresponding to data in the graph. (b) The relationship between fluorescence enhancement and target concentration. Insert shows the responses of sensing system to target at low concentration. F_0 and F are the fluorescence intensity of the sensor in the absence and presence of target, respectively.

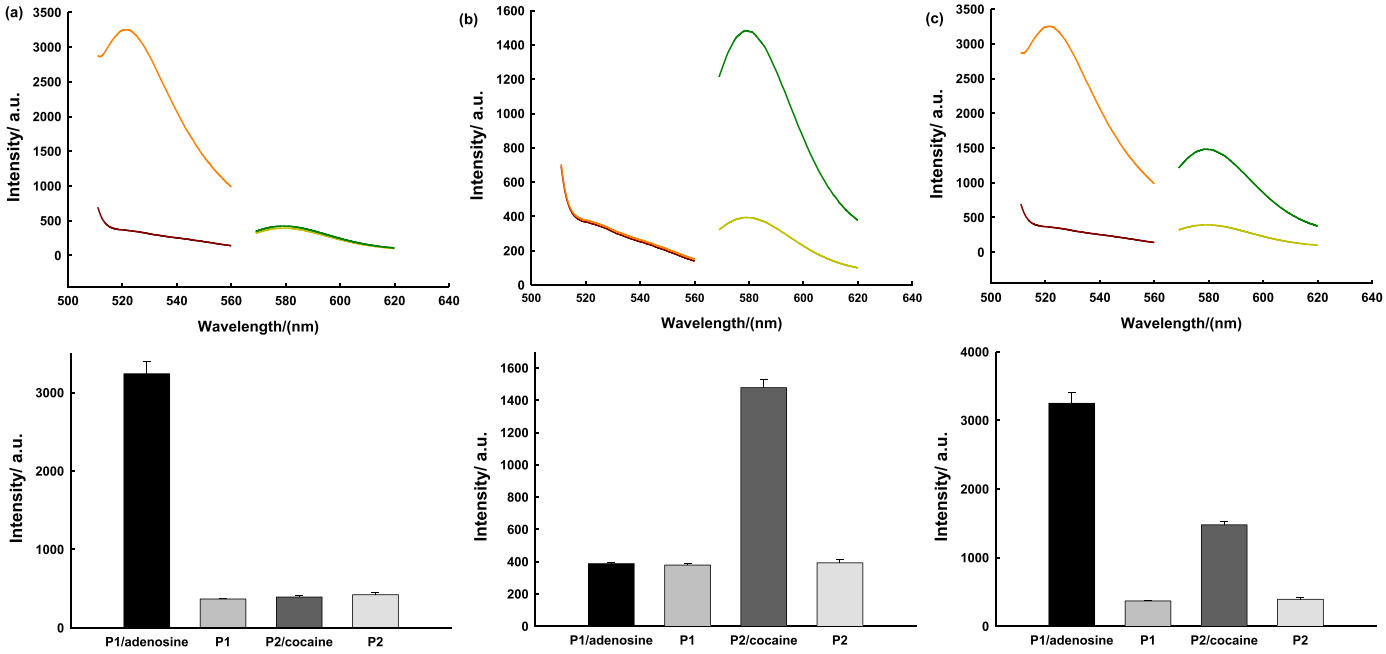

Fig. 4. Multiplex and selective analysis of small molecules.

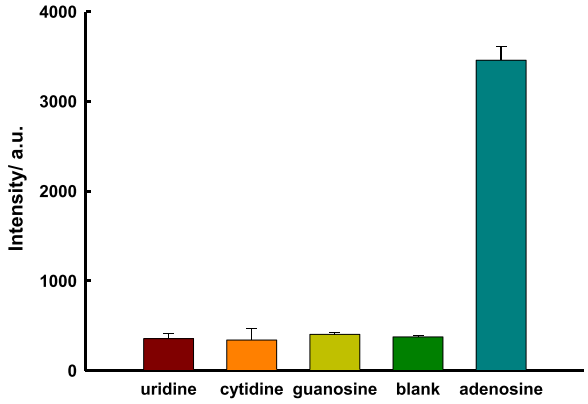

Fig. 6. Selectivity of the assay for adenosine (500 μM) over other potential interferences (5 mM).

cocaine were added to the mixed solution, the fluorescence enhancements for both FAM and TAMRA channels were observed (Fig. 4c). These results clearly demonstrate the feasibility of our strategy for multiplexed detection of small molecules.

3.5. Analytical performance

Whether two approaches (pre-addition and post-addition) would influence the accurate quantification of small molecules was next investigated. Compared with the pre-addition approach, the post-addition approach is simpler and easier to accomplish, which could afford *in situ* and real-time information for targets. As seen in Fig. 5, the fluorescence intensity increased gradually with adenosine concentration increase, irrespective of whether the post-addition (Fig. 5) or pre-addition approach were used (Figs. S6 and S7). The dynamic range of the sensing system for post-addition was from 1000 μM to 2 μM. The limit of detection (LOD) was 400 nM obtained by 3σ rule. Fig. S7 shows that the dynamic range of sensing system for pre-addition was from 1000 μM to 1 μM, and the limit of detection (LOD) was 200 nM. Almost the same detection limit for adenosine detection was obtained from either of these approaches, suggesting that the aggregated PDI did not influence the activity of the nucleases. Thus, the "post-addition" strategy was performed in the experiments. And for post-addition, the detection limit of cocaine was 30 nM obtained by 3σ rule (Fig. S8). Compared with other strategies used for homogeneous fluorescence multiplexed detection of small molecules (Table S2), the perylene derivative-based assay shows shorter assay time and a comparable, or even lower, detection limit (Zhang et al., 2012, 2009; Wei et al., 2015; Xiang et al., 2009; Zhu et al., 2013).

Selectivity is another important factor to assess the performance of a biosensor. The current responses induced by several potential interferents were also studied under the same conditions as those involved for small molecule detection. As shown in Fig. 6, only adenosine (500 μM) caused a dramatic fluorescence response, while other interferents (5 mM) did not induced obvious response. In other words, our design biosensor has good specificity towards other molecules.

The practical application of the designed sensor was evaluated by the determination of the recovery of spiked target in real human serum samples, a realistically complex medium containing a variety of proteins and other biomolecules. The results were listed in Table S3. From the results one can see that the obtained in real human serum samples showed good recovery values. This result indicated our sensing system could be used for real sample detection.

4. Conclusion

In conclusion, a new kind of PDI compound was synthesized, and this aggregated PDI not only could quench ssDNA-labeled fluorophores, but also quench the dsDNA-labeled fluorophores with high quenching efficiency which emit over a wide wavelength range from the visible to NIR region. Based on the unique proprieties of PDI, a label-free aptamer fluorescent sensor based on the taking advantage of double-stranded DNA (dsDNA)/ PDI as the signal probe for multiplexed detection of small molecules was developed. Adenosine and cocaine were used as the model analytes, because adenosine plays important roles in a number of physiological and pathological processes in the brain. Cocaine is a powerfully addictive stimulant drug. While abusing cocaine has a variety of adverse effects on the body. An exonuclease-assisted autocatalytic target recycling amplification was integrated into the sensing system. High quenching efficiency combined with autocatalytic target recycling amplification afforded the biosensor with high sensitivity towards target. The detection limit of 400 nM for adenosine was obtained. Simultaneous and multicolor analysis of several small molecules in homogeneous solution was also achieved by the proposed sensing system, thus demonstrating its potential application in the rapid screening of multiple biotargets. The PDI quencher did not interfere with the catalytic activity of nuclease. No matter in pre-addition or post-addition manner, the biosensor could be manipulated with similar sensitivity. Finally, our designed strategy is universal and may be useful in the detection of other target analytes.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2017.01.051.

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