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Rational design of fluorescent light-up probes based on an AIE luminogen for targeted intracellular thiol imaging†

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A water-soluble fluorescent light-up bioprobe based on a luminogen with aggregation-induced emission characteristics was developed for targeted intracellular thiol imaging.

Cellular thiols are essential biomolecules that play a critical role in many biological processes including antioxidant defense, cell signaling and cell proliferation.[1] Among them, glutathione (GSH) is the most abundant (1–10 mM) thiolated tripeptide found within the human cellular system.[2] It is reported that the changes in the cellular GSH level are associated with many health problems such as Alzheimer's disease, leucocyte loss, psoriasis, liver damage and cancer.[3] In addition, GSH levels in cancer cells also affect the effectiveness of chemotherapy.[4] Many intelligent drug or gene delivery systems have been developed by taking advantages of the differences between intracellular and extracellular thiol concentrations in cancer cells.[5] Detection of intracellular thiol levels is thus of high importance for early diagnosis of diseases, evaluation of disease progression and therapy efficiency of new potential drugs.

In recent years, a number of fluorescent sensors have been developed for the detection of free or intracellular thiols.[6] One design strategy is to utilize the strong nucleophilicity of the thiols to react with unsaturated carbon in electrophiles through thiol-addition reactions.[6a] Many of these probes have shown poor water solubility or high background signal with limited biological applications. Another design is based on dual-labeled fluorescent probes, and the signal is generated upon separation of dye–quencher pairs or reducing energy/charge transfer due to the cleavage of the disulfide bond upon exposure to thiols.[6b] Some of these probes have been used for intracellular thiol imaging, but lack cell specificity. In addition, the traditional fluorophores used for these probe designs often show aggregation-caused quenching (ACQ), which reduces their brightness and sensitivity in cellular imaging.[7] Considering the importance of thiols in biological functions, it is highly desirable to develop simple, noninvasive, and specific probes with high signal-to-noise ratios for targeted imaging of intracellular thiol levels.

Aggregation-induced emission (AIE), a unique phenomenon that is exactly opposite to the ACQ effect, has emerged as a powerful and versatile strategy for the design of novel fluorescent probes.[8] Propeller-shaped luminogens, such as tetraphenylethene (TPE) and silole derivatives, are non-emissive when molecularly dissolved but are induced to emit efficiently upon aggregation.[8b] We have rationalized the AIE mechanism as restriction of intramolecular rotations (RIR), which prohibits energy dissipation *via* non-radiative channels, leading to high quantum yields in aggregated states.[8] These advantages make AIE luminogens particularly attractive for biological applications.[8c]

In this contribution, we designed an integrin $\alpha_v\beta_3$ targeted light-up probe for cell specific intracellular thiol imaging. The probe is composed of a targeted cyclic RGD (cRGD) peptide, a highly water soluble peptide with five aspartic acids (Asp, D5), a TPE fluorogen and a thiol-specific cleavable disulfide linker. cRGD exhibits high binding affinity towards $\alpha_v\beta_3$ integrin which is a unique molecular biomarker for early detection and treatment of rapidly growing solid tumors. The probe is highly water soluble and is almost non-fluorescent in aqueous media. The cleavage of the disulfide group by thiols leads to enhanced fluorescence signal output (Scheme 1). This probe has the potential for real-time monitoring of thiol levels in specific tumor cells.

TPE is an iconic AIE fluorogen.[8a] It is non-emissive in the molecularly dissolved state, but shows intense fluorescence in the aggregates. It is known that water solubility is a prerequisite for bioprobes, which is typically achieved by attaching hydrophilic groups to the organic fluorophores. Recently, we have developed AIE luminogen based light-up probes for monitoring of cellular proteins.[9] The first generation of probe design is limited to peptide

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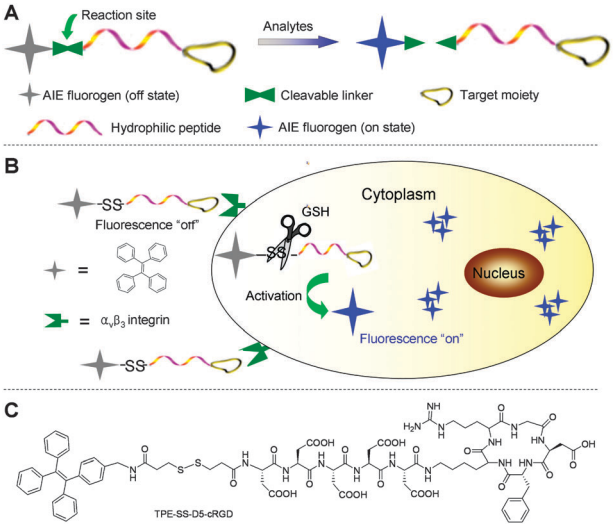

Scheme 1 (A) General probe design strategy and (B) schematic illustration of cRGD targeted imaging of intracellular thiols through $\alpha_v\beta_3$ integrin mediated cellular uptake and cleavage of the disulfide bond to induce fluorescence "turn on". (C) Chemical structure of the probe.

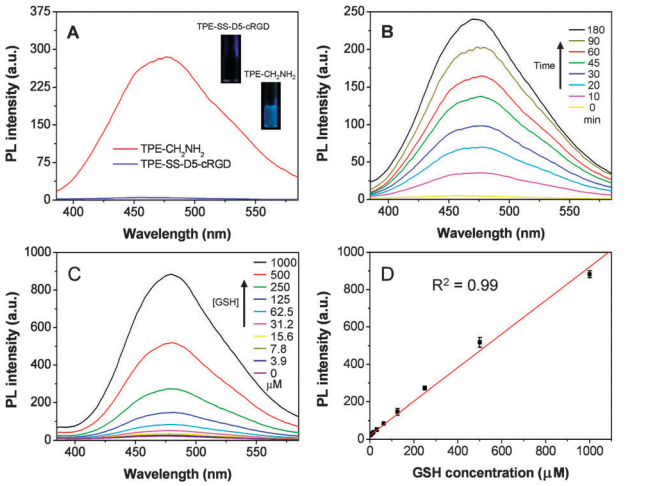

Fig. 1 (A) PL spectra of TPE-CH_2NH_2 and TPE-SS-D5-cRGD in DMSO–PBS (v/v = 1 : 199). Inset: the corresponding photographs taken under illumination of a UV lamp. (B) Time-dependent PL spectra of TPE-SS-D5-cRGD treated with GSH. (C) PL spectra of TPE-SS-D5-cRGD (1.0 mM) in the presence of different concentrations of GSH. (D) Plot of PL intensity at 470 nm *versus* concentrations of GSH (mean ± SD, n = 3).

based recognition elements with high water solubility. To extend the design principle to include a broader range of hydrophobic recognition elements, it is necessary to develop a more general strategy. In this work, a thiol specific cleavable disulfide linker dithiobis(succinimidyl propionate) (DSP) was utilized to conjugate aminated TPE and a cRGD using D5 to ensure good water solubility of the probe. We found that the incorporation of D5 can efficiently transfer TPE into a non-emissive molecular species in aqueous media. Using intracellular thiol imaging as an example, we here report a general platform for designing specific light-up probes for imaging of intracellular molecules or cellular processes.

Amine-functionalized TPE (TPE-CH_2NH_2) was synthesized by reducing azide-TPE (TPE-CH_2N_3) in methanol (Scheme S1 and Fig. S1–S3, ESI†). Asymmetric functionalization of a DSP linker with TPE-CH_2NH_2 and NH_2 terminated D5-cRGD in the presence of *N*,*N*-diisopropylethylamine (DIEA) in anhydrous dimethyl sulfoxide (DMSO) afforded the probe TPE-SS-D5-cRGD in 45% yield (Scheme S2, ESI†). A control probe TPE-SS-D5 with a similar structure but without a cRGD moiety was also synthesized in 49% yield (Scheme S3, ESI†). In addition, a non-activatable control probe TPE-CC-D5 was prepared in 44% yield by using disuccinimidyl suberate to replace DSP in the coupling reaction (Scheme S4, ESI†). NMR and MS characterization confirmed the right structures with high purity of the three probes (Fig. S4–S12, ESI†).

The photoluminescence (PL) spectra of TPE-CH_2NH_2 and TPE-SS-D5-cRGD in DMSO and phosphate buffered saline (PBS, pH = 7.4) mixtures (v/v = 1/199) are shown in Fig. 1A. The hydrophobic TPE-CH_2NH_2 shows intense fluorescence as nanoaggregates with a quantum yield (Φ) of 0.23 ± 0.01 when using quinoline sulfate as the standard.[10] The TPE-SS-D5-cRGD probe is almost non-fluorescent in the same medium (Φ = 0.001), due to the easy intramolecular rotations of the TPE phenyl rings in aqueous media. The significant difference in the PL intensities of TPE-CH_2NH_2 and TPE-SS-D5-cRGD offers opportunity for the probe to be used for specific light-up imaging of thiols. The PL spectrum of TPE-SS-D5-cRGD shows no response to NaCl in the concentration range of 0 to 960 mM (Fig. S13, ESI†). Its PL profile also does not change in the presence of the commonly used cell culture medium, Dulbecco's Modified Eagle Medium (DMEM). The probe maintains an "off" state in the complex environment and thus has great potential to serve as a specific light-up probe with minimum background interference.

To study the response of the probe to free thiols, GSH was chosen as the representative thiol due to its high concentration in the human cellular system.[1] GSH (1 mM) was incubated with 10 μM TPE-SS-D5-cRGD in DMSO–PBS mixtures (v/v = 1/199), and the fluorescence spectra were measured at different time points. As shown in Fig. 1B, the emission intensity of TPE-SS-D5-cRGD increases significantly with time, reaching the maximum within 3 h, which is 68-fold higher than the intrinsic emission of the probe. The TPE-SS-D5 shows a similar time dependent fluorescence increase after incubation with GSH (Fig. S14A, ESI†), while a negligible signal is observed for TPE-CC-D5 (Fig. S14B, ESI†). TPE-SS-D5-cRGD is further demonstrated to respond to GSH under acidic conditions (Fig. S15, ESI†).

Next, we investigated the effect of GSH concentration on the emission of the probe. GSH at different concentrations ranging from 3.9 μM to 1.0 mM was incubated with TPE-SS-D5-cRGD for 3 h, and the corresponding spectra are shown in Fig. 1C. With increasing GSH concentration, the fluorescence is gradually intensified due to the increased amount of TPE aggregates formed in aqueous media. The molecular dissolution of the probe and the aggregate formation of the cleaved products were confirmed by laser light scattering (LLS) measurements. In the aqueous mixture, no LLS signals could be detected from the solution of TPE-SS-D5-cRGD. However, after incubation with GSH, the residual hydrophobic AIE luminogen tends to cluster into aggregates (Fig. S16A, ESI†). The formation of aggregates was further confirmed by AFM (Fig. S16B, ESI†). Under the same experimental conditions, a similar increase in fluorescence intensity is observed for TPE-SS-D5 (Fig. S17A, ESI†), but not for TPE-CC-D5 (Fig. S17B, ESI†). In addition, plotting the PL intensities at

470 nm for TPE-SS-D5-cRGD against the GSH concentration gives a perfect linear line (Fig. 1D), suggesting the possibility of using the probe for GSH quantification with a detection limit of 1.0 μM.

To monitor the GSH-induced fluorescence activation of TPE-SS-D5-cRGD, reverse-phase HPLC and MS analyses were used to monitor the exposure of the probe to GSH. After incubation of TPE-SS-D5-cRGD with GSH for 3 h, the mixture was subjected to HPLC analysis. As shown in Fig. S18 (ESI†), in addition to the TPE-SS-D5-cRGD peak observed at 10.83 min, two new peaks at 10.68 min for GSS-TPE and 11.58 min for TPE-SH are observed and the peaks show mass-to-charge ratios (*m*/*z*) of 755.217 and 472.164 analyzed by IT-TOF, respectively. The fragments of TPE-SH and GSS-TPE tend to aggregate in DMSO–PBS (v/v = 1/199), which show blue fluorescence with quantum yields of 19 ± 1% and 12 ± 1%, respectively, using quinoline sulfate as a reference (Table S1, ESI†). These results clearly demonstrate that the observed GSH-induced fluorescence intensity change of TPE-SS-D5-cRGD is due to cleavage of the disulfide bond, which leads to solubility difference between the probe and the fragment. Further titration of TPE-SS-D5-cRGD with cysteine (Cys), glycine (Gly) and glutamate (Glu), the three amino acids present in GSH, reveals that the fluorescence turn-on is due to the interaction of free thiol in Cys with the disulfide bond (Fig. S19, ESI†).

To explore the capability of TPE-SS-D5-cRGD as a specific bioprobe for monitoring intracellular thiol levels in cancer cells, the probe is incubated with U87-MG human glioblastoma and MCF-7 breast cancer cell lines. The confocal imaging results are shown in Fig. 2. U87-MG cells with overexpressed integrin $\alpha_v\beta_3$ on the cellular membrane were chosen as integrin-positive cancer cells, while breast cancer cells (MCF-7) with a low level of integrin $\alpha_v\beta_3$ expression were used as the negative control. After incubation with TPE-SS-D5-cRGD, a strong blue fluorescence is observed for U87-MG cells (Fig. 2A), whereas for MCF-7 cells only a weak fluorescence signal could be found even after incubation for 6 h (Fig. 2D). In contrast, TPE-SS-D5 displays weak fluorescence intensity with essentially identical behavior for both cell lines (Fig. 2B and E). When U87-MG cells were pretreated with free cRGD prior to TPE-SS-D5-cRGD incubation, weak fluorescence was observed (Fig. S20, ESI†). The marked difference reveals that the selective uptake of TPE-SS-D5-cRGD by U87-MG cells is due to the integrin receptor-mediated process. For the control probe TPE-CC-D5, no detectable fluorescence was observed even after incubation for 6 h (Fig. 2C and F). It should be noted that the probe could also be used for live cell imaging and the images are shown in Fig. S21 (ESI†).

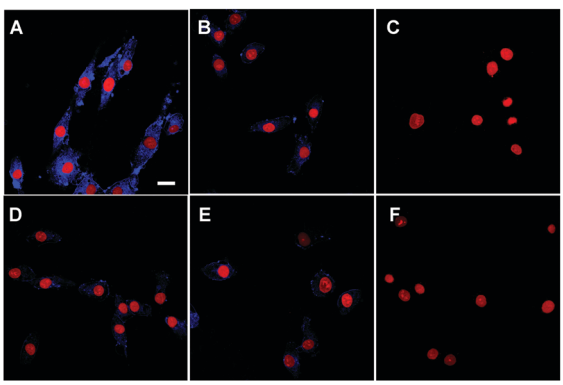

Fig. 2 Confocal microscopy images of U87-MG (A–C) and MCF-7 (D–F) cells after incubation with TPE-SS-D5-cRGD (A, D), TPE-SS-D5 (B, E) and TPE-CC-D5 (C, F). The nuclei were stained with propidium iodide. All images share the same scale bar (20 μm).

To provide further evidence for thiol-induced disulfide bond cleavage as the trigger of fluorescence turn-on, the U87-MG cells were also pretreated with buthionine sulfoximine (BSO) before incubation with TPE-SS-D5-cRGD. BSO is an inhibitor of g-glutamylcysteine synthetase which can prevent the cells from synthesizing GSH.[11] As shown in Fig. S22 (ESI†), the fluorescence of TPE-SS-D5-cRGD treated U87-MG cells decreases as the concentration of BSO increases from 25 to 100 μM. The significantly reduced fluorescence shown in Fig. S22C (ESI†) as compared to that shown in Fig. 2A reveals that the probe fluorescence is directly related to GSH concentration in the cells. These results indicate that despite the existence of other free thiols in cells, TPE-SS-D5-cRGD could be used as an indicator for intracellular GSH imaging. *In vitro* cytotoxicity studies also show that the TPE-SS-D5-cRGD probe is biocompatible (Fig. S23, ESI†).

In conclusion, we report the synthesis and biological applications of a light-up GSH responsive AIE probe. Thanks to the unique nature of the AIE luminogen, the probe is non-fluorescent in aqueous media but becomes highly emissive when cleaved by thiols. The probe enables light-up monitoring of free thiols in solution and in cells with a high signal-to-noise ratio. The cRGD functionalized peptide allows for selective targeting of $\alpha_v\beta_3$ integrin of many angiogenic cancers using U87-MG as an example, which opens a new opportunity for specific intracellular thiol imaging. Our AIE probe strategy can be generalized to perform various tasks by simply changing the disulfide groups with other cleavable linkers in chemical biology. Further development of AIE fluorogens with long wavelength emission will facilitate the development of specific bioprobes for *in vivo* applications. The probe design thus opens new avenues for the construction of various selective targeting probes for diagnosis, imaging and drug screening applications.

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