Light-Up Probe for Targeted and Activatable Photodynamic Therapy with Real-Time In Situ Reporting of Sensitizer Activation and Therapeutic Responses

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1. Introduction

Targeted delivery, accurate assessment of active therapeutic agents, and in situ reporting of their therapeutic effects are of high importance in cancer therapy.¹ The capability to visualize the whole process will undoubtedly provide more useful information to further optimize and advance the therapy. However, our capability is still limited in answering precisely when, where and how the therapeutic agents are delivered as well as what is their therapeutic effect. To realize targeted delivery, drug conjugates or drug encapsulated carriers with surface targeting moieties have been developed.² Fluorescent dyes and quantum dots have been further used to label these carriers or drugs to study their targeting effect and biodistribution.³,⁴ Recently, fluorophore and drug conjugates with tumor-responsive linkers have been developed for real-time monitoring of activated drug concentration in cancer cells.⁵–¹¹ In addition, by incorporation of apoptosis sensors or apoptotic markers (e.g., ethidium) into prodrugs or nanoparticles, in situ monitoring of the drug therapeutic effect has also been realized.¹²,¹³ Despite of the great efforts made toward smart probe design for traceable cancer therapy, most of the approaches can only monitor one of the processes. It remains challenge to develop a single molecular probe that is able to simultaneously detect, image, ablate cancer cells, and report the therapeutic response in situ.

Among the various therapeutic drugs under study, photosensitizer (PS) drugs have recently attracted increasing attention because photodynamic therapy (PDT) does not acquire drug resistance and it can be controlled with light irradiation.¹⁴,¹⁵ PDT is based on the concept that PSs generate cytotoxic reactive oxygen species (ROS) upon light irradiation to induce cell death which is a powerful strategy for cancer treatment.¹⁴,¹⁵ Over the years, although some fluorescent probes have been developed for ROS detection,¹⁶–¹⁸ it remains challenge to monitor ROS generation in real-time and in situ in living systems due to the extremely short lifetime (<40 ns)
and small radius of action (<20 nm) of ROS. There is thus very limited strategies reported to provide in situ monitoring of the therapeutic effect of PDT. In addition, the currently available PSs frequently cause unwanted normal cell death due to their intrinsic phototoxicity and lack of selectivity for cancer cells. As a consequence, targeted and/or activatable PSs with high therapeutic efficiency to cancer cells but minimized side effects to normal cells are subsequently reported. However, the integration of all the desired properties into a single molecular probe that is able to simultaneously target and image cancer cells, report the PS activation and predict the therapeutic response in situ remains a grand challenge.

To visualize multiple processes in cancer therapy, fluorophores exhibiting different fluorescence upon a single wavelength excitation are highly desirable as they can minimize the complexity of fluorescence imaging by offering simultaneous detection. The design principle of traditional fluorescent probes often requires a pair of fluorescent dyes (energy donor/acceptor or donor/quencher) or relies on photoinduced electron transfer (PET) mechanism to visualize a single biological event, which makes it difficult to develop one molecular probe for monitoring multiple processes. This calls for the development of multifunctional materials, where a single molecule can be used to report an event or even also born with therapeutic functions. It is fortunate that the recent development of fluorogens with aggregation-induced emission characteristics (AIEgens) offers such an opportunity. AIEgens show almost no fluorescence as molecular species, but bright fluorescence in aggregate state due to restriction of intramolecular motions and prohibition of energy dissipation via nonradiative channels. Taking advantages of the unique properties of AIEgens, we have developed light-up probes without incorporation of any quencher moiety or energy transfer pairs. More recently, we and other groups also reported functional AIEgens with tunable emissions, and demonstrated that they could be used as potential chemo-drugs or PSs for chemotherapy and PDT.

In this contribution, we developed an AIEgen based probe, namely TPETP-SS-DEVD-TPS-cRGD (Figure 1), for targeted imaging, activatable and traceable cancer cell PDT with additional capacities of real-time self-reporting of PS activation and therapeutic responses upon a single wavelength excitation. Tetraphenylethene-thiophene (TPETP) is a red-emissive AIEgen with photosensitization property, while tetraphenylsilole (TPS) is an iconic green emissive AIEgen and both could be excited with a 405 nm laser to yield distinct emission peaks. As ROS could cause cancer cell death through apoptosis pathway within a short period of time and caspases-3/7 are key mediators of cell apoptosis, a caspase-3/-7 responsive peptide substrate DEVD based apoptosis sensor is incorporated into the probe. In addition, c-RGD and –S–S– are used as the targeting ligand and linker to realize dual-targeted activatable PDT. Upon c-RGD mediated cellular uptake, the –S–S– bond can be cleaved by glutathione (GSH) to yield red fluorescence turn-on of the PS and release the apoptosis sensor. Consequently, the activated caspases cleave the DEVD substrate on the probe and release hydrophobic TPS residue with intense green fluorescence to report the therapeutic effect. As compared to the recent report of a lysotracker and PS coencapsulated nanoparticles for monitoring of the therapeutic responses of PDT via a fluorescence-off signal, our distinct fluorescence turn-on signal is more desirable in complicated biological systems.

Figure 1. Schematic illustration of the dual-targeted probe for real-time and in situ monitoring of PS activation and the therapeutic responses. A) The chemical structure of the probe TPETP-SS-DEVD-TPS-cRGD; B) The probe functions in four steps: (1) receptor mediated endocytosis of the probe; (2) cleavage of the disulfide bond through intracellular reduction by glutathione to release the nonemissive apoptosis sensor and the PS with red fluorescence; (3) upon light irradiation, ROS generation to induce cell apoptosis and activate caspase enzymes; (4) cleavage of the DEVD substrate by the activated caspases on the apoptosis sensor to result in green fluorescence turn-on. The red fluorescence of the TPETP residue can be used for image-guided photodynamic therapy while the green fluorescence of TPS can be used for the therapeutic response imaging. The structures of TPETP and TPS residues are shown in the Scheme S1, Supporting Information.
2. Results and Discussion

2.1. Design Principle of the AIEgen PS and the Probe

The design principle of the AIEgen PS is based on the following two considerations: AIE characteristics with efficient ROS generation, and the same excitation but distinct emission wavelengths from those of TPS. Build upon tetraphenylenylene (TPE), one of the most commonly used AIEgens with UV absorption and weak ROS generation,[42] introducing dicyanovinyl group as an electron acceptor (A) and methoxy group as an electron donor (D) into a TPE core forms a D–π–A structure, which is expected to yield a new AIEgen with red shifted absorption/emission maxima. In addition, the D–π–A structure can lead to small $\Delta E_{S1,T1}$ (energy gap between the lowest singlet excited state ($S_1$) and the lowest triplet excited state ($T_1$) states), which is favorable for intersystem crossing (ISC) to yield efficient ROS generation.[43] The molecular structures, HOMO and LUMO distribution and $\Delta E_{S1,T1}$ values of TPE (1.22 eV), TPS (1.27 eV) and TPETP (0.29 eV) are summarized in Figure 2. TPETP exhibits a much smaller $\Delta E_{S1,T1}$ compared with TPE, suggesting a potentially high ISC rate and thus possibly efficient ROS generation upon light irradiation.[44] The probe design principle is illustrated in Figure 1. The probe is composed of five components: (1) a red emissive TPETP to serve as a visualizing agent and a PS; (2) a disulfide linker, which can be cleaved by GSH overexpressed in cancer cells; (3) a highly hydrophilic DEVD substrate that can be specifically cleaved by caspase-3/-7; (4) a green emissive TPS molecule to serve as the signal reporter for the apoptosis sensor; and (5) a hydrophilic DEVD substrate that can be specifically cleaved by GSH overexpressed in cancer cells. The probe thus offers a unique opportunity for targeted and activated cancer therapy with a single wavelength excitation, the probe thus offers a real-time reporting of PS activation and therapeutic responses.

2.2. Synthesis of TPETP-NH$_2$ and Identification of the Isomer

The two isomers of TPETP-NH$_2$ (cis-TPETP-NH$_2$ and trans-TPETP-NH$_2$) were synthesized as depicted in Figure 3. Briefly, compound 1 was treated with n-butyllithium, trimethyl borate, and diluted HCl to yield compound 2 with boronic acid functionality, which underwent palladium-catalyzed coupling with 2-thiophencarbonyl chloride to yield compound 3. Compound 3 was further treated with TiCl$_4$ and malononitrile to generate compound 4 with a dicyanovinyl group, which was subsequently treated with BBr$_3$ to yield compound 5 with one free hydroxyl group. The reaction between compound 5 and 3-(boc-amino) propyl bromide furnished compound 6, which was reacted with trifluoroacetic acid (TFA) to deprotect the Boc group and yield the mixture of cis and trans isomers of TPETP-NH$_2$. The compounds 1–6 were characterized by NMR and mass spectroscopy (Figures S1–S4, Supporting Information). The two isomers of TPETP-NH$_2$ were separated by preparative high-performance liquid chromatography (HPLC) as red powders after freeze drying (Figures S5 and S6, Supporting Information). After measuring $^1$H COSY and NOESY NMR for the major isomer, all the peaks with different chemical shifts were assigned (Figures S7–S9, Supporting Information). As shown in the NOESY spectrum (Figure 4), the H-3&5 have strong correlations with the H-21&25, which suggest that the rings A and C are on the same side. Since there was no significant difference in fluorescence and ROS generation ability between the two isomers (Figure S10A,B, Supporting Information), the major isomer of cis-TPETP-NH$_2$ was used for the subsequent modification.

2.3. Synthesis of the Probe

Azide-functionalized TPS (TPS-2N$_3$) was prepared according to our previous report.[45] The double “click” reactions between TPS-2N$_3$ and alkyne-functionalized cRGD as well as alkyne-functionalized DEVD were catalyzed by CuSO$_4$/sodium ascorbate in DMSO/water mixture ($v/v = 10/1$), which afforded the apoptosis sensor DEVD-TPS-cRGD in 42% yield after HPLC purification. Furthermore, asymmetric functionalization of dithiobis(succinimidyl propionate) (DSP) with TPETP-NH$_2$ and the DEVD-TPS-cRGD afforded the target probe, TPETP-SS-DEVD-TPS-cRGD in 32% yield as red powders. The HPLC and mass spectra confirmed the right structures of the probe and intermediates with high purity (Figures S11–S14, Supporting Information).
2.4. Optical Properties of the Probe and its Activation by GSH

The absorption and emission spectra of \textit{cis}-TPETP-NH$_2$ in DMSO/water mixtures (v/v = 1/199) are shown in Figure 5A. The UV–vis absorption of \textit{cis}-TPETP-NH$_2$ covers the range of 400–500 nm with a shoulder peak at 430 nm. The PL spectrum is ranged from 550 to 850 nm with an emission maximum at 650 nm and the quantum yield ($\Phi$) is determined to be 0.13 using 4-(dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)-4$H$-pyran (DCM) as the reference ($\Phi$ = 0.43 in water). To study whether \textit{cis}-TPETP-NH$_2$ retains the AIE property, its PL spectra in DMSO/water mixtures were monitored at the same compound concentration but with different water fractions ($f_w$). As shown in Figure 5B, \textit{cis}-TPETP-NH$_2$ is almost nonemissive in its benign solvent DMSO. This is attributed to the free motions of \textit{cis}-TPETP-NH$_2$ in molecularly dissolved state. The fluorescence of \textit{cis}-TPETP-NH$_2$ increases quickly when the $f_w$ is higher than 50 vol%. At $f_w$ = 99 vol%, the fluorescence intensity of \textit{cis}-TPETP-NH$_2$ is 120-fold higher than its intrinsic fluorescence in pure DMSO. The PL results demonstrate that \textit{cis}-TPETP-NH$_2$ is AIE-active.

The PL spectra of \textit{cis}-TPETP-NH$_2$ and the probe in DMSO/PBS mixtures (v/v = 1/199) are shown in Figure 5C. \textit{cis}-TPETP-NH$_2$ shows intense red fluorescence. However, the probe TPETP-SS-DEVD-TPS-cRGD is almost nonemissive because of its good water-solubility due to the attached hydrophilic DEVD and cRGD peptides. The significant difference in the PL spectra of the probe and \textit{cis}-TPETP-NH$_2$ inspired us to develop a cancer cell specific light-up probe based on cleavage of the disulfide bond by intracellular thiols. The fluorescence response of the probe toward GSH was studied first. As shown in Figure 5D, the red fluorescence of TPETP residue is increased steadily over 2 h upon addition of GSH to the probe solution. The fluorescence intensity reaches a plateau after 90 min incubation, which is 14-fold higher than the original intensity of the probe. The gradual enhancement of red fluorescence is ascribed to the increased amount of TPETP residue formed by cleavage of disulfide bond of the probe. Furthermore, the formation of TPETP aggregates was confirmed by laser light scattering (LLS) measurements. No LLS signals could be detected from the probe initially, while aggregates with an average diameter of 148 ± 12 nm were detected after GSH treatment (Figure S15A, Supporting Information). Subsequently, the probe at different concentrations was incubated with GSH for 90 min and the corresponding fluorescence changes were recorded. As shown
in Figure 5E, the plot of the PL intensities at 650 nm shows a linear relationship with the probe concentrations ($R^2 = 0.98$), indicating that the red fluorescence intensity could be used to quantify the amount of activated PS. The selectivity of the probe was subsequently studied, and the PL results revealed that the red fluorescence is only turned-on in the presence of reducing agents such as GSH and ascorbic acid while other bioacids and proteins show negligible fluorescence changes (Figure 5F). These results further support that the increase of red fluorescence in Figure 5D is due to the cleavage of the disulfide bond by the reducing agents.

2.5. Caspase-3/-7 Activation of the Released Apoptosis Sensor

The absorption and emission spectra of TPS-2N$_3$ in DMSO/PBS (v/v = 1/199) buffer are shown in Figure 6A. TPS-2N$_3$ has absorption and emission maxima at 365 and 480 nm, respectively. The quantum yield ($\Phi$) of TPS-2N$_3$ in DMSO/PBS (v/v = 1/199) is determined to be 0.28 using quinoline sulfate as the reference ($\Phi = 0.54$ in 0.5 M $\text{H}_2\text{SO}_4$). The ROS generation from the TPETP residue was also evaluated by measuring the absorbance of the solution containing the GSH pretreated probe and a ROS indicator, 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA), in DMSO/PBS mixtures (v/v = 1/199) under light irradiation. As depicted in Figure S10C, Supporting Information, the absorption peaks of ABDA at 358, 378, and 400 nm decrease gradually upon light irradiation, as a result of fast reaction between the ROS generated by the TPETP residue and the anthracene moiety in ABDA. After 12 min irradiation, the absorbance at 400 nm is decreased to 22% of its original value. When vitamin C (a well-known ROS scavenger) is added, only 6% of the original ABDA absorbance is decreased under the same treatment, further confirming that ROS is generated by the TPETP residue upon light irradiation.
green fluorescence (the broad emission above 600 nm is attributed to the TPETP residue), indicating that the release of the apoptosis sensor alone will not yield TPS fluorescence. However, a quick green fluorescence increase is observed when the GSH-pretreated probe (10^{-6} M) is further treated with caspase-3/-7 due to the release and aggregation of the hydrophobic TPS residue (Figure 6D), confirmed by LLS results (Figure S15B, Supporting Information). The TPS residue fluorescence reaches plateau after 60 min treatment of caspase-3 (100 \times 10^{-12} M), which is 18-fold higher than the intrinsic fluorescence of the GSH-pretreated probe TPETP-SS-DEVD-TPS-cRGD (Figure 6D) upon excitation at 365 nm. In addition, the green fluorescence enhancement is prohibited in the presence of a highly specific caspase-3/-7 inhibitor 5-[(S)-(+)-2-(methoxymethyl)pyrrolidino]sulfonylisatin. \[47\] It is important to note that the fluorescence of GSH-pretreated probe at 480 nm intensifies upon incubation with increased concentrations of caspase-3 (Figure 6E). The plot of the PL intensities at 480 nm against caspase-3 concentrations gives a linear fit (R^2 = 0.97), suggesting that it is possible to semiquantify the concentration of caspase-3 through PL intensity changes. The detection limit of caspase-3 is 2.3 \times 10^{-12} M estimated by three times standard deviation (3\sigma) method.

The selectivity of the apoptosis sensor DEVD-TPS-cRGD was evaluated by incubating the GSH-pretreated probe with pepsin, lysozyme, bovine serum albumin (BSA), and other caspases. As shown in Figure 6F, only caspase-3/-7 treated groups trigger fluorescence of the apoptosis sensor, indicating that the DEVD substrate is specifically cleaved by caspase-3/-7 when the cells undergo apoptosis. Since there are many kinds of enzymes in the cells, we also incubated the probe with cellular lysates of untreated and apoptotic (induced with 2 \times 10^{-6} M staurosporine, STS) MDA-MB-231 cancer cells. \[48\] The fluorescence intensities at 480 and 650 nm were monitored after different periods of incubation. As shown in Figure S16, Supporting Information, the fluorescence intensities at 650 nm increase quickly in both the untreated and apoptotic cells confirming that the intracellular GSH can cleave the disulfide bond. On the other hand, the fluorescence enhancement at 480 nm is only observed when the probe is incubated with the apoptotic cell lysate.

2.6. Monitoring of Intracellular PS Release

To demonstrate the light-up imaging of specific \(\alpha_\beta\) integrin overexpressed cancer cells, the probe was incubated with \(\alpha_\beta\) integrin overexpressed MDA-MB-231 breast cancer cells and low \(\alpha_\beta\) integrin expressed MCF-7 breast cancer cells as well as 293T normal cells. \[49\] As shown in Figure 7, the red fluorescence from TPETP residues in MDA-MB-231 cells intensifies gradually with the increase of incubation time. In addition, the red fluorescence in MDA-MB-231 is much stronger than those in MCF-7 and 293T cells under the identical conditions. It is important to note that free TPETP can also be uptaken by
MDA-MB-231 cells to yield strong red fluorescence (Figure 7H), and the fluorescence pattern is similar to that in Figure 7E. When MDA-MB-231 cells are pretreated with free cRGD prior to probe incubation, the red fluorescence in the cells is dramatically reduced. Similarly, the MDA-MB-231 cells show much weaker red fluorescence when the cells are treated with g-glutamylcysteine synthetase buthionine sulfoximine (BSO), which could inhibit the cells to produce GSH. Moreover, flow cytometry analysis (Figure S17, Supporting Information) is used to further evaluate the probe specificity and the results are in well accordance with the confocal images shown in Figure 7. These results clearly demonstrate that the probe is specifically internalized by MDA-MB-231 cells through receptor mediated endocytosis and the red fluorescence is turned on by intracellular GSH, which can be used for PS activation monitoring and specific cancer cell imaging.

2.7. Imaging of Cell Apoptosis Induced by PDT

The intracellular ROS generation upon light irradiation in MDA-MB-231 cells was first evaluated by using 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) as the indicator (Figure S18, Supporting Information). To explore the capability of using DEVD-TPS-cRGD as an apoptosis sensor, the MDA-MB-231 cells were pretreated with cis-TPETP-NH$_2$ (10 × 10$^{-6}$ M) with light irradiation (1 min) or STS (1 × 10$^{-6}$ M), then the cells were further incubated with DEVD-TPS-cRGD for 4 h. As shown in Figure S19, Supporting Information, strong green fluorescence of the TPS residue is observed in both STS and cis-TPETP-NH$_2$ treated MDA-MB-231 cells with light irradiation for the latter, indicating that apoptosis occurs in both cases. It should be noted that the DEVD-TPS-cRGD itself is of low cytotoxicity (Figure S20, Supporting Information), which does not induce cell apoptosis.

To further explore the possibility of the probe for PDT and in situ apoptosis imaging in αvβ3 integrin rich cancer cells, we incubated the probe with MDA-MB-231, MCF-7, and 293T cells for 4 h with light irradiation. The fluorescence changes of TPS in cells were monitored by confocal images. As shown in Figure 8, the green fluorescence of TPS residue in probe incubated MDA-MB-231 cells increases gradually with time when the cells are exposed to light irradiation. Together with that shown in Figure S16, Supporting Information, these results clearly indicate that the ROS generation of TPETP residue upon light irradiation can cause cell apoptosis and activate caspase-3/-7 to cleave the DEVD substrate. This is further confirmed by TPETP and cRGD-TPS-DEVDP coincubated MDA-MB-231 cells, which show strong green fluorescence after light irradiation. On the contrary, only a weak fluorescent signal is detected in MCF-7 or 293T cells for more than 4 h of light irradiation. When MDA-MB-231 cells were pretreated with free cRGD or vitamin C prior to the probe incubation, the cells showed much weaker green fluorescence. This is because the free cRGD competes with the αvβ3 integrin receptor to the probe while vitamin C serves as a ROS scavenger to consume the ROS generated by the activated PS.

2.8. Imaging of Cell Apoptosis

To further confirm that the probe can image cell apoptosis, the probe incubated MDA-MB-231 cells after light irradiation were stained with anticaspase-3 primary antibody and a Texas Red-labeled secondary antibody. As shown in Figure 9A, the green fluorescence of the TPS residue and the red immunofluorescence signals from Texas Red are overlapped with high coefficient. When the cells are incubated with a caspase-3/7 inhibitor of S-(2-(methoxymethyl)pyrrolidino)sulfonylisatin, followed by light irradiation, the green fluorescence of TPS residue is greatly reduced and only red fluorescence of Texas Red remains (Figure 9B). Overall, these results demonstrate that the ROS generation of TPETP residue can induce cell apoptosis and the activation of the caspase-3/7 has led to the green fluorescence turn-on of TPS residue, which can be used for self-reporting of cell apoptosis in situ.

2.9. Cell Death Prediction

The CytoTox-Fluor cytotoxicity assay was used to evaluate the correlation between cell viability and apoptosis after cell
incubation with the probe and light irradiation. The CytoTox-Fluor cytotoxicity assay can evaluate the cell viability using a fluorogenic peptide substrate (bis-alanyl-alanyl-phenylanlanyl-rhodamine 110; bis-AAF-R110). We studied the correlation between the R110 fluorescence intensity evaluated by the assay and the TPS residue fluorescence of the probe in MDA-MB-231 cells after light irradiation for different period of time. After the cells were incubated with the probe for 4 h and exposed to light irradiation for different time durations, the fluorescence intensities of R110 and the TPS residue were recorded by a microplate reader. As shown in Figure 10, the fluorescence intensity of R110 after different time of light irradiation correlates well with the apoptosis induced TPS residue fluorescence change, suggesting that the probe is capable of predicting the PDT-induced cell viability by measuring the TPS residue fluorescence intensity noninvasively. Therefore, the probe can be used to report its therapeutic response in situ.

3. Conclusions

We developed a multifunctional probe based on two AIEgens with distinguishable emissions for targeted cancer cell ablation and simultaneous real-time reporting of PS activation and therapeutic responses upon a single wavelength excitation. The probe itself is almost nonemissive in aqueous media due to the attachment of hydrophilic peptides, and the molecularly dissolved state facilitates free rotation of the phenyl rings contained in both AIEgens. Upon cRGD-mediated cellular uptake, the red fluorescence of TPETP residue is turned on as a result of the cleavage of the disulfide group by intracellular GSH, which has been used for cellular imaging and monitoring of the PS activation. Upon image-guided light illumination, the green fluorescence of TPS residue is generated by apoptosis induced caspase-3/-7 activation, which is used for monitoring of the cell apoptosis and evaluation of the therapeutic responses. As compared with the
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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Figure 10. Cytotoxicity fluorescence intensity monitored using CytoToxFluor cytotoxicity assay and the intrinsic TPS residue fluorescence intensity change of MDA-MB-231 cells upon treatment with the probe (10 × 10^−6 M) for 4 h with different durations of light irradiation.

Previously reported molecular probes that can only monitor a single process of drug activation or therapeutic response, simultaneous monitoring of the whole processes is able to provide a clearer picture for further improved cancer therapy. We anticipate that the integrated probe design will offer new opportunities for cancer therapy and early evaluation of the therapeutic response will help to forecast specific therapeutic regimes for individual patient to help realize the ultimate goal of personalized medicine. Based on the proof-of-concept probe design in this work, further development of new AIEgens through molecular design with longer absorption and emission wavelengths for direct in vivo application is our next goal.

Supporting Information

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