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An aggregated perylene-based broad-spectrum, efficient and label-free quencher for multiplexed fluorescent bioassays

Tao Liu, Rong Hu, Yi-Fan Lv, Yuan Wu, Hao Liang, Shuang-Yan Huan, Xiao-Bing Zhang*, Weihong Tan*, Ru-Qin Yu

Molecular Science and Biomedicine Laboratory, State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, College of Biology, Hunan University, Changsha 410082, China

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ABSTRACT

Fluorescent sensing systems based on the quenching of fluorophores have found wide applications in bioassays. An efficient quencher will endow the sensing system a high sensitivity. The frequently used quenchers are based on organic molecules or nanomaterials, which usually need tedious synthesizing and modifying steps, and exhibit different quenching efficiencies to different fluorophores. In this work, we for the first time report that aggregated perylene derivative can serve as a broad-spectrum and label-free quencher that is able to efficiently quench a variety of fluorophores, such as green, red and far red dyes labeled on DNA. By choosing nucleases as model biomolecules, such a broad-spectrum quencher was then employed to construct a multiplexed bioassay platform through a label-free manner. Due to the high quenching efficiency of the aggregated perylene, the proposed platform could detect nuclease with high sensitivity, with a detection limit of 0.03 U/mL for EcoRV, and 0.05 U/mL for EcoRI. The perylene quencher does not affect the activity of nuclease, which makes it possible to design post-addition type bioassay platform. Moreover, the proposed platform allows simultaneous and multicolor analysis of nucleases in homogeneous solution, demonstrating its value of potential application in rapid screening of multiple bio-targets.

1. Introduction

Fluorescence methods exhibit unique advantages such as high sensitivity, rapid analysis with spatial resolution, and little proclivity to sample or cell damage (Martinez-Manez, and Sancenon, 2003; Borisov and Wolfbeis2008; Liu et al., 2009; Gale, 2010). Subsequently, past decade has seen considerable interest in development of fluorescent sensing systems for biological molecules. Most of these sensing systems are based on the quenching of a fluorophore by a non-fluorescent quencher. To date, the most frequently used commercial quenchers are small organic molecules. However, they usually need to be conjugated on the probe through tedious modifying steps, and suffer from low quenching efficiency or variant quenching efficiency for fluorophores that emit at different wavelengths (Tyagi et al., 1998; Dubertret et al., 2001).

In recent years, a variety of nanomaterials, including carbon nanomaterials and gold nanoparticles (AuNPs), have been developed which can quench various fluorophores with high quenching efficiency (Yang et al., 2008; Pautler et al., 2013; Song et al., 2009; Zhao et al., 2011). For example, AuNPs are reported to be able to quench FAM with quenching efficiency 100-fold higher than that of organic quenchers (Song et al., 2010). Nevertheless, these AuNP-based methods generally possess poor salt stability and thermal stability, thus limiting their practical applications (Song et al., 2010; Elghanian et al., 1997). It has also been proven that the fluorescence of different kinds of dyes could be efficiently quenched by carbon nanotubes (CNTs) or graphene. However, proteins and other biomolecules are prone to nonspecifically adsorb on CNTs or graphene, which might inhibit the activity of these biomolecules to some extent (Mann et al., 2013). Chen et al. reported a broad-spectrum nanoquencher by incorporating a series of dark quenchers into mesoporous silica nanoparticles (MSNs) (Huang et al., 2012), which can efficiently quench a broad range of visible and near infrared (NIR) fluorophores. To obtain such a nanoquencher, several tedious preparing steps are necessary. The development of a broad-spectrum fluorescent quencher which is easy to synthesize, exhibits high quenching efficiency with high biocompatibility and stability, is therefore highly desirable.

Recently, aggregated cationic perylene diimide derivative (compound **1**, Fig. 1) was reported to be able to act as a superquencher to quench the fluorescence of the adjacent oligonucleotide-labeled

* Corresponding authors. Tel./fax: +86 731 88821894.
E-mail addresses: xbzhang@hnu.edu.cn (X.-B. Zhang), tan@ufl.chem.edu (W. Tan).

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FAM (Wang et al., 2011; Fu et al., 2013). Such a superquencher can be formed "in situ" in a label-free manner, which make it an attractive, universal and convenient quencher for constructing FAM-based fluorescence biosensors. In this work, we for the first time report that cationic aggregated perylene **1** can act as a broad-spectrum and label-free quencher which is able to efficiently quench a variety of anionic oligonucleotide-labeled fluorophores that emit at a wide wavelength range from 520 to 670 nm via the strong electrostatic interactions. In comparison with classic small organic molecule-based quenchers, compound **1** is label-free quencher that can efficiently quench a variety of adjacent anionic oligonucleotide-labeled fluorophores that emit at a wide wavelength range from the visible to NIR region via strong electrostatic interactions. It avoids to be conjugated on the probe through tedious modifying steps. By choosing nucleases as model biomolecules, such a broad-spectrum quencher was then employed to construct a multiplexed bioassay platform through a label-free and post-addition manner with satisfactory results (Fig. 1).

2. Materials and methods

2.1. Reagents

DNA oligonucleotides used in this work were synthesized and purified by Sangon Biotechnology Co., Ltd. (Shanghai, China), and their sequences are shown in Table 1. Perylenetetracarboxylic dianhydride and N, N-Dimethyl-1, 3-propanediamine were purchased from Alfa Aesar. Methyl iodide and other compounds were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). All chemicals were of analytical grade and used without further purification. The restriction nucleases EcoRI, EcoRV and NEB buffer 3 were purchased from New England Biolabs. All solutions were prepared in Milli-Q water (resistance > 18 MΩ cm) from a Millipore system.

2.2. Sensing system preparation

2.2.1. Quenching efficiency investigation

Different concentrations of compound **1** were added into solution containing 10 μLof 1 μM various fluorophores-labeled DNA (3-FAM-A, TA-A, TE-A, and Cy5-A). The experiments were performed in 100 μL reaction solution which contained 1 × NEB buffer 3 (50 mM Tris–HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9). After incubating for

Table 1
Sequences of oligonucleotides used in this work.

Name	Sequences (from 5′ to 3′)
3-FAM-A	FAM-CTGAATTCTGGATGTAGTTAGTAGTC
3-FAM-B	GACTACTAACTACATCCAGAATTCAG
5-FAM-A	FAM-CACTGAATTCTGGATGTAGTTAGTAGTC
5-FAM-B	GACTACTAACTACATCCAGAATTCAGTG
7-FAM-A	FAM-CTCACTGAATTCTGGATGTAGTTAGTAGTC
7-FAM-B	GACTACTAACTACATCCAGAATTCAGTGAG
TA-A	TAMRA-CAGATATCGTTAAGTAGGTGCATGTGTC
TA-B	GACACATGCACCTACTTAACGATATCTG
TE-A	Texas Red-ATGAAGGACGATGTATGCTTGAGGTC
Cy5-A	Cy5-CAGATATCGTTAAGTAGGTGCATGTGTC

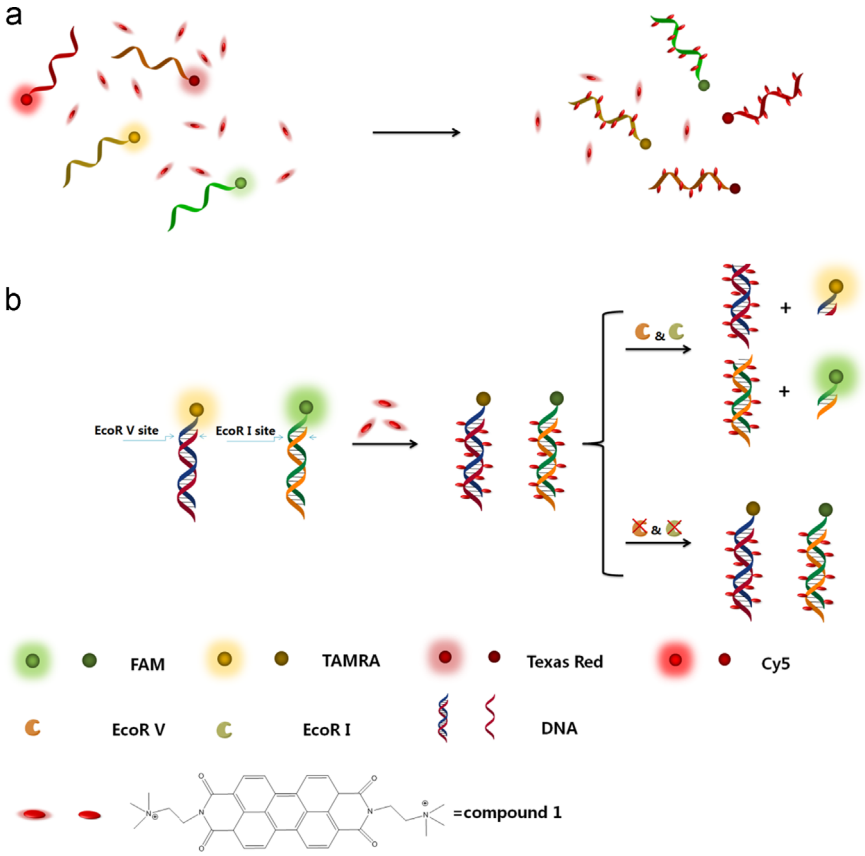

Fig. 1. (a) Schematic illustration of quenching properties of perylene derivative against various dyes. The fluorescence of the fluorophore was quenched by the compound **1**. (b) Schematic illustration of the multiplexed bioassay system for nucleases based on the broad-spectrum quencher of aggregated cationic perylene. Oligonucleotides labeled with FAM and TAMRA were designed as substrates for nuclease EcoRI and EcoRV, whose fluorescence was first quenched by compound **1**. The addition of EcoRI or EcoRV would induce the cleavage of the double strand DNA, releasing a short FAM-linked or TAMRA-linked oligonucleotide fragment, with their fluorescence signal being recovered.

10 min, the fluorescence was measured by a FluoroMax-4 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ).

2.2.2. Procedures for nuclease EcoRI activity detection with post-addition manner

For nuclease EcoRI activity assay, 100 nM of 5-FAM-A DNA probe was first incubated with 100 nM of 5-FAM-B DNA at room temperature for 10 min in a buffer solution (50 mM Tris–HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9). Then, 8 μL of 30 μM compound **1** was added to the above mixture. After 10 min, different concentrations of nuclease EcoRI were added into the sample solution. When the mixture was incubated at 37 °C for 1 h, the FluoroMax-4 spectrofluorometer was used to record the fluorescence intensity change at 518 nm upon excitation at 494 nm.

2.2.3. Procedures for nuclease EcoRI activity detection with pre-addition manner

For nuclease EcoRI activity assay, 100 nM of 5-FAM-A DNA probe was first incubated with 100 nM of 5-FAM-B DNA at room temperature for 10 min in a buffer solution (50 mM Tris–HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9). After 10 min, different concentrations of nuclease EcoRI were added into the sample solution. When the mixture was incubated at 37 °C for 1 h, 8 μL of 30 μM compound **1** was added to the above mixture. The FluoroMax-4 spectrofluorometer was used to record the fluorescence intensity change at 518 nm upon excitation at 494 nm.

2.2.4. Procedures for nuclease EcoRV activity detection

To detect EcoRV activity, 100 nM of TA-A DNA probe was first incubated with 100 nM of TA-B DNA at room temperature for 10 min in a buffer solution (50 mM Tris–HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9). Then 3.6 μM (final concentration) of compound **1** was added to the mixture to incubate at room temperature for 10 min. After the addition of varying concentrations of nuclease EcoRV into the buffer solution and incubation at 37 °C for another 1 h, the experiments were performed in 100 μL solution. The FluoroMax-4 spectrofluorometer was used to record the fluorescence spectra from 569 to 650 nm upon excitation at 559 nm.

2.2.5. Multiplexed detection nuclease activity

To detect two nucleases, the compound **1** (4.8 μM) was first incubated with 5-FAM-A DNA (150 nM), 5-FAM-B DNA (150 nM), TA-A DNA (100 nM) and TA-B DNA (100 nM) for 10 min. Then, the restriction nucleases 0.3 U/μL (final concentration) EcoRI and 0.2 U/μL (final concentration) EcoRV were added into the above solution. After incubating at 37 °C for another 1 h, the measurement conditions of FAM were excitation at 494 nm, excitation and emission slits of 3 nm and 4 nm, respectively. The measurement conditions of TAMRA were excitation at 559 nm, excitation and emission slits of 5 nm each.

2.3. Apparatus

All fluorescence measurements were carried out using a FluoroMax-4 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ). All measurements were carried out at room temperature unless stated otherwise.

3. Results and discussion

3.1. Design and working mechanism

To demonstrate the broad-spectrum quenching capability of the proposed quencher, the quenching efficiencies of the aggregated perylene derivative **1** to the oligonucleotide-labeled fluorophores emitted at different wavelengths were first investigated. As shown in Fig. 2, all the fluorophores show gradual decrease in fluorescence intensity with the addition of increased concentration of perylene **1**, with a final quenching efficiency of 98.3% observed for FAM, 97% for TAMRA, 98.5% for Texas red and 98.2%

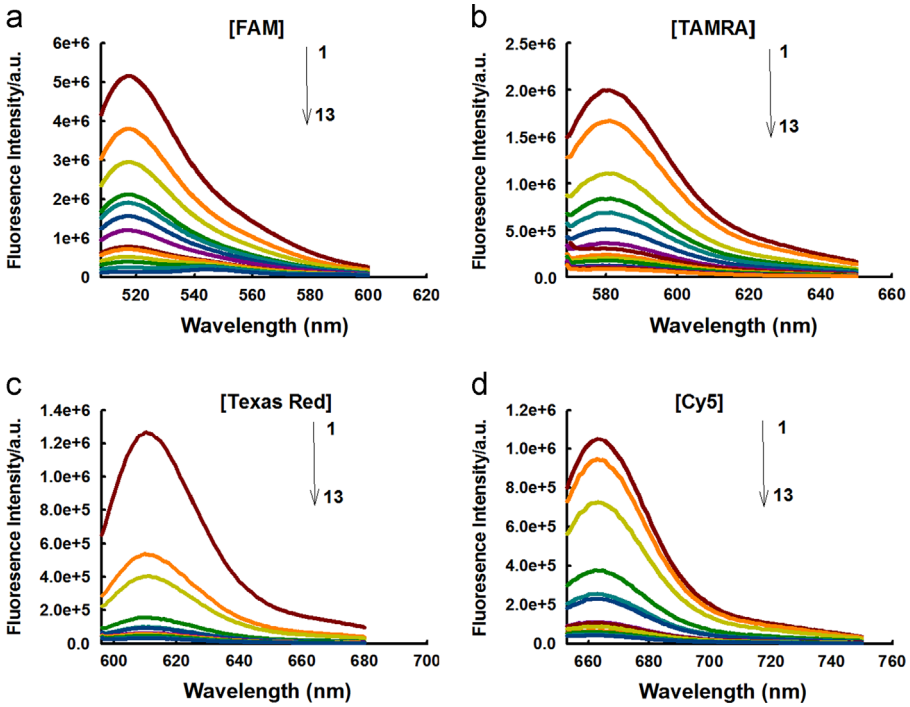

Fig. 2. Fluorescence emission spectra of different oligonucleotide-labeled fluorophores on exposure to perylene derivative **1** solutions of different concentrations. (a–c) (1) 0, (2) 0.1, (3) 0.2, (4) 0.3, (5) 0.4, (6) 0.5, (7) 0.6, (8) 0.7, (9) 0.8, (10) 0.9, (11) 1.0, (12) 1.1, and (13) 1.2 μM; (d) (1) 0, (2) 0.25, (3) 0.5, (4) 0.75, (5) 1.0, (6) 1.25, (7) 1.50, (8) 1.75, (9) 2.0, (10) 2.25, (11) 2.50, (12) 2.75 and (13) 3.0 μM. All concentrations of single-stranded labeled DNA were 100 nM.

for Cy5. The quencher noncovalently interacts with the dye labeled DNA. As ssDNA is a polyanion, the strong electrostatic interactions between the positively charged perylene derivative **1** and the polyanionic DNA will result in the rapid binding of compound **1** to the dye labeled oligonucleotide, and the aggregation of compound **1.** And it could be assumed that the fluorescence quenching of fluorophore-labeled DNA can occur by strong intermolecular hydrophobic and π–π stacking interactions between aggregated compound **1** and the fluorophore. The quenching efficiency of aggregated compound **1** is much higher than that of the reported AuNPs or CNTs (90%). The effect of metal ions on the sensing system was also investigated. As shown in Fig. S7 (see ESI†), we can see that the metal ions have no obvious effect on the response of the sensing system. The quenching kinetic was fairly fast, and the fluorescence was mostly quenched within several minutes after the addition of compound **1** into the sensing system (see Fig. S1 in ESI†). In addition, the self-assembly complex of oligonucleotide with compound **1** also possessed a highly thermal stability (date is not shown). All these unique features make aggregated compound **1** an ideal quencher for constructing multiplexed bioassay platform in a label-free manner.

To verify our hypothesis, a multiplexed bioassay platform was then constructed with nucleases chosen as model biomolecules. The design strategy is depicted in Fig. 1b. Oligonucleotides labeled with FAM and TAMRA were first designed as substrates for nuclease EcoRI and EcoRV, respectively. In the absence of nuclease, the fluorescence of the DNA-labeled fluorophore will be efficiently quenched. We found that both FAM-labeled single-stranded (ssDNA) and duplex-stranded DNA (dsDNA) could induce the aggregation of the compound **1** at a pH of 7.9, with similar quenching efficiency observed (see Fig. S2 and Fig. S3 in ESI†). Upon addition of the nuclease, the oligonucleotide probe will be cleaved on the cleavage site, releasing a short fluorophore-conjugated ssDNA. Since the short fluorophore-linked oligonucleotide only contains 5 bases, which could bind a few molecules of derivative **1**, therefore, compound **1** will exhibit weak quenching effect on the fluorophore's fluorescence. The fluorescence intensity will gradually increase with the addition of increasing concentration of nuclease.

3.2. Optimization of probes

In order to achieve the system's best sensing performance, the length of the base pairs on the FAM-labeled end away from the cleavage site of the oligonucleotide probe was first optimized (see Fig. S4 in ESI†). Too long base pairs will result in strong aggregation of compound **1** on the cleavage-produced ssDNA segments, and result in low target nuclease-triggered fluorescence restoration of the sensing system. One can observe that an obviously larger signal-to-background ratio (SBR) was observed for the probe containing 5 bases on the FAM-labeled end away from the cleavage site than that of the probe containing 7 bases. Low SBR was also observed when 3 bases on the FAM-labeled end away from the cleavage site, as too short base pairs might cause the steric hindrance of the adjacent fluorophore to lower the activity of nuclease. Therefore, a probe containing 5 bases was chosen as the optimum probe. In comparison with carbon nanomaterials, another significant advantage of our broad-spectrum quencher is that it did not affect the activity of the nucleases. As shown in Fig. S4 (see ESI†), no obvious difference of SBR for nuclease was observed between the pre-addition and post-addition approach (for the pre-addition approach, the quencher **1** was added after the completion of enzymatic reaction; while for the post-addition approach, all components were added together to induce the enzymatic reaction), which make the platform favorable for post-addition assay. In comparison with pre-addition approach, post-addition approach is simple and easy for operation, and could provide in situ and real-time information for targets.

3.3. Multiplexed detection of nucleases with high sensitivity and selectivity

Under the optimized condition, the above mentioned two DNA probes were then employed for multiplexed assay of nucleases via

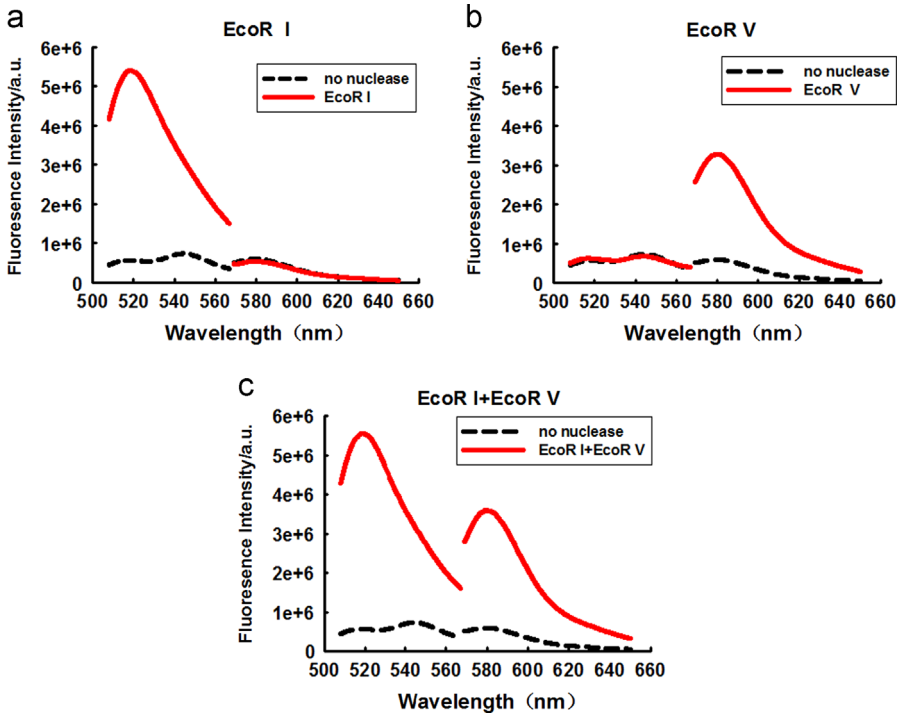

Fig. 3. Fluorescence emission spectra of the sensing system after treating with one of the two nucleases (a, b), and after treatment with two nucleases (c). Concentrations for the two nucleases: 300 U/mL for EcoRI and 200 U/mL for EcoRV.

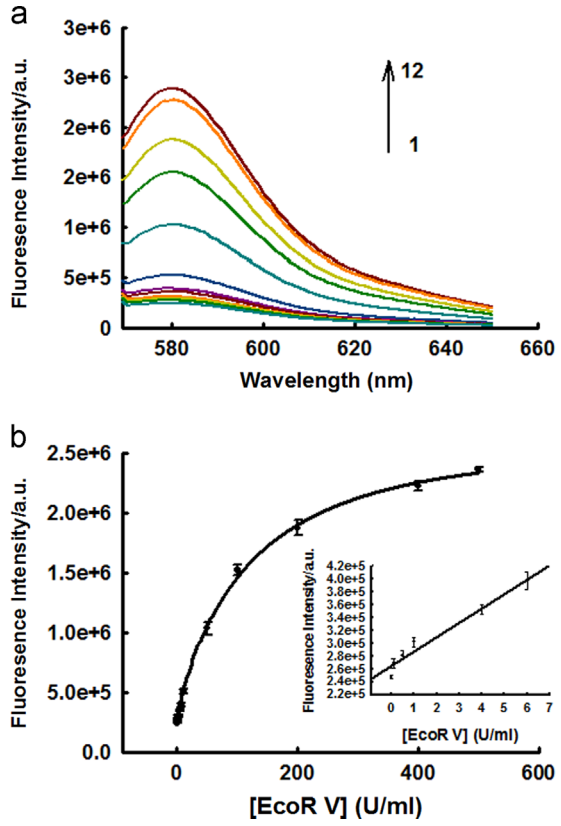

Fig. 4. (a) Fluorescence emission spectra of the sensing system on exposure to nuclease EcoRV solutions of different concentrations: (1) 0, (2) 0.1, (3) 0.5, (4) 1.0, (5) 4.0, (6) 6.0, (7) 10, (8) 50, (9) 100, (10) 200, (11) 400, and (12) 500 U/mL. (b) Calibration curve of the sensing system for the nuclease EcoRV activity. Inset shows the linear responses at low EcoRV concentrations.

the post-addition approach. The two DNA probes were first mixed in the same solution. It could be expected that the addition of certain nuclease will induce the cleavage of the corresponding probe, and result in the significant fluorescence enhancement of the corresponding fluorophore (FAM or TAMRA). Fig. 3a demonstrates the capability of the platform to detect a single nuclease. Upon the addition of EcoRI, FAM-labeled probe will be cleaved into two separate parts, which induce the significant fluorescence increase of FAM at 518 nm. At the same time, no obvious change was observed for the fluorescence of TAMRA (Fig. 3a), indicating negligible cross-interferences between the two nucleases. The method can also be used for the specific detection of EcoRV in a similar way, with only the fluorescence enhancement of TAMRA at 583 nm observed (Fig. 3b). The simultaneous detection of the two nucleases was then carried out. Upon the addition of EcoRI and EcoRV into the mixed solution, the fluorescence enhancements for both FAM at 518 nm and TAMRA at 583 nm were observed (Fig. 3c), indicating that the proposed platform is useful for multiplexed detection of nucleases.

The analytical performances of the sensing system for nucleases were then investigated. Fig. 4a shows the fluorescence-emission spectra of the TAMRA-labeled oligonucleotide probe upon the addition of EcoRV at different concentrations. A dramatic increase in TAMRA fluorescence intensity was observed as the EcoRV concentration increased from 0.1 U/mL to 500 U/mL. Fig. 4b depicts the relationship between the fluorescence intensity and the concentration of EcoRV. It shows a very low detection limit of 0.03 U/mL for EcoRV (based on 3δ/slope), which is much lower than previously reported fluorescence assays (Lu et al., 2011; Lee et al., 2011). A similar high sensitivity was also achieved for the detection of EcoRI (see Fig. S5 in ESI†), with a detection limit of 0.05 U/mL observed.

The selectivity of the platform was also investigated by testing the assay in response to several non-targeted endonucleases such as Nt.AlwI and Nt.BbvCI. with results shown in Fig. S6 (see ESI†). It could be found that the fluorescence response of non-targeted nuclease is almost the same as that of the blank solution, whereas a significant fluorescence increase was observed for targeted nuclease. These results clearly demonstrate that the proposed method can be applied for detection of EcoRI or EcoRV with high specificity.

4. Conclusions

In conclusion, we for the first time report that aggregated perylene derivative **1** can act as a broad-spectrum and label-free quencher to efficiently quench a variety of fluorophores, such as green, red and far red dyes labeled on DNA. Such a broad-spectrum quencher was employed to construct a multiplexed bioassay platform through a label-free manner with nucleases chosen as model biomolecules. By taking advantage of the high quenching efficiency of the aggregated perylene, the proposed platform exhibits high sensitivity to nuclease with a detection limit of 0.03 U/mL for EcoRV and 0.05 U/mL for EcoRI. The sensing platform can be carried out via a post-addition approach, and allows for simultaneous and multicolor analysis of nucleases in homogeneous solution. Significantly, the perylene derivative possesses several unique advantages: high stability, favorable biocompatibility, high quenching efficiency to various kinds of fluorophores, and not affecting the activity of the enzymes. It might find wide application in constructing multiplexed oligonucleotide-based bioassay platform for rapid screening of bio-targets.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.02.083.

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