A smart perylene derived photosensitizer for lysosome-targeted and self-assessed photodynamic therapy†

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A perylene probe PC4 is explored as a smart lysosome-targeted photosensitizer. It can efficiently kill cancer cells and also mark dead cells with bright fluorescence emission in nuclei for real-time monitoring and assessing of the photodynamic therapeutic efficiency.

With the increase of the mortality rate caused by cancer, considerable efforts have been devoted to seeking effective treatment.1 In recent years, photodynamic therapy (PDT) has attracted much attention as a non-invasive medical method to treat cancer.2 The general procedure of PDT involves the use of a dye known as a photosensitizer (PS) and the illumination with light of appropriate wavelength. In the absence of light, the PS is non-toxic towards cancer cells. With light illumination, the PS can absorb photon energy and transfer its energy to generate reactive oxygen species (ROS), mainly singlet oxygen (1O2).3 The generated 1O2 molecules consequently induce damage and cause cell death. Because of the short half-life (<0.04 μs) and action distance (<0.02 μm) in the biological system,4 the photo damage caused by 1O2 is quite close to the PS location. Thus the intracellular location of the PS is of vital importance in determining the therapeutic efficiency.

A lysosome is a dynamic organelle containing a variety of hydrolytic enzymes and serves as an intracellular digestive system.5 Its function relies on lysosomal membrane integrity. Disruption of the integrity can cause permeabilization, which can initiate cell death in various pathways.6 Therefore, lysosomes are emerging as an attractive target for PDT agents,7 and the search for PSs specifically targeting towards lysosomes is of great importance.

When exposed to light, a PS can generate toxic 1O2, and it may also emit fluorescent light due to relaxation from the excited state to the ground state. A fluorescent PS, for photosensitizer fluorescence detection (PFD), is of significant use for effective PDT treatment.8 Fluorescent PSs can facilitate the determination of intracellular location and the degree of uptake of the photosensitizer.7 And if cancer cells retain the PS, they will be lighted up, and thus provide an imaging guide for the therapy.9 In situ and real-time monitoring and assessing the therapeutic effects may then be realized.10,11 These characteristics of the fluorescent PSs can provide a facile way to develop a smart, intuitive, and convenient therapy. However, to assess the therapeutic effect, the current major focus is the use of fluorescence emission intensity or life time changes, which are not as apparent as the fluorescent PS intracellular location changes. And to meet the need for real time monitoring, good photostability and fluorescence quantum yield are also required. Hence, the search for target directed, stable and brightly fluorescent PSs is highly demanded.

Perylene derivatives containing a rigid and planar aromatic scaffold are known for their outstanding chemical and photostability for many years. Perylene derived PSs have been reported in the past.12,13 And highly fluorescent perylene imaging agents used as DNA intercalators and membrane dyes are also reported.14,15 However, the integration of a PS and an imaging dye into a single perylene probe to our knowledge is not reported.

Herein, a cationic perylene probe PC4 was explored as a photosensitizer (Scheme 1). It exhibits excellent water solubility, high chemical and photostability, and effective 1O2 generation capability. It can specifically target lysosomes with negligible dark toxicity (IC50 > 500 μM), and it shows excellent photo-toxicity (IC50 = 2.8 μM) upon low dose light irradiation (4.2 J cm−2). More importantly, the movement of PC4 from lysosomes in live cells to the nuclei in dead cells provides a facile and efficient way to monitor and assess the treatment effect. To the best of our knowledge, this is the first perylene probe reported as a smart lysosome-targeted photosensitizer and PDT effect imaging/assessing agent. Given its excellent properties, PC4 shows high potential for future PDT theranostic applications.

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Probe PC4 was prepared via a simple synthetic procedure with high yield.16 Four cationic quaternary ammonium functional groups were attached to the perylene core structure which gave the probe good water solubility (> 5 mM) and enhanced binding affinity to the negatively charged cell membrane. The photophysical properties of PC4 were measured. As depicted in Fig. S1a (ESI†), PC4 shows an absorption maximum at 470 nm and an emission maximum at 490 nm. The molar absorption coefficient \( (\epsilon) \) was calculated to be 35,000 cm\(^{-1}\) mol\(^{-1}\), which is an excellent feature for a PDT agent.17 The photostability was investigated. PC4 was subjected to continuous irradiation for 20 min, and the emission intensity remained unchanged (Fig. S1b, ESI†). Thus PC4 shows excellent photostability and very little photo-bleaching effect. In addition, as the lipophilicity/hydrophilicity of the probe is related to the cellular uptake efficiency, partition of PC4 in an octanol/water solvent mixture was investigated.7 Direct visual inspection clearly showed that the bright emission was only observed in the aqueous phase, and the emission intensity differences in the two phases indicate that PC4 is highly hydrophilic (Fig. S2, ESI†).

The capability to generate ROS is an important parameter for a PDT PS. Using diphenylisobenzofuran (DPBF) as an indicator, the generation of ROS was detected by monitoring the absorption changes of DPBF at 412 nm.18 As demonstrated in Fig. 1a and b, the time-dependent degradation of DPBF was only observed in the presence of both PC4 and light irradiation. The results confirmed the generation of ROS by PC4. Electron spin resonance (ESR) spectroscopy was used to verify the generation of ROS species.20 The \( ^1\text{O}_2 \) and \( \text{OH}^\bullet \) were used as \( ^1\text{O}_2 \) and \( \text{OH}^\bullet \) traps, respectively.19 As illustrated in Fig. 1c, characteristic SO induced spectral signals were detected, but no \( \text{OH}^\bullet \) signal was observed. The intensity of the \( ^1\text{O}_2 \) signal increased at prolonged irradiation time, again no \( \text{OH}^\bullet \) signal was detected (Fig. S3, ESI†). To further confirm the generation of \( ^1\text{O}_2 \), the NIR fluorescence spectrum of \( ^1\text{O}_2 \) was recorded (Fig. 1d). An emission band with a band maximum at 1270 nm was observed, which is consistent with the reported \( ^1\text{O}_2 \) spectra.20 The \( ^1\text{O}_2 \) quantum yield of 0.13 was obtained using an indirect method with \( \text{Ru(bpy)}_3^2\) as the standard (Fig. S4, ESI†).

Inspired by the good photostability and bright fluorescence emission of PC4, cellular uptake and the intracellular location were investigated using human lung cancer A549 cells and confocal laser scanning microscopy (CLSM). As shown in Fig. S5 (ESI†), after 30 min of incubation, the cells were clearly lighted up by PC4. When the incubation time was prolonged, more fluorescent spots were observed, suggesting the time-dependent uptake behaviour. To examine the subcellular location of PC4, the cells were co-incubated with Hoechst 33342, Mitochondria Tracker@Red (MTR) and Lysosome Tracker@Deep Red (LTDR), respectively. As illustrated in Fig. 2, the emission signal of PC4 inside the cells overlapped well with LTDR, and a high Pearson's coefficient value of 0.83 was obtained.7,11 And negligible co-localization with MTR and Hoechst 33342 was observed. These results indicate that PC4 can specifically target lysosomes. In addition, no emission signal of PC4 in A549 cells at low temperature (4 °C) was observed (Fig. S6, ESI†), suggesting that the uptake took an energy-dependent endocytosis pathway.21 Based on these results, it can be concluded that PC4 first bound to the cell membrane, then was engulfed by the endosome, and finally reached the lysosome.6,22
To assess whether PC4 is toxic towards cells in the dark, the cytotoxicity of PC4 against A549 cells was investigated using the traditional MTT assay. As shown in Fig. 3a, cell viability remained > 80% after incubation with various concentrations of PC4 (50–500 μM) for 24 h in the dark, which clearly indicates that PC4 showed little dark toxicity. Furthermore, remarkably increased cytotoxicity was observed when the cells were irradiated with 450 nm light (4.2 J cm⁻²) in the presence of PC4, and a light IC₅₀ value of 2.8 μM was estimated. It is worth noting that the traditional photosensitizers PpIX (dark IC₅₀ = 1.8 μM, light IC₅₀ = 0.9 μM) and Ru(bpy)₃²⁺ (dark IC₅₀ > 500 μM, light IC₅₀ = 150 μM) show various dark and light toxicity. Compared with these photosensitizers, PC4 shows much lower dark toxicity than PpIX and much higher light toxicity than Ru(bpy)₃²⁺, indicating that PC4 can serve as an excellent photosensitizer for PDT. It is also interesting to note that the ¹O₂ quantum yields of PpIX (0.45) and Ru(bpy)₃²⁺ (0.73) are considerably higher than that of PC4. The results thus indicate that besides ¹O₂ quantum yield, the photodynamic efficiency is also strongly influenced by several other factors including the intracellular location, cell uptake efficiency, etc. Cell morphology changes after PDT in the presence of PC4 were studied (Fig. S7, ESI†). The cell membrane was obviously ruptured and bright green fluorescence emission was observed in the nuclei. There is an interesting phenomenon that the intracellular location of PC4 drastically changed from the lysosome to nuclei during the photodynamic treatment. In contrast, the cells treated with Ru(bpy)₃²⁺ and PpIX showed no apparent morphology changes, and a dim fluorescence signal due to the low fluorescence quantum yield of the PSSs was observed. In addition, the toxicity of PC4 towards other cancer cell lines was also tested. As shown in Fig. S8 (ESI†), PC4 showed neglected toxicity towards both HepG-2 and MCF-7 cells in the dark, whilst high toxicity was observed after light irradiation (450 nm, 4.2 J cm⁻²). Light IC₅₀ values of 2.2 and 4.1 μM for HepG-2 and MCF-7 cells, respectively, were obtained. The results confirm that PC4 is an effective photosensitizer for various cancer cells.

On the basis of high phototoxicity and intracellular location changes during the PDT treatment, it was suggested that PC4 can be utilized for the real-time monitoring of the PDT process and assessing of the treatment efficiency. Real-time cellular location changes of PC4 during the PDT treatment were monitored by CLSM (Movies S1 and S2, ESI†). Before continuous light irradiation (488 nm, 50 mW, 6%), PC4 (8 μM) was specifically located in the lysosome with a bright green emission signal and gradually moved from the lysosome to nuclei in the presence of continuous light irradiation. And lots of blebs formed as well, indicating that the cell integrity was totally disrupted. In comparison, cells without incubation with PC4 were also monitored under continuous light irradiation (Movie S3, ESI†). Neither the emission signal nor morphology changes were detected, suggesting that the phototoxicity of 488 nm laser light alone was negligible. To confirm the movement of PC4 into nuclei, cells were labelled with Hoechst 33342, a known nuclear dye. As shown in Fig. 4, the green signal of PC4 did not overlap with the blue signal of Hoechst 33342 before light irradiation. However, after 2 min light irradiation, these two signals overlapped well with each other. And the signal intensity of PC4 increased at longer irradiation time (4 min), suggesting that more PC4 entered the nuclei. The results demonstrate that PC4 was localized in the lysosome in live cells, but moved into the nuclei of the dead cells with bright green emission. Thus the discrimination of live/dead cells after PDT treatment is easily accomplished. Therefore, PC4 can be used as a reliable and convenient therapeutic PS and as a fluorescence dye for the monitoring and assessing of the PDT treatment effect.

Finally, the therapeutic mechanism of PC4 was further investigated. Firstly, the cause of cell death was explored using a known ¹O₂ scavenger Vitamin C (VC). Cells were co-incubated with PC4 and VC, and then observed by CLSM. As depicted in Fig. S9 (ESI†), remarkable inhibition of cell death was observed under a condition of continuous light irradiation. The results suggest that cell death was mainly induced by PC4 generated ¹O₂ upon light irradiation, which is consistent with the previous report. As mentioned above, cell membrane rupture was observed during the therapy process. To certify that the cause of membrane damage is oxidation during PDT treatment, malondialdehyde (MDA), a lipid peroxidation product, was measured after PDT treatment. As shown in Fig. S10 (ESI†), the content of MDA in cells after treatment with PC4 (8 μM) and light (450 nm, 4.2 J cm⁻²) was about 1.9-fold high than the control, whilst the cells treated with only PC4 or light showed no obvious increase. The results indicate that the membrane damage was attributed to the generated ¹O₂. Moreover, the cell death pathway induced by PC4 was quantitatively studied. An Annexin V-Alexa Fluor 647/Propidium Iodide (PI) apoptotic/necrotic detection kit was used to double-label the cells.
As shown in Fig. S11 (ESI†), most of the cells treated with light (4.2 J cm⁻²) were located at the (Annexin V⁻, PI⁻) quadrant, denoting high cell viability. However, cells treated with PC4 (8 μM) and light (4.2 J cm⁻²) were mainly located at the (Annexin V⁺, PI⁺) quadrant, indicating that necrosis was the primary pathway induced by the PC4 phototherapy. These results suggest the possible cause of the movement of PC4 into the cell nuclei during therapy. As the nuclear membrane of necrotic cells became permeable to small molecules, PC4 entered cell nuclei and bound to the negatively charged DNA with bright emission. To test the binding of PC4 to DNA, cells incubated with PC4 were further stained with PI. PI, a known nuclear dye, can enter dead cell nuclei and bind to DNA with turn-on red emission. As depicted in Fig. S12 (ESI†), the cells stained with PC4 (8 μM) only showed emission in plasma. And after treatment with PC4 (8 μM) and light irradiation (4.2 J cm⁻²), the emission of both PC4 and PI was found in the nuclei, suggesting excellent binding affinity of PC4 to DNA. In addition, since the 470 nm light appears to be short for the nuclei, suggesting excellent binding affinity of PC4 to DNA. The study clearly demonstrates the potential capability of PC4 as a smart PDT theranostic agent for the PDT treatment of cancer cells and in situ monitoring and assessing of the therapeutic effect.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

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