A Charge Reversible Self-Delivery Chimeric Peptide with Cell Membrane-Targeting Properties for Enhanced Photodynamic Therapy

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The cell membrane is the most important protective barrier in living cells and cell membrane targeted therapy may be a high-performance therapeutic modality for tumor treatment. Here, a novel charge reversible self-delivery chimeric peptide C_{16}–PRP–DMA is developed for long-term cell membrane targeted photodynamic therapy (PDT). The self-assembled C_{16}–PRP–DMA nanoparticles can effectively target to tumor by enhanced permeability and retention effect without additional carriers. After undergoing charge reverse in acidic tumor microenvironment, C_{16}–PRP–DMA inserts into the tumor cell membrane with a long retention time of more than 14 h, which is very helpful for in vivo applications. It is found that under light irradiation, the reactive oxygen species generated by the inserted C_{16}–PRP–DMA would directly disrupt cell membrane and rapidly induce cell necrosis, which remarkably increases the PDT effect in vitro and in vivo. This novel self-delivery chimeric peptide with a long-term cell membrane targeting property provides a new prospect for effective PDT of cancer.

1. Introduction

Carrier-assistant drug delivery systems (DDSs) for tumor therapy have received tremendous attention in the past decades. Many nanoscale carriers such as liposomes, polymers, inorganic nanoparticles (such as mesoporous silica nanoparticles), Au nanoparticles, upconversion nanoparticles, or quantum dots, and metal-organic frameworks have been developed for DDSs. While the further development of carrier-assistant DDSs is retarded by many drawbacks such as the complex synthesis, low drug loading efficiency, carrier-induced toxicity and immunogenicity etc. To address these issues, drug self-delivery systems (DSDSs) have been proposed as a novel strategy for high performance tumor therapy. DSDSs could realize systemic delivery of active drugs into disease sites without additional nanocarriers, which could avoid carrier-induced toxicity and immunogenicity. Moreover, DSDSs are easily prepared with excellent drug loading capacities. However, to date, the reported DSDSs are very limited. Besides, most reported DSDSs tried to improve the therapeutic effect by improving drug loading capacity or combining other drugs. Other efficient strategies are still highly desirable.

Very recently, a promising approach to improve therapeutic efficacy is to precisely deliver drug to specific subcellular organelles such as mitochondrion, and nuclei. In our previous study, we reported a self-delivery chimeric peptide PPK for dual-stage-light guided and mitochondrion-targeted photodynamic therapy (PDT). Han et al. also reported a nuclei-targeted self-delivery chimeric peptide PAPP–DMA to realize in situ PDT in nuclei. It is well known that cell membrane is the most important protective barrier in living cells, which provides a stable environment for the efficient intracellular cell metabolism processes. Cell membrane is in charge of the nutrients exchange between the cells and external environment. What's more, many membrane proteins (such as ATP-binding cassette transporter) and the reversal of the pH gradient at plasma membrane (ΔpHcm), might promote resistance of cancer cells to many chemotherapy drugs. Considering the unique feature of cell membrane, direct destruction of tumor cell membrane would be a novel and efficient approach for tumor inhibition. Comparing with the conventional antitumor strategy, cell membrane-targeted therapy can be more efficient since it could take effect without the cell endocytosis of drugs. Moreover, many drawbacks in the conventional chemotherapy such as the degradation of drug intracellular and drug resistance induced trial failures can be avoided. Unfortunately, to develop a cell membrane-targeted therapy system is extremely difficult, mainly due to the lack of effective strategy to long-time anchor the drugs or dyes on cell membrane. To our best knowledge, the reported cell membrane-targeted therapy systems are very rare.

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In this work, a charge reversible self-delivery chimeric peptide \( \text{C}_{16}\text{–K(PpIX)RRK(DMA)K(DMA)}\text{–PEG–COOH}, \) designated as \( \text{C}_{16}\text{–PRP–DMA} \), was developed for tumor therapy by cell membrane-targeted PDT. As shown in Scheme 1, this chimeric peptide \( \text{C}_{16}\text{–PRP–DMA} \) allows the integration of four functional segments. Palmitic acid (PA) is the first one that functioned as a lipophilic component for membrane insertion as well as a hydrophobic part for the self-assembly of \( \text{C}_{16}\text{–PRP–DMA} \). DMA modified tetra-peptide sequence (RRKK) is the second segment, in which RRKK is a positively charged hydrophilic moiety that may improve the membrane affinity of the chimeric peptide by electrostatic interaction.\(^{[18]}\) Negative DMA group is employed to disguise the electropositivity of RRKK, thus can reduce the nonspecific adsorption of \( \text{C}_{16}\text{–PRP–DMA} \) in vivo. DMA group is reported with acidic sensitivity,\(^{[19]}\) which can be detached from the peptide at the acidic environment in tumor. It is expected that synergistic effect of lipophilic PA and positively charged RRKK peptide would contribute to a long-time drug retention of \( \text{C}_{16}\text{–PRP–DMA} \) on cell membrane. PEG is the third segment that not only serves as a hydrophilic segment for the self-assembly of \( \text{C}_{16}\text{–PRP–DMA} \) but also acts as a hydrophilic shell layer to enhance the biocompatibility and prolong the half-life for \( \text{C}_{16}\text{–PRP–DMA} \) in blood.\(^{[20]}\) The last part: photosensitizer (PS) protoporphyrin IX (PpIX) is a PDT therapeutic agent. In this paper, the acidity induced charge reverse and disintegration of \( \text{C}_{16}\text{–PRP–DMA} \) nanoparticles, cell membrane retention ability as well as the antitumor effect of the chimeric peptide \( \text{C}_{16}\text{–PRP–DMA} \) was investigated in detail.

2. Results and Discussion

### 2.1. Synthesis and Characterization of Chimeric Peptide \( \text{C}_{16}\text{–PRP–DMA} \)

The synthesis process of \( \text{C}_{16}\text{–PRP–DMA} \) was demonstrated in Scheme S1 (Supporting Information) and was detailedly described in Experimental Section. Briefly, chimeric peptide \( \text{C}_{16}\text{–PRP} \) (\( \text{C}_{16}\text{–K(PpIX)RRKK–PEG–COOH} \)) was first prepared via the standard solid-phase peptide synthesis (SPPS) method using 2-chlorotrityl chloride resin. Subsequently, the primary amines in RRKK sequence were reacted with DMA group to obtain acid-labile \( \text{C}_{16}\text{–PRP–DMA} \) (\( \text{C}_{16}\text{–K(PpIX)RRK(DMA)K(DMA)}\text{–PEG–COOH} \)). In this study, chimeric peptide \( \text{C}_{2}\text{–PRP–DMA} \) (\( \text{C}_{2}\text{–K(PpIX)RRK(DMA)K(DMA)}\text{–PEG–COOH} \)) was designed as a negative control, because this peptide was prone to be internalized by cell. Succinic anhydride (SA) modified chimeric peptide \( \text{C}_{16}\text{–PRP–SA} \) (\( \text{C}_{16}\text{–K(PpIX)RRK(SA)K(SA)}\text{–PEG–COOH} \)) was also designed as a control, because SA group could not detach from peptide backbone at acidic tumor environment (pH 6.8–7.2).\(^{[21]}\) The detailed synthesis procedures of chimeric peptides \( \text{C}_{16}\text{–PRP–DMA} \), \( \text{C}_{2}\text{–PRP–DMA} \) and \( \text{C}_{16}\text{–PRP–SA} \) were confirmed by the electrospray ionization mass spectrometry (ESI-MS, Figures S1–S4, Supporting Information) or matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (Figures S5, Supporting Information).

During the DMA modification process, the zeta potential of \( \text{C}_{16}\text{–PRP–DMA} \) nanoparticles in \( 10 \times 10^{-3} \) m phosphate buffer solution (PBS, pH 7.4) changed from 0.67 mV (\( \text{C}_{16}\text{–PRP} \)) to \(-17.6 \) mV (\( \text{C}_{16}\text{–PRP–DMA} \)). As shown in transmission electron microscope (TEM) image in Figure 1A, \( \text{C}_{16}\text{–PRP–DMA} \) could form well-dispersed uniform spherical nanoparticle with a diameter size of about 30 nm in PBS 7.4. Dynamic light scattering (DLS) analysis result showed that the hydrodynamic size of \( \text{C}_{16}\text{–PRP–DMA} \) nanoparticles was \( 54.9 \pm 3.7 \) nm (Figure 1A), which was similar to the TEM result. The small discrepancy between DLS and TEM results was attributed to the shrinkage of nanoparticles in vacuum state during TEM sample preparation process.\(^{[22]}\) The \( \pi–\pi \) stacking interaction and hydrophobic interaction between PpIX molecules and the hydrophobic interaction between many PA alkyl chains induced the formation of hydrophobic core in \( \text{C}_{16}\text{–PRP–DMA} \). The compact stacking of PpIX and PA in hydrophobic core and well hydrophilic PEGylated peptide (RRK(DMA)K(DMA)–PEG–COOH) in shell constructed a relative stable micelle system. The zeta potential and DLS of \( \text{C}_{2}\text{–PRP–DMA} \) and \( \text{C}_{16}\text{–PRP–SA} \) nanoparticles in PBS (pH 7.4) were also obtained, which were showed in Figure S6 (Supporting Information). From Figure 1B,
the blue shift of sharp Soret band in UV–vis spectrum of C16–PRP–DMA from around 406 nm in DMSO (indicated the formation of PpIX monomer) to 382 nm indicated the formation of PpIX dimer in C16–PRP–DMA nanoparticles in PBS 7.4, which meant that their existed π–π interaction in the self-assembly structure of C16–PRP–DMA. For PpIX in PBS 7.4, a broaden split Soret band with the maxima wavelength at 364 and 457 nm was observed, correlating with the formation of PpIX dimer in C16–PRP–DMA nanoparticles in PBS 7.4, which meant that there existed π–π interaction in the self-assembly structure of C16–PRP–DMA. For PpIX in PBS 7.4, a broaden split Soret band with the maxima wavelength at 364 and 457 nm was observed, correlating with the formation of PpIX dimer in C16–PRP–DMA nanoparticles in PBS 7.4.

of extended aggregates. Compared to the extended π-π stacking effects in PpIX aggregates, the formation of PpIX dimer in C16–PRP–DMA nanoparticles would remarkably improve the reactive oxygen species (ROS) generation efficacy of PpIX.

2.2. Acidity-Triggered Charge Reverse and Nanoparticle Disintegration

To demonstrate the acidity-triggered charge reversal behavior of C16–PRP–DMA, zeta potential changes of C16–PRP–DMA nanoparticles with prolonged incubation time at pH 6.8 and pH 7.4 were studied. As shown in Figure 1C, with the incubation time prolonging to 4 h, zeta potentials of C16–PRP–DMA nanoparticles at pH 6.8 were rapidly increased from about −20 mV to +2.19 mV, mainly attributed to the rapidly degradation of DMA amide at acidic environment. At a more acidic environment (pH 5.0), the zeta potential changes of C16–PRP–DMA nanoparticles were much faster than that at pH 6.8, indicating the acidity responsive DMA detachment of C16–PRP–DMA. In comparison, the zeta potential changes of C16–PRP–DMA nanoparticles at pH 7.4 and pH 8.0 were much slower than that at pH 6.8. The zeta potential was still below −10 mV at pH 7.4 and below −19 mV at pH 8.0 even at the 4.5th h. Similar phenomenon was also reported in many literatures. These results clearly demonstrated the appreciable acidity-triggered charge reversible ability of C16–PRP–DMA nanoparticles. The different zeta potential values of C16–PRP–DMA at 1 min at different pHs were due to the different ionization state of the guanidine groups in the side chain of RRKK sequence.

In the acidic environment, DMA group detachment from C16–PRP–DMA nanoparticles resulted in zeta potential changes (Figure 1C). The charge changes in peptide side chains may also lead to structure change or instability. Then, the acidity-triggered C16–PRP–DMA nanoparticles disintegration was evaluated by comparing the critical micelle concentration (CMC) of C16–PRP–DMA nanoparticles at pH 6.8 and pH 7.4 utilizing a hydrophobic pyrene fluorescent probe. It was reported that pyrene would preferentially diffuse into the hydrophobic core of nanoparticles, resulting in photophysical property changes. As shown in Figure 1D,E, the CMC value of C16–PRP–DMA nanoparticles was 3.58 mg L\(^{-1}\) (1.69 × 10\(^{-6}\) \(\mu\)m) at pH 7.4, while it was 46.1 mg L\(^{-1}\) (21.7 × 10\(^{-6}\) \(\mu\)m) at pH 6.8 (12.9-fold of the CMC value at pH 7.4), which was very close to the CMC value of DMA unmodified C16–PRP nanoparticles (52.9 mg L\(^{-1}\), 27.5 × 10\(^{-6}\) \(\mu\)m, Figure 1F) at pH 6.8. The increased CMC value with increased acidity indicated that C16–PRP–DMA nanoparticles were prone to disintegration with the DMA group degradation in RRKK peptide at acidic condition. At the low concentration (<20 × 10\(^{-6}\) \(\mu\)m), after the hydrolysis of the protecting group, C16–PRP–DMA nanoparticles were prone to form peptide monomer. For C16–PRP–SA nanoparticles that could not undergo charge reverse in weakly acidic condition, the CMC values were not changed much at different pHs (38.43 mg L\(^{-1}\) at pH 7.4 and 44.15 mg L\(^{-1}\) at pH 6.8, Figure 1G,H). TEM image and DLS for the nanoparticles of C16–PRP–DMA (50 × 10\(^{-6}\) \(\mu\)m) after incubation at pH 6.8 were also obtained to study the size changes of C16–PRP–DMA between acidic and neutral environment (Figure S8, Supporting Information). It was found that the Z-average diameter of C16–PRP–DMA nanoparticles in DLS result was 79.7 ± 3.3 nm, which was larger than the Z-average diameter at pH 7.4 (54.9 ± 3.7 nm, Figure 1A). The TEM image of C16–PRP–DMA after incubation at pH 6.8 for 3 h showed spherical nanoparticles with a average diameter size of about 40 nm. The enlarged nanoparticle size of C16–PRP–DMA at acidic environment might be ascribed to the extension of the corona scale induced by the enhanced electrostatic repulsion interaction between positively charged guanidine and amine group in the side chain of C16–PRP after DMA group detachment. The hydrophilic diameter and CMC value differences between C16–PRP–DMA and C16–PRP–SA might be attributed to the electrostatic interaction discrepancy between charged groups of side chain in both chimeric peptides at pH 7.4. The acid–base titration profiles of C16–PRP–DMA and C16–PRP–SA were also presented in Figure S9 (Supporting Information). The acid–base titration section from pH 5.0 to pH 8.0 was analyzed. Apparent pKa values in this section were ≈7.2 for C16–PRP–DMA and ≈6.5 for C16–PRP–SA respectively. These indicated that at pH 7.4, electrostatic repulsion interaction between negatively charged SA molecules was predominant in C16–PRP–SA nanoparticles. Enhanced electrostatic repulsion in hydrophilic shell might extend the corona of micelles and make C16–PRP–SA more soluble, which increased the CMC value. For C16–PRP–DMA, part of amine groups were positively charged at pH 7.4 (might be attributed to the hydrolyzation of primary amide’s amine of DMA). Thus, the electrostatic attraction interaction between DMA molecules and positively charged amine groups also played an important role in the self-assembly of C16–PRP–DMA nanoparticles, which made C16–PRP–DMA nanoparticles more compact.

2.3. In Vitro ROS Generation

The ROS generation capability of C16–PRP–DMA upon light irradiation was then investigated using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). It was reported that the presence of ROS, the transfected 2′,7′-dichlorodihydrofluorescein (DCFH) by DCFH-DA would be rapidly oxidized, and form fluorescent dichlorofluorescein (DCF). As shown in Figure 2B, the fluorescence intensity of C16–PRP–DMA solution (5 × 10\(^{-6}\) \(\mu\)m) was rapidly increased upon 630 nm light irradiation (30 mW cm\(^{-2}\)), reaching a 11-fold fluorescence enhancement at the time point of 320 s. While for PBS buffer (pH 7.4) without C16–PRP–DMA, the fluorescence intensity remained very low. When an ROS scavenger, vitamin C (VC, 100 × 10\(^{-6}\) \(\mu\)m) was added into C16–PRP–DMA solution, the fluorescence increase in solution was significantly inhibited, which further confirmed the ROS generation of C16–PRP–DMA upon light irradiation. Besides, comparing to C16–PRP–DMA, the fluorescence increase of free PpIX (5 × 10\(^{-6}\) \(\mu\)m, 0.5% DMSO) solution was much slow. These data clearly demonstrated that the self-assembled amphiphilic C16–PRP–DMA nanoparticles could significantly reduce self-quenching of hydrophobic PpIX and improve ROS generation efficiency.

After confirming the appreciated ROS generation ability of C16–PRP–DMA in aqueous solution, its intracellular ROS
production in mouse mammary carcinoma cells (4T1 cells) was then tested. 4T1 cells were first incubated with drug contained mediums at pH 6.8 for 3 h and then stained with ROS sensitive probe DCFH-DA for 0.5 h. After being irradiated with 630 nm light (30 mW cm\(^{-2}\)) for 100 s, cell samples were observed using confocal laser scanning microscopy (CLSM). As shown in Figure 2A, nearly no green fluorescence was observed in untreated blank cells. However, apparent green fluorescence was observed in C\(_{2}\)-PRP–DMA treated cells, indicating that the appearance of green fluorescence was due to the generated ROS by PpIX modified peptide under red light irradiation. In contrast, very bright green fluorescence was shown in C\(_{16}\)-PRP–DMA treated cells. Moreover, DCF fluorescence observed in C\(_{16}\)-PRP–SA treated cells was much weaker than C\(_{16}\)-PRP–DMA group. The intracellular ROS generation by different chimeric peptides upon irradiation was also investigated by flow cytometry (Figure 2C), and the obtained mean DCF fluorescence in each cell samples were consistent with the results in Figure 2A. This enhanced intracellular ROS detected in C\(_{16}\)-PRP–DMA than C\(_{2}\)-PRP–DMA treated cells was mainly attributed to the improved cell membrane targeting ability of C\(_{16}\)-PRP–DMA brought by the cell membrane attachable PA. The enhanced intracellular ROS generation of C\(_{16}\)-PRP–DMA than C\(_{16}\)-PRP–SA might be ascribed to the enhanced membrane affinity by positively charged RRKK sequence after charge reverse, which was verified by the PpIX fluorescence discrepancy detected in each chimeric peptides treated cells by flow cytometry analysis (Figure 2D). It was found that the mean PpIX fluorescence intensity in C\(_{16}\)-PRP–DMA treated cell sample was about 2.2-fold of C\(_{2}\)-PRP–DMA and C\(_{16}\)-PRP–SA treated cell samples, which indicated that both alkyl chain PA and positively charged hydrophilic tetra-peptide sequence RRKK played important roles in the improved cell membrane affinity for C\(_{16}\)-PRP–DMA.

2.4. Cell Membrane Targeting Study

Above studies clearly demonstrated the great ROS generation capability of chimeric peptide C\(_{16}\)-PRP–DMA in vitro. Then, the cell membrane targeting property of this chimeric peptide was tested using 4T1 cells. C\(_{2}\)-PRP–DMA and C\(_{16}\)-PRP–SA were used as controls. In this experiment, cell mask green plasma membrane stain was used to label the cell membrane.\(^{[30]}\) The inherent red fluorescence of PpIX was used to track the location of chimeric peptides. As shown in Figure 3, most of red fluorescence of C\(_{2}\)-PRP–DMA was found at the cell membrane, with a small part of red fluorescence observed in cytoplasm. The red fluorescence of C\(_{16}\)-PRP–DMA was well overlapped with the cell membrane in bright field and the green fluorescence of cell membrane tracker. In contrast, red fluorescence of C\(_{2}\)-PRP–DMA was only observed in the cytoplasm, nearly no overlap between red fluorescence and cell membrane in bright field was found. Also, the red fluorescence intensity of C\(_{16}\)-PRP–SA at the cell membrane was lower than that of C\(_{16}\)-PRP–DMA.
The cell membrane affinity of chimeric peptide C16–PRP–DMA to other cancer cells was also studied. As shown in Figure 4, after 3 h incubation of C16–PRP–DMA contained medium at pH 6.8, red fluorescence of PpIX was mainly found around the cell membrane of human cervical cancer cells (HeLa cells), human lung adenocarcinoma cells (A549 cells) and human breast cancer cells (MCF 7 cells). These results clearly revealed the satisfied cell membrane targeting property of chimeric peptide C16–PRP–DMA for many kinds of cancer cells. Alkyl chain PA and positively charged RRKK sequence were designed to enhance membrane insertion, and improve membrane affinity for C16–PRP–DMA. Thus, the prolonged cellular location of C16–PRP–DMA in cell membrane was then investigated. Peptide C16–PRP and C16–PRP–SA were used as positive and negative control. RRKK sequence was not modified in C16–PRP and SA group in C16–PRP–SA could not detach from peptide backbone at pH 6.8–7.2. In this experiment, 4T1 cells were first incubated with different drug mediums at

Figure 3. Confocal microscopic images of 4T1 cells treated with C2–PRP–DMA, C16–PRP–DMA, and C16–PRP–SA (30 × 10^{-6} \text{m}) for 3 h. C/P Merged indicated the merge field of cell membrane (green) and PpIX (red). B/P Merged indicated the merge field of bright field and PpIX (red). Scale bar: 20 × 10^{-6} \text{m}.

Figure 4. Confocal microscopic images of A549, HeLa, and MCF-7 cancer cells treated with C16–PRP–DMA (30 × 10^{-6} \text{m}) for 3 h. Scale bar: 20 × 10^{-6} \text{m}.  

pH 6.8 for 3 h. After replacing the incubation mediums, the cells were further cultured for different time intervals, and observed using confocal laser scanning microscopy (CLSM). As shown in Figure 5, at 0 h (after medium replacing), all three peptides (represented by red fluorescence) were mainly found in the cell membrane, but as the incubation time prolonged to 6 h, most C16–PRP–SA was located at cell cytoplasm with a small part in cell membrane. When the incubation time prolonged to 14 h, all of C16–PRP–SA was located at cell cytoplasm. In contrast, peptide C16–PRP and C16–PRP–DMA showed good co-location with cell membrane even after 14 h incubation. Considerable amount of C16–PRP and C16–PRP–DMA peptides was observed in cell membrane with the incubation time prolonged to 20 h. It was very surprised to find that even after 28 h cell culturing, certain amount of these peptides was still observed in cell membrane, although most of these peptides were endocytosed into cell cytoplasm. From above observations, it could be verified that chimeric peptide C16–PRP–DMA achieved a satisfying long cell membrane retention, attributed to the synergistic effect of PA and RRKK peptide. There were not much difference in the PS retention time in cell membrane between C16–PRP and C16–PRP–DMA, since negative DMA group would be rapidly detached at acidic environment (at pH 6.8). Comparing to C16–PRP–DMA, C16–PRP–SA showed a very low time retention in cell membrane, mainly because of the reduced membrane affinity by negative charged and nondetachable SA modification in RRKK sequence. It should be noticed that the retention time of commercial cell plasma membrane mask dye was lower than 2.5 h (Figure S10, Supporting Information), which further demonstrated the enhanced cell membrane targeting capability of C16–PRP–DMA (more than fivefold retention time of commercial cell plasma membrane mask dye).

2.5. PDT Cytotoxicity In Vitro

Subcellular localization of PSs greatly affects the therapeutic efficacy in PDT. The membrane located PS molecules would lead to rapid membrane disruption upon light irradiation and subsequently induce cell necrosis, which greatly increase the PDT outcome. Then, the improved PDT efficacy by cell membrane targeted chimeric peptide C16–PRP–DMA was investigated using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyldtetrazolium-bromide (MTT) assay. 4T1 cells were incubated...
with different drug contained mediums at pH 6.8 for 3 h to allow the complete charge reverse of peptides. As expected, C<sub>16</sub>–PRP–DMA showed significantly higher photo-toxicity than C<sub>16</sub>–PRP–SA and C<sub>2</sub>–PRP–DMA (Figure 6A). 32% cell viability was observed for C<sub>16</sub>–PRP–DMA at the concentration of 0.5 × 10<sup>−6</sup> m, while there were about 80% cell viability for C<sub>16</sub>–PRP–SA and C<sub>2</sub>–PRP–DMA. Negligible dark cytotoxicity was observed in all three chimeric peptides (Figure 6B), suggested that the photo-toxicity of these peptides were induced by the ROS generated by PSs under irradiation. Considering the extremely low dark cytotoxicities of these peptides, they might be sufficiently biocompatible for in vivo usage.

The effective plasma membrane disruption by the ROS generated in C<sub>16</sub>–PRP–DMA was further investigated. Propidium iodide (PI) was used as an indicator in this experiment. PI is a cell-impermeable nucleic acid dye, which exhibit weak fluorescence but rapidly emit bright red fluorescence when it is bound to the DNA of cell membrane disrupted cell. 4T1 cells were first incubated with different drug contained mediums at pH 6.8 for 3 h, after replacing with free mediums, the cells were treated with 630 nm light for 100 s (30 mW cm<sup>−2</sup>). C<sub>16</sub>–PRP–DMA incubated cells in darkness were used as a control. Then the cells were stained with PI, and visualized by CLSM. As shown in Figure 7, negligible red fluorescence (represented the PI) was found in the cells of C<sub>16</sub>–PRP–DMA/dark group, while bright PI fluorescence in nuclei was observed in the C<sub>16</sub>–PRP–DMA/hv group, indicating that the ROS generated by the membrane located C<sub>16</sub>–PRP–DMA could disrupt the plasma membrane and allow the free diffusion of cell-impermeable PI into cell nuclei. In contrast, negligible PI fluorescence was found in the cells of C<sub>16</sub>–PRP–SA/hv and C<sub>2</sub>–PRP–DMA/hv group.

Figure 6. Cell viability of C<sub>2</sub>–PRP–DMA, C<sub>16</sub>–PRP–DMA, and C<sub>16</sub>–PRP–SA A) with or B) without light irradiation (30 mW cm<sup>−2</sup> for 100 s) in 4T1 cells.

Figure 7. Confocal microscopic images of C<sub>16</sub>–PRP–DMA, C<sub>2</sub>–PRP–DMA, and C<sub>16</sub>–PRP–SA (1 × 10<sup>−6</sup> m) treated 4T1 cells with/without light irradiation (30 mW cm<sup>−2</sup> for 100 s) stained with cell impermeable PI.
suggesting the weak plasma membrane damage induced by C16–PRP–SA and C2–PRP–DMA under irradiation.

In addition, the cell apoptosis and necrosis analyses of the chimeric peptides treated cells were investigated by CLSM. 4T1 cells were first incubated with different drug contained mediums (1 × 10^−6 m) at pH 6.8 for 3 h, after replacing with free mediums, the cells were treated with 630 nm light for 100 s (30 mW cm^−2). After 6 h further incubation, the cells were stained with annexin V-FITC and PI, and visualized by CLSM. Cells without drug treatment were used as blank control. As shown in Figure 8, C16–PRP–DMA treated group showed remarkable cell necrosis with most cells stained with PI while less cells stained with annexin V-FITC. While C16–PRP–SA and C2–PRP–DMA treated groups just showed low degree of cell apoptosis and necrosis. These results above clearly demonstrated the enhanced PDT effect of plasma membrane targeted chimeric peptide C16–PRP–DMA than other peptides, attributing to the cell membrane damage and seriously cell necrosis induced by ROS.

2.6. Pharmacokinetic Study and In Vivo Tumor Imaging

The feasibility of the chimeric peptide C16–PRP–DMA nanoparticles for in vivo usage was then explored. In this section, elimination rate of C16–PRP–DMA nanoparticles in blood was investigated. DMA unmodified peptide C16–PRP was used as a negative control. C16–PRP–DMA and C16–PRP nanoparticles contained PBS solutions were intravenously injected into mice respectively, and then fixed volume of mice blood samples were obtained in the pre-set time. The fluorescence intensity changes of PpIX were used to evaluate the concentration changes of peptide C16–PRP–DMA in blood. As shown in Figure 9D, eliminating rate of C16–PRP–DMA nanoparticles was much slower than C16–PRP nanoparticles. The concentration of C16–PRP–DMA nanoparticles in blood was still high at 6 h (6.28 mg L^−1) but it was 1.38 mg L^−1 for C16–PRP nanoparticles. The relatively long blood retention time of C16–PRP–DMA than C16–PRP nanoparticles was probably due to the negative DMA group modification in the RRKK peptide sequence, which reduced the nonspecific adsorption of C16–PRP–DMA nanoparticles in vivo. PEG segment also played an important role in enhancing the biocompatibility and prolonging the half-life of C16–PRP–DMA in blood.[34] These results strongly verified the good biocompatibility and blood retention of C16–PRP–DMA nanoparticles, which was favored for in vivo PDT.

Further, the EPR-effect-mediated accumulation process of C16–PRP–DMA nanoparticles in the tumor tissue was studied. The fluorescence emission of PpIX at 650 nm was utilized to trace the bio-distribution of C16–PRP–DMA nanoparticles. After intravenously injection of drug contained PBS solution, fluorescence in mice was visualized via a small animal imaging system at different times. It was found that C16–PRP–DMA nanoparticles were gradually accumulated in tumor site and reached a maximum at about 10 h after administration (Figure 9A). Although the fluorescence in tumor was decreasing with time.
prolonging due to drug metabolism, it was still observable even after 48 h post-injection. Meanwhile, the tissue distribution of C16–PRP–DMA nanoparticles at 48 h was studied by comparing the fluorescence in tumor and other organs. As shown in Figure 9B, strong fluorescence was found in tumor tissue, weak fluorescence was observed in the liver, and nearly no fluorescence was found in the spleen, lung, kidney and heart. Figure 9C showed the mean fluorescence signal of C16–PRP–DMA nanoparticles in tumor and organs, and the results were consistent with that observed in Figure 9B. These results indicated that the C16–PRP–DMA nanoparticles could selectively accumulate to tumor tissue. In addition, the ex vivo fluorescence images of various organs were also obtained at 0.5, 2, 4, and 10 h (Figures S11, Supporting Information). The EPR-effect-mediated tumor accumulation of C2–PRP–DMA and C16–PRP–SA nanoparticles was also observed (Figure S12A, Supporting Information). But there were apparent distribution discrepancies for these peptide nanoparticles. C2–PRP–DMA and C16–PRP–SA nanoparticles showed maximum red fluorescence in tumor at about 4 and 36 h, respectively after administration. Red fluorescence was found in tumor and kidney for C2–PRP–DMA administrated mice, while it was mainly found in tumor tissue and liver for C16–PRP–SA administrated mice after 48 h postinjection (Figure S12B–E, Supporting Information). The drug distribution discrepancy between different peptide nanoparticles might be ascribed to the different particle sizes and self-assembly behavior (Figure 1 and Figure S6, Supporting Information).[35]

2.7. PDT Efficacy and Systemic Toxicity Evaluation In Vivo

The enhanced in vivo PDT efficacy for charge reversible cell membrane targeted chimeric peptide C16–PRP–DMA nanoparticles was investigated using 4T1-bearing mouse animal mode. C2–PRP–DMA and C16–PRP–SA nanoparticles were used as controls. Considering the pharmacokinetic discrepancy among different peptides used (Figure 9 and Figure S12, Supporting Information), intratumor injection was used for this study to ensure the controlled dosages of various samples in tumor site. Before that, intratumor fluorescence changes of different peptide nanoparticles with time were studied (Figure S13, Supporting Information). Light irradiation was chosen at 4 h after injection to allow the well drug absorption by tumor tissue without apparent drug metabolism. As shown in Figure 10A, compared with PBS group, C16–PRP–DMA group under light irradiation (5 min, 340 mW cm\(^{-2}\)) showed significant tumor inhibition at the second day, indicating the remarkable tumor suppression by the tumor acidity triggered cell membrane targeted PDT effect of C16–PRP–DMA. In contrast, negligible therapeutic efficacy was found in the mice administrated with C16–PRP–DMA without irradiation, owing to the lack of ROS generation without light. Weaker therapeutic efficacy was observed in the C2–PRP–DMA and C16–PRP–SA administrated mice, mainly because of the poor cellular uptake of C2–PRP–DMA and limited cell membrane affinity of C16–PRP–SA respectively. No apparent body weight loss was found in each group among the 14 d treatment (Figure 10B), indicating the negligible systemic toxicity of each peptide. At the 14th day, significant differences in the tumor weights between the mice group treated with C16–PRP–DMA and other groups were observed (Figure 10C), illustrating the enhanced PDT efficacy of the cell membrane targeted chimeric peptide C16–PRP–DMA in vivo. This good antitumor efficacy of C16–PRP–DMA was also visually demonstrated by the representative tumor images of each groups in Figure 10I (All mice were sacrificed on the 14th day because the tumors in PBS groups were very big). Further, hematoxylin and eosin (H&E) staining analysis of tumor tissues for each group was also conducted at the 14th day (Figure 10D). Few apoptotic or necrotic tumor cells were found...
in the tumor tissues of PBS/hv group and C16–PRP–DMA/dark group, and more damaged tumor cells were observed in the tumor tissues of C2–PRP–DMA and C16–PRP–SA treated mice with light irradiation, while in sharply contrast, serious damage was observed in the tumor tissue of C16–PRP–DMA and light treated mice. These results were in well accordance with the data in Figure 10A–C. All these results clearly verified the efficient antitumor activity of C16–PRP–DMA under irradiation in vivo. H&E staining for organs were also performed (Figure S14, Supporting Information). Compared with the PBS/hv groups, there were no apparent physiological morphology abnormalities observed in the organs (heart, liver, lung, spleen, and kidney) from peptides treated mice.

Encouraged by the efficient C16–PRP–DMA nanoparticles accumulation in tumor (Figure 9A), the feasibility of C16–PRP–DMA in antitumor therapy in vivo via intravenous injection was also explored utilizing 4T1-bearing mouse animal mode. Because of the pharmacokinetic discrepancy among peptides C16–PRP–DMA, C16–PRP–SA, and C16–PRP–DMA without hv groups were compared with C16–PRP–DMA with hv group, respectively. *p was analyzed by Student’s t-test when PBS, C16–PRP–DMA, C16–PRP–SA, and C16–PRP–DMA without hv groups were compared with C16–PRP–DMA with hv group, respectively. **p was analyzed by Student’s t-test when PBS, C16–PRP–DMA without hv groups were compared with C16–PRP–DMA with hv group, respectively.

Figure 10. A) Relative tumor volume, B) body weight, C) tumor weight, and D) H&E staining images of tumor tissues in the mice after the intratumor injection of different samples: PBS, C16–PRP–DMA, C2–PRP–DMA, and C16–PRP–SA nanoparticles with or without light irradiation (630 nm, 340 mW cm$^{-2}$ for 5 min). E) Relative tumor volume, F) body weight, G) tumor weight, and H) H&E staining images of tumor tissues in the mice after different treatments: intravenous injection of PBS and C16–PRP–DMA nanoparticles with or without light irradiation (630 nm, 340 mW cm$^{-2}$ for 5 min). Representative tumor images of the different groups after I) 14 d intratumor injection treatment and J) 14 d intravenous injection treatment. *p was analyzed by Student’s t-test when PBS, C16–PRP–DMA, C16–PRP–SA, and C16–PRP–DMA without hv groups were compared with C16–PRP–DMA with hv group, respectively. **p was analyzed by Student’s t-test when PBS, C16–PRP–DMA without hv groups were compared with C16–PRP–DMA with hv group, respectively.
would accumulate in tumor tissue at a maximum at 10 h and stay in tumor tissue for a long time (>36 h). Then to optimize the therapeutic efficacy of C16–PRP–DMA nanoparticles, we decided to give light illumination twice to tumor site at 10th and 24th h post-injection, respectively. As shown in Figure 10E, comparing with PBS group, C16–PRP–DMA nanoparticles absolutely could not suppress tumor growth when mice were kept in darkness. But once the C16–PRP–DMA nanoparticles administrated mice received 5 min light irradiation (340 mW cm⁻², C16–PRP–DMA/hv group), tumor growth was noticeably retarded. At the day of 14, the tumor volume of light treated C16–PRP–DMA nanoparticles injected mice was about 1/3 of the light untreated one. These substantially conformed to the in situ PDT efficacy of C16–PRP–DMA nanoparticles. In addition, no obviously body weight changes were found in each group among 14 d treatment (Figure 10F), suggesting the negligible systemic toxicity of C16–PRP–DMA nanoparticles. At the 14th day, mice were sacrificed, and tumors and organs were obtained. Apparent differences in the tumor weights between the mice group treated with C16–PRP–DMA/hv and other groups were observed (Figure 10G), illustrating the enhanced PDT efficacy of C16–PRP–DMA nanoparticles in vivo. These were in accordance with the tumor images in Figure 10J. Then, the enhanced therapeutically effect was further confirmed via H&E staining (Figure 10H). It was found that tumor cells in PBS/hv and C16–PRP–DMA/dark groups were compact, but in sharply contrast, tumor cells in C16–PRP–DMA/hv group were very sparse, indicating that a lot of tumor cells were dead. H&E staining for organs were also performed (Figure S15, Supporting Information). Compared with the PBS/hv groups, there were no apparent physiological morphology changes showed in the organs (heart, liver, lung, spleen, and kidney) from peptides treated mice. Clearly, C16–PRP–DMA nanoparticles were biocompatible in vivo in darkness but could exhibit good anti-tumor efficacy by light induced ROS generation.

3. Conclusion

In summary, a charge reversible self-delivery chimeric peptide, C16–PRP–DMA, was developed to realize the cell membrane targeted PDT. C16–PRP–DMA was easily prepared with excellent PpIX loading capacity (25.1%). After undergoing charge reverse in tumor tissue, C16–PRP–DMA could stay on cancer cell membrane in a long time (more than 14 h) due to synergetic effect of cell membrane attachable alkyl chain PA and positively charged RRKK. C16–PRP–DMA could exhibit good antitumor effect attributed to the ROS induced effective plasma membrane disruption demonstrated in in vitro experiments. The strategy of enhancing PDT efficacy by directly targeting cancer cell membrane using charge reversible self-delivery nanoparticles demonstrated here simultaneously settled many bottlenecks in PDT, such as the inherent drawbacks of \( \text{O}_2 \) restricted toxicity due to the extremely short lifetime (about 40 ns) and very limited diffusion distance (20–200 nm), additional side effect of carriers, the complex synthesis processes, and the requirement of cell internalization for common PS delivery systems. This novel PS self-delivery system provides a new prospect for effective PDT of cancer.

4. Experimental Section

Materials: 2-chlorotriyl chloride resin (100–200 mesh, loading: 1.08 mmol g⁻¹), N-fluorenyl-9-methoxycarbonyl (Fmoc)-protected \( \text{l} \)-amino acids (Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Dde)-OH), 1-hydroxybenzotriazole (HOBt), and \( \text{o} \)-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) were purchased from GL BioChem Ltd. (Shanghai, China). Fmoc-protected PEG8 was purchased from Biomatik Biotechnology Co., Ltd. (Jiaxing, China). N-methylmorpholine (NMM) was purchased from Shanghai Aladdin Biotechnology Co., Ltd. (China). Trifluoroacetic acid (TFA); N,N,N'-dimethylformamide (DMF); piperidine, methanol, disopropylethlyamine (DIEA); dichloromethane (DCM); anhydrous ether; PA; SA; acetic anhydride; and VC were obtained from Shanghai Chemical Co. (China). Hoechst 33342 were provided by Sigma-Aldrich (USA). DmA was purchased by Ark Pharm Inc. (USA). DCFH-DA was purchased from Beyotime Biotechnology Co., Ltd. (China). CellMask green plasma membrane stain was purchased from Life Technologies (USA). Annexin V-FITC/PI apoptosis and necrosis detection kit was purchased from 4A Biotech Co., Ltd. (Beijing, China). Dulbecco’s modified Eagle’s medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, trypsin, fetal bovine serum (FBS), and Penicillin-streptomycin were provided by Biological Industries (USA). MTI was purchased from Cerview Scientific Inc. (USA). All other reagents were used without further purification.

Synthesis of C16–PRP and C2–PRP Peptides: Peptides C16–K(PpIX)RRKK–PEG–COOH (C16–PRP) and C2–K(PpIX)RRKK–PEG–COOH (C2–PRP) were synthesized by standard solid phase peptide synthesis (SPPS) using 2-chlorotriyl chloride resin and Fmoc-amino acids: i) The resins were soaked in anhydrous DMF for 0.5 h, and then 2 equiv. (relative to the loading content of resins) of Fmoc-PEG20-OH and 6 equiv. of DIEA in a DMF solution were added to the resins and reacted for 3 h. ii) The unreacted mixture solution was then removed and the resins were washed thrice by anhydrous DMF, untreated sites of resins were capped by a 30 min incubation with a mixture of CH3OH and DIEA in DMF (v/v/v = 1.5:2:6.5). iii) The resins were treated with 20% piperidine in DMF (v/v) for 10 min twice to remove the Fmoc protecting group in the attached Fmoc protected acids before, and the highly reactive NH₂ groups were exposed. Next Fmoc protected amide acid was coupled to resin by the reacting with 4 equiv. of Fmoc-protected amino acid, 4.8 equiv. of HBTU, 4.8 equiv. of HOBt, and 8 equiv. of DIEA for 2 to 2.5 h. Then ii) was repeated until lys(Dde)(Arg(Pbf)Arg(Pbf)Lys(Boc)Lys(Boc)) was coupled to the resin. Then resins were treated with 20% piperidine in DMF (v/v) for 10 min twice, and PA (C16) or acetic anhydride (C2) was coupled to the resins. After removing the Dde protecting group by 2% hydrazine hydrate in DMF, the resins were reacted with 2 equiv. of PpIX, 4.8 equiv. of PyBOP, and 8 equiv. of NMM for 12 h. Then, the resins were washed with DMF for ten times, then washed with methanol and DCM for four times, respectively, and dried under vacuum overnight. Peptides C16–PRP and C2–PRP were cleaved from the resins by incubating the resins with a mixture of TFA/thioanisole/H₂O (95:2.5:2.5) for 1.5 h at room temperature. The filtrations were collected and concentrated by rotary evaporation. Then the concentrates were precipitated in cold ether, the precipitates were collected by centrifugation, and dried under vacuum. The successful synthesis of peptides C16–PRP and C2–PRP were confirmed by ESI-MS (Figure S1 and S2, Supporting Information), calculated mass: 1920.2, found [M+H]+ = 1921.3 for C16–PRP, calculated mass: 1725.1, found [M+H]+ = 1726.0 for C2–PRP.

Synthesis of C16–PRP–DMA and C2–PRP–DMA Peptides: C16–PRP and C2–PRP (20 mg) were first dissolved in 5 ml pure water, then 1 mol L⁻¹ NaOH was slowly added to the solutions to make the pH value around 8.5. Later, 16 equiv. of DMA were added dropwise, and solutions were monitored to keep the pH value always around 8.5. After 12 h stirring in darkness, the solutions were dialyzed against H₂O solutions around pH 8.5 for 24 h (MWC 1000 Da). The purified solutions were then freeze-dried and obtained C16–PRP–DMA and C2–PRP–DMA peptides. The successful synthesis of peptides C16–PRP–DMA and C2–PRP–DMA were confirmed by ESI-MS (Figures S3 and S4, Supporting Information).

Synthesis of C16–PRP–SA Peptide: C16–PRP (20 mg) was first dissolved in 5 mL anhydrous DMF, then 100 μL DIEA was added to the solution. Later, 16 equiv. of SA was added, and solution was stirred in dark for 12 h, the solution was then dialyzed against water for 24 h (MWCO ≤ 1000 Da). The purified solution was then freeze-dried and obtained C16–PRP–SA peptide. The successful synthesis of peptide C16–PRP–SA was confirmed by MALDI-TOF MS (Figure S5, Supporting Information).

UV–Vis Spectrum Analysis of Peptide: UV–vis spectra of C16–PRP–DMA and C2–PRP–DMA were obtained using UV–VIS Spectrometer Lambda 35 (Perkin-Elmer). PpIX dissolved in PBS 7.4 containing 1% DMSO were obtained using preset times at the concentration of 30 μg mL\(^{-1}\). The UV–vis spectra of C16–PRP–DMA and C2–PRP–DMA nanoparticles in PBS solutions (10 × 10\(^{-3}\) M, pH 7.4 and 6.8) were measured by Zetasizer Nano ZS (Malvern Instruments) at 25 °C. Morphology of C16–PRP–DMA nanoparticles was observed by TEM (JE-M-2100 microscope). The concentrations of C16–PRP–DMA or C2–PRP–DMA nanoparticles in these studies were 30 × 10\(^{-6}\) M.

Intracellular ROS Generation Investigated by Flow Cytometry: 4T1 cells were seeded on a 6-well plate (2 × 10\(^5\) cells per well) cultured in RPMI 1640 containing 10% FBS and further incubated for 24 h (37 °C, 5% CO2), then the C16–PRP–DMA, C2–PRP–DMA, and C16–PRP–SA contained 1640 mediums (10 × 10\(^{-6}\) M) were added into the cells. Light irradiation (30 mW cm\(^{-2}\) for 100 s) was performed after 30 min incubation. Then the cells were observed by CLSM soon at the excitation wavelength of 488 nm (emission band pass: 500–550 nm).

Intracellular ROS Generation Investigated by Flow Cytometry: 4T1 cells were seeded on a 6-well plate (2 × 10\(^5\) cells per well) cultured in RPMI 1640 containing 10% FBS and further incubated for 24 h (37 °C, 5% CO2), then the C16–PRP–DMA, C2–PRP–DMA, and C16–PRP–SA contained 1640 mediums (10 × 10\(^{-6}\) M, pH 6.8, free of FBS) were added, and incubated for 3 h. After replacing the drug contained medium and washed the cells thrice, DCFH-DA contained 1640 mediums (10 × 10\(^{-6}\) M) were added into the cells. Light irradiation (30 mW cm\(^{-2}\) for 100 s) was performed after 30 min incubation. Then the cells were digested by trypsin and collected in centrifuge tubes. After washed with 0.5 mL PBS, cells were analyzed by Flow Cytometry (BD FACS Aria TM III).

Mean PpIX Fluorescence in Cells Test by Flow Cytometry: 4T1 cells were seeded on a 6-well plate (2 × 10\(^5\) cells per well) cultured in RPMI 1640 containing 10% FBS and further incubated for 24 h (37 °C, 5% CO2), then were incubated with RPMI 1640 mediums containing C16–PRP–DMA, C2–PRP–DMA, and C16–PRP–SA (10 × 10\(^{-6}\) M, pH 6.8, free of FBS) for 3 h. After being washed thrice using PBS, the cells were stained with CellMask green plasma membrane stain (10 × 10\(^{-6}\) M) for 10 min, and then stained with Hoechst 33342 (10 μg mL\(^{-1}\)) for 15 min, and then the cells were washed and observed via CLSM (LSM 710, two-photo excitation wavelength: 780 nm, emission band pass: 450–480 nm, excitation wavelength: 488 nm, emission band pass: 500–530 nm, excitation wavelength: 630 nm, emission band pass: 650–700 nm).

Cell Membrane Targeting Study: 4T1 cells were seeded into three petri dishes and cultured in RPMI 1640 containing 10% FBS and further incubated for 24 h (37 °C, 5% CO2), then were incubated with RPMI 1640 mediums containing C16–PRP–DMA, C2–PRP–DMA, and C16–PRP–SA (10 × 10\(^{-6}\) M, pH 6.8, free of FBS) for 3 h. After being washed thrice using PBS, the cells were stained with CellMask green plasma membrane stain (10 × 10\(^{-6}\) M) for 10 min, and then stained with Hoechst 33342 (10 μg mL\(^{-1}\)) for 15 min, and then the cells were washed and observed via CLSM (Nikon, excitation wavelength: 408 nm, emission band pass: 450–480 nm, excitation wavelength: 488 nm, emission band pass: 630–680 nm).

4T1 cells were seeded into 15 petri dishes and cultured in RPMI 1640 containing 10% FBS and further incubated for 24 h (37 °C, 5% CO2), then were incubated with RPMI 1640 mediums containing C16–PRP–DMA, C16–PRP–PR, or C16–PRP–SA (30 × 10\(^{-6}\) M at pH 6.8, free of FBS) for 3 h. After being washed thrice using PBS, the cells were stained with Hoechst 33342 (10 μg mL\(^{-1}\)) for 15 min, and then the cells were washed and further cultured for preset times (0, 6, 14, 20, and 28 h) and then observed via CLSM (Nikon, excitation wavelength: 408 nm, emission band pass: 450–480 nm, excitation wavelength: 488 nm, emission band pass: 630–680 nm).

For the evaluation of cell membrane retention effect of commercial CellMask green plasma membrane stain, 4T1 cells were seeded into a petri dish and cultured in RPMI 1640 containing 10% FBS and further incubated for 24 h (37 °C, 5% CO2), then the cells were stained with CellMask green plasma membrane stain (10 × 10\(^{-6}\) M) for 10 min, stained with Hoechst 33342 for 15 min, and then the cells were washed and further cultured for preset times (0, 2.5, and 6 h) and observed via CLSM (Nikon, excitation wavelength: 408 nm, emission band pass: 450–480 nm, excitation wavelength: 488 nm, emission band pass: 500–530 nm).
Cytotoxicity In Vitro: 4T1 cells were seeded into 96-well plates (5000 cells per well) cultured in RPMI 1640 containing 10% FBS incubated for 24 h (37 °C, 5% CO₂), and then RPMI 1640 (100 µL, pH 6.8, without FBS) containing a fixed concentration of the C₁₆–PRP–DMA, C₂–PRP–DMA, or C₁₆–PRP–SA solutions were added into wells. Cells were further incubated for 24 h for dark toxicity. For phototoxicity, drug contained mediums were replaced after 3 h incubation, and the cells were irradiated by a 630 nm diode laser (30 mW cm⁻², 100 s) and further incubated for 24 h at 37 °C. MTT solution (5 mg mL⁻¹, 20 µL) was then added to each well and incubated for 4 h. After that, mediums were removed and 150 µL of DMSO was added to each well. The optical density (OD) in each well was measured at 570 nm by a microplate reader (Thermo Scientific Multiskan Go). Relative cell viability was calculated as (ODample/ODcontrol) × 100%. The ODsample was the OD value of the cells treated with a specific concentration of drug, and ODcontrol was the OD value of the cells without drug treatment.

Cell Membrane Damage Visualized by PI Stain: 4T1 cells were seeded into four petri dishes and cultured in RPMI 1640 containing 10% FBS and further incubated for 24 h (37 °C, 5% CO₂), then they were incubated with RPMI 1640 mediums containing C₁₆–PRP–DMA, C₂–PRP–DMA, or C₁₆–PRP–SA (1 × 10⁻⁶ M at pH 6.8, free of FBS) for 3 h. After being washed thrice using PBS, the cells were treated with light irradiation (30 mW cm⁻²) or kept in dark, then were stained with PI (10 µg mL⁻¹) for 15 min, and then the cells were washed and observed via CLSM (excitation wavelength: 488 nm, emission band pass: 570–680 nm).

Annexin V-FITC/PI Assay In Vitro: 4T1 cells were seeded into four petri dishes and cultured in RPMI 1640 containing 10% FBS and further incubated for 24 h (37 °C, 5% CO₂), then they were incubated with RPMI 1640 mediums containing C₁₆–PRP–DMA, C₂–PRP–DMA, or C₁₆–PRP–SA (1 × 10⁻⁶ M at pH 6.8, free of FBS) for 3 h. After being washed thrice using PBS, the cells were treated with light irradiation (30 mW cm⁻²) for 100 s, and then the cells were further cultured for 4 h and then stained with Annexin V-FITC and propidium iodide (PI) in binding buffer for 15 min before observed via CLSM (excitation wavelength: 488 nm, emission band pass: 500–530 nm, 570–680 nm).

Pharmacokinetic Study: All animal experiments were conducted in accordance with the laboratory animals guidelines established by the Wuhan University Center for Animal Center Experiment/A3-Lab. For pharmacokinetics study, blood samples of BALB/c mice were obtained at preset times after intravenous injection of C₁₆–PRP–DMA nanoparticles. The blood samples were then diluted with PBS, and were repeatedly freeze-thawed. Subsequently, cells were under ultrasound for 5 min, the fluorescence intensities of samples were recorded at the excitation wavelength of 409 nm and emission wavelength of 630 nm.

In Vivo Tumor Imaging and Tissue Distributions: When tumor volume reached around 200 mm³, 4T1 tumor-bearing mice were intravenously injected with C₁₆–PRP–DMA, C₂–PRP–DMA, or C₁₆–PRP–SA solutions (0.5 × 10⁻³ M, 100 µL) respectively at the first and third day. 4 h later, PBS/hv, C₁₆–PRP–DMA/hv, C₂–PRP–DMA/hv, or C₁₆–PRP–SA/hv groups received 5 min 630 nm light irradiation at the power density of 340 mW cm⁻². The tumor volume and mice weight of each group were measured every day. The tumor volume was calculated as following: V = 0.5 × (tumor length) × (tumor width)². Relative tumor volume was calculated as V/V₀, V₀ was the tumor volume on the first day before treatment. The mice were sacrificed on the 14th day, the solid tumors and organs were peeled for histological observation by standard H&E staining.

In Vivo Antitumor Study by Intravenous Injection: When tumor volume was reached around 100 mm³, 4T1 tumor-bearing mice were randomly divided into three groups (PBS/hv, C₁₆–PRP–DMA/hv, and C₁₆–PRP–SA/dark groups). Mice were intravenously injected with PBS or C₁₆–PRP–DMA solutions (1 × 10⁻³ M, 100 µL) respectively at the first, fourth, seventh, and tenth days via tail vein. PBS/hv and C₁₆–PRP–DMA/hv groups received 5 min light irradiation (630 nm, 340 mW cm⁻²) twice at 10 and 24 h after injection. The tumor volume and mice weight of each group were measured every day. The tumor volume was calculated as following: V = 0.5 × (tumor length) × (tumor width)². Relative tumor volume was calculated as V/V₀, V₀ was the tumor volume on the first day before treatment. The mice were sacrificed on the 14th day, and the solid tumors and organs were peeled for histological observation by standard H&E staining.

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

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Conflict of Interest
The authors declare no conflict of interest.

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