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A ratiometric fluorescent system for carboxylesterase detection with AIE dots as FRET donors†

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A ratiometric fluorescent system for CaE detection with AIE dots as the FRET donors was designed. Upon enzymatic reaction, electrostatic interaction between the cationic TPE-N$^+$ dots and the enzymatic reaction product – the negatively charged fluorescein molecules – allows the FRET process to proceed, thus affording the ratiometric fluorescence CaE assay.

Fluorescent sensors are attractive and versatile tools for both analytical sensing and optical imaging because of their high sensitivity, fast response and technical simplicity.[1] Many fluorescent sensors employ increase or decrease in a single emission intensity as the sensing signal that responds to the target analyte(s).[2] However, a single fluorescence signal is readily interfered by external factors and this sensing mode is sometimes problematic for precise analysis. While ratiometric sensing, which involves the simultaneous measurement of two fluorescence signals at different wavelengths followed by the calculation of their intensity ratio, was devised to circumvent these unfavorable effects. Currently, several mechanisms have been exploited to realize ratiometric detection,[3] and the fluorescence resonance energy transfer (FRET) process has been widely adopted for ratiometric detection because of its facile control as a sensing mode and its basis on well-established theory.[3c–e]

With the development of nanoparticles, more and more researchers established FRET systems using them,[3d,e] such as quantum dots, and silica and polymer particles. However, this architecture is sometimes problematic for obtaining ideal ratiometric sensors, because most fluorophores usually display aggregation-caused quenching (ACQ) properties,[4] hence it is hard to achieve strong donor emission and high energy transfer efficiency by using the ACQ fluorophores as donors in nanoparticle-based FRET systems. Contrary to conventional fluorophores, some organic compounds are only highly emissive when aggregated due to the restricted intramolecular rotation/vibration, which was referred to as "aggregation-induced emission" (AIE).[5] To date, a variety of AIE fluorophores have attracted widespread interest in chemical sensors, biological imaging and optoelectronic devices. However, AIE dots are rarely adopted as donors in FRET systems,[5o–r] which can avoid the limitations of ACQ-fluorophore-based donors and would be beneficial for nanoparticle-based systems. Therefore, it is our primary interest to investigate the employment of AIE dots as donors in the nanoparticle-based FRET systems.

Carboxylesterase (CaE) is a group of enzymes that catalyze the hydrolysis of fatty acid esters into acids and alcohols with water. Due to its broad substrate tolerance, CaE are widely used in organic synthesis and industrial production.[6] It is also one of the main enzymes involved in the detoxification of organophosphorus (OP) compounds and serves as a determinant of individual sensitivity to these agents.[7] Recently, it was discovered that human plasma carboxylesterase could be a novel serologic biomarker candidate for hepatocellular carcinoma.[8] And the apparent CaE activity/level in human plasma (colorimetric method, determined with 1-naphthyl acetate) is reported to be 0.019 ± 0.001 U mL^{-1}.[9] Up to now, several fluorescent sensors for detecting CaE have been reported,[10] but most of them focused on imaging it in cells. While, establishing a reliable fluorescent system for detecting carboxylesterase in serum is of great importance in terms of clinical applications.

Herein we designed and fabricated a FRET-based ratiometric fluorescent system as CaE assay, by taking advantage of the efficient energy transfer between tetraphenylethene derivative (TPE-N$^+$) nanoaggregate (AIE dots or TPE-N$^+$ dots) and fluorescein. In this assay system, AIE dots serve as donors in the FRET system. As a proof of concept, we choose fluorescein diacetate (FDA) as the substrate for esterase activity study. The schematic illustration for the ratiometric fluorescent detection is shown in Fig. 1. The FDA substrate is neutral and nonfluorescent. In the

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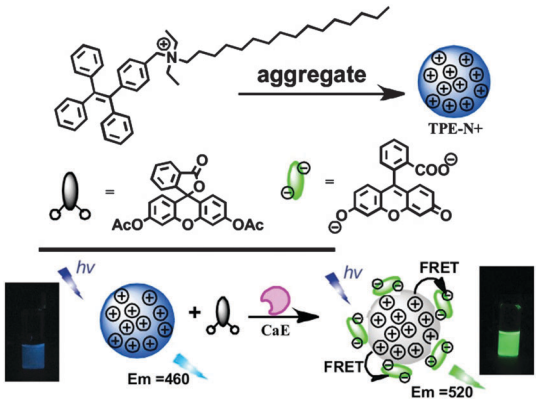

Fig. 1 Schematic illustration for the assay system and its ratiometric fluorescence response to CaE. Photographs were taken under hand-held UV light (365 nm).

absence of CaE, there is no electrostatic interaction between the TPE-N^+ dots and the non-fluorescent FDA. No FRET occurs and only the TPE-N^+ dots' emission is detectable upon excitation at 355 nm. After addition of CaE, FDA undergoes catalytic hydrolysis to yield fluorescent fluorescein molecules, which have two negative charges at pH = 7.4.[10a] Electrostatic attraction between the cationic TPE-N^+ dots and anionic fluorescein molecules brings them into close proximity so that the FRET process readily occurs, and the ratio of the two fluorescence intensities serves as the sensing signal for the enzyme activity/level. The assay herein is applied to the detection of CaE activity/level in such biological fluid as human serum.

The positively charged TPE derivative (TPE-N^+) and fluorescein diacetate were synthesized according to the synthetic routes as shown in Scheme S1 (ESI†). And these compounds were characterized by ^{1}H NMR and mass spectrometry (Fig. S1–S7, ESI†). Afterwards, the TPE-N^+ dots (AIE dots) were prepared by dissolving TPE-N^+ in small amount of DMSO, and then diluting it with PBS (1 mM, pH = 7.4) under stirring; TPE-N^+ nanoaggregates readily formed due to hydrophobic interaction. Then fluorescein diacetate (FDA) solution was added to TPE-N^+ dots suspension, thus forming the sensing system. The AIE behavior of TPE-N^+ is shown in Fig. S8 (ESI†). In the DMSO/water mixture solution, as the water fraction is increased, the emission of TPE-N^+ enhances.

Before investigating the fluorescence response of the sensing system towards CaE, we first studied the spectral properties of TPE-N^+ dots and fluorescein. The normalized absorption and emission spectra of TPE-N^+ dots and fluorescein are shown in Fig. S9 (ESI†). It can be seen that TPE-N^+ has absorption and emission at 300–360 nm and 430–480 nm, respectively, whereas fluorescein exhibits absorption and emission at 460–500 nm and 510–560 nm, respectively. Hence, there is a good spectral overlap between the emission of TPE-N^+ dots and the absorption of fluorescein, which should favor the energy transfer between them. In addition, upon varying the amount of fluorescein added into the TPE-N^+ dots dispersions, the energy transfer efficiency of the system can be adjusted (Fig. S10, ESI†). Also, we measured the quantum yields of TPE-N^+ in the solutions containing different water fractions. It reaches 8.75% in the 95% water/DMSO mixture and 9.65% in the powder state, which is ~30-fold higher than that of its DMSO solution (molecularly dissolved state), as shown in Fig. S11 and Table S1 (ESI†). In contrast, the quantum yield of dansyl-*N*-butylamine (DNS-NA, a typical non-AIE fluorophore (ACQ fluorophore) exhibits similar spectral properties as the AIE dots) dramatically decreases in the 90% water/DMSO mixture (Table S1, ESI†). In addition, the photostability of the AIE dots was also investigated and compared it with that of non-AIE fluorophores; the results are shown in Fig. S12 (ESI†). It can be seen that upon continuous illumination at 365 nm (15 W handheld UV lamp) for 2 h, only 4.4% fluorescence intensity loss for AIE dots has been recorded, while the other two non-AIE fluorophores whose spectral properties are similar to that of the AIE dots show obvious photobleaching phenomenon (Fig. S12, ESI†). This observation indicates that the AIE dots have good photostability and can serve as promising energy donors in nanoparticle-based FRET systems.

To investigate the fluorescence response of the assay system towards CaE, we measured the fluorescence spectra of the sensing system (TPE-N^+ 30 μM and FDA 0.3 μM in PBS containing 15% DMSO) in the absence or presence of CaE. The fluorescence spectra of the system were periodically recorded during the incubation of the assay system with CaE at 37 °C, and the results are shown in Fig. 2a–c. In the absence of CaE, the assay system shows intense blue emission at 460 nm from TPE-N^+ dots. Upon addition of CaE and with increased CaE incubation time, the blue emission intensity of TPE-N^+ dots at 460 nm gradually decreases over the incubating time from 0 to 30 min. Meanwhile, the emission of fluorescein at 520 nm shows a gradual increase. After 30 min, the fluorescence intensity of the system and the fluorescence intensity ratio I_{520}/I_{460} level off, as shown in Fig. 2b. In the meantime, we also studied the size change of TPE-N^+ dots before and after CaE incubation using TEM and DLS. The dots have an average diameter of about 40 nm before CaE addition.

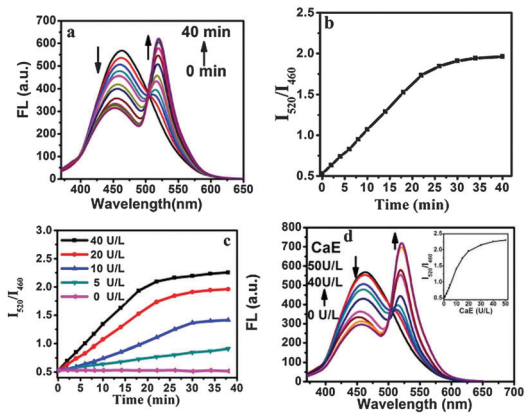

Fig. 2 Time-dependent emission spectra (a) and fluorescence intensity as a function of time (b) in the presence of CaE 20 U L^{-1}; the fluorescence intensity ratio as a function of time (c) and fluorescence spectra in the presence of different CaE levels at 30 min upon addition of CaE (d) of the sensing system (TPE-N^+ 30 μM, FDA 0.3 μM). Inset in (d): fluorescence intensity ratio as a function of CaE level. Excitation wavelength: 355 nm.

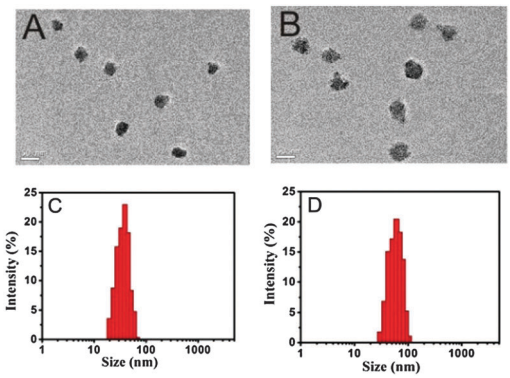

Fig. 3 HR-TEM images of the assay systems before (A) and after (B) the addition of 20 U L^{-1} CaE (placed on copper grids). Scale bar: 50 nm. Size distribution of the assay system determined by dynamic light scattering (DLS) before (C) and after (D) the addition of 20 U L^{-1} CaE.

After the addition of CaE and with the incubation of 30 min, the size of the nanoaggregates becomes a little larger, about 50 nm as shown in Fig. 3. The size changes determined by DLS (~45 nm and 70 nm) are slightly larger than those determined using TEM.

Also, we measured the fluorescence spectra of the sensing system in the absence or presence the different concentrations of CaE, as shown in Fig. 2d. It is clear that, as the enzyme level is increased, the emission of the TPE-N^+ dots decreases, while that of the fluorescein enhances; therefore, this sensing system can detect the enzyme level in a ratiometric way, and the detection limit is determined as 0.26 U L^{-1} (Fig. S13, ESI†).

Moreover, the fluorescence intensity of the assay system with AEBSF (a potent CaE inhibitor, 1 mM) in the presence of CaE (20 U L^{-1}) was measured (Fig. S14, ESI†). The enzyme inhibition by AEBSF demonstrates that the fluorescence change is indeed induced by CaE. To study the selectivity of the system, various potential interfering species were examined in parallel under the same conditions. As shown in Fig. S15, ESI,† the system shows a high selectivity towards CaE over the other species tested.

To evaluate the efficacy of this assay system in real samples, the system was applied to measure the level of carboxylesterase in the serum. The serum samples were diluted 50-fold for the measurements, and the determined carboxylesterase levels are listed in Table 1. The endogenous (originally existing) CaE in the diluted serum sample was determined by the assay system herein using the calibration curve (Fig. 3D, inset) as the standard, and the endogenous CaE level for the undiluted serum sample is then calculated as 17.2 U L^{-1}. Furthermore, the assay system was also compared with a commercially available colorimetric method, by which the naphthyl acetate hydrolyzed product was measured at 321 nm (ε_{321} = 2200 M^{-1} cm^{-1}).[9] The endogenous esterase level in serum determined using the colorimetric method is 16.4 U L^{-1}, which is close to that determined by our system. For the colorimetric assay, the detection process requires complex multiple procedures, whereas for the assay system herein, the detection process is quite convenient and could serve as a one-step straightforward assay.

In addition, the recovery of the added known amounts of CaE into the serum samples is in general more than 94% by the assay system, which suggests the accuracy and reliability of the present method for esterase determination. Furthermore, the precision of the assay system was also investigated (Table 1), which was determined using the relative standard deviation. It is obvious that the assay system herein displays quite good precision.

Compared with other reported fluorescent probes for carboxylesterase (as shown in Table S2, ESI†), the probe system herein can realize carboxylesterase detection in the fluorescence ratiometric mode, while current fluorescent probes for carboxylesterase mostly function in turn-on mode; furthermore, the probe system can detect carboxylesterase in human serum samples and could serve as a convenient straightforward one-step fluorescence assay for carboxylesterase.

In summary, we have successfully developed a novel ratiometric fluorescence assay system based on AIE dots as the FRET donors, which can avoid the limitations of the donors consisting of aggregation-caused quenching fluorophores in nanoparticle-based FRET systems. The obvious advantages of employing AIE dots include: first, they exhibit strong emission in the aggregated state, which is beneficial for the nanoparticle-based systems; second, they have better photostability, which is good for applications for a longer period. Also, the assay system features sensitive and selective detection of carboxylesterase in aqueous media and biological milieus. This strategy may provide a new and effective approach for establishing new FRET systems and developing other enzyme assays.

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Table 1 Determination of carboxylesterase (CaE) in serum (50-fold diluted)

Determined endogenous CaE (U L^{-1}) by colorimetry	Determined endogenous CaE (U L^{-1}) by this probe	Added CaE (U L^{-1})	Combined CaE (U L^{-1})	Measured (U L^{-1}) by this probe[c]	Recovery (%)
0.328 ± 0.012[a]	0.344 ± 0.007[b]				104.9
		6.000	6.344	6.001 ± 0.010	94.6
		10.000	10.344	10.365 ± 0.005	100.2
		20.000	20.344	20.649 ± 0.014	101.5
		30.000	30.344	29.980 ± 0.008	98.8

[a] The carboxylesterase level in the 50-fold diluted serum sample is 0.328 U L^{-1}, which means the endogenous carboxylesterase concentration in undiluted serum is *ca.* 0.0164 U mL^{-1}, determined by the colormetric method. [b] The carboxylesterase level in the 50-fold diluted serum sample is 0.344 U L^{-1}, which means the endogenous carboxylesterase concentration in undiluted serum is *ca.* 0.0172 U mL^{-1}, determined by the assay system herein. [c] The data are summarized as mean ± standard deviation (SD).

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