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Dual imaging-guided photothermal/photodynamic therapy using micelles

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Abstract

We report a type of photosensitizer (PS)-loaded micelles integrating cyanine dye as potential theranostic micelles for precise anatomical tumor localization *via* dual photoacoustic (PA)/near-infrared fluorescent (NIRF) imaging modalities, and simultaneously superior cancer therapy *via* sequential synergistic photothermal therapy (PTT)/photodynamic therapy (PDT). The micelles exhibit enhanced photostability, cell internalization and tumor accumulation. The dual NIRF/PA imaging modalities of the micelles cause the high imaging contrast and spatial resolution of tumors, which provide precise anatomical localization of the tumor and its inner vasculature for guiding PTT/PDT treatments. Moreover, the micelles can generate severe photothermal damage on cancer cells and destabilization of the lysosomes upon PTT photo-irradiation, which subsequently facilitate synergistic photodynamic injury *via* PS under PDT treatment. The sequential treatments of PTT/PDT trigger the enhanced cytoplasmic delivery of PS, which contributes to the synergistic anticancer efficacy of PS. Our strategy provides a dual-modal cancer

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imaging with high imaging contrast and spatial resolution, and subsequent therapeutic synergy of PTT/PDT for potential multimodal theranostic application.

Keywords

Micelles; Photosensitizer; Near infrared fluorescent imaging; Photoacoustic imaging; Photothermal therapy; Photodynamic therapy

1. Introduction

Photodynamic therapy (PDT) is extensively considered as an intriguing treatment modality in the field of cancer therapy [1,2]. However, there are still several issues that restrict its clinical potential. Firstly, PDT generally requires to a pinpoint light irradiation at tumor, and thus tumor localization and monitoring are essential to achieve efficient PDT with low side effect. Secondly, singlet oxygen has a short half-life of <40 ns and diffusion range of 10–20 nm in biological system [3], which requires efficient subcellular trans-location of photosensitizer (PS) to improve the accessibility of singlet oxygen to the cytoplasm and target organelles in cancer cells [1,3,4]. Additionally, photo-bleaching of PS under photoradiation and limited penetration depth of light in the excitation range of 600–700 nm may also hinder their anticancer efficacy owing to their insufficient photocytotoxicity [2]. Therefore, to overcome these limitations of PDT, it is highly necessary to develop a multimodal nanoplatform for achieving both precise tumor localization and enhanced PDT efficacy of PS.

Recently, various imaging or anticancer agents have extensively been integrated with PS using nanoparticles for achieving cancer imaging and enhanced PDT efficacy *via* combinational treatments [5–12]. However, the generation of imaging modality and enhanced efficacy demands the incorporations of additional imaging agent and anticancer drugs within nanoparticles, which thus result in a ternary or more complex composition. Moreover, so far there are only a few strategies to synergize efficacy on anticancer agents *via* intracellular synergistic mechanisms in addition to some inorganic nanomaterials such as gold nanoparticles and superparamagnetic iron oxide nanoparticles in our previous studies [5,13]. Consequently, it is highly desired to develop a simple strategy that can provide both cancer imaging and synergistic effect on PDT.

Near-infrared organic cyanine dyes such as indocyanine green and Cypate have been considered as clinically potential theranostic agents, which possess promising multiple properties including near-infrared fluorescence (NIRF) emission above 800 nm, photoacoustic (PA) effect, photothermal therapy (PTT) after excitation by near-infrared light [14–21]. Cyanine dyes exhibit NIRF and PA imaging modalities with good imaging sensitivity and spatial resolution, respectively [14,20,21], and are also able to generate obvious photothermal damage *via* hyperthermia [6,22–24]. To date, cyanine dyes have been integrated into theranostic nanoparticles for both cancer imaging and therapy [5,14,25–31]. In our previous study, polymeric micelles encapsulating cyanine dye were found to generate severe photothermal damage on tumor and simultaneously induce the disruption of

lysosomal membranes upon photoirradiation [32], which are presumably potential to facilitate the cytoplasmic delivery of anticancer agents.

In this study, we report a PS (Ce6)-loaded micelle system encapsulating cyanine dye (Cypate) (Cy/Ce6-Micelles) (Scheme 1), which was evaluated for the NIRF and PA imaging modalities with precise anatomical tumor localization and high contrast. The synergistic anticancer efficacy of Cy/Ce6-Micelles was further elucidated under sequential PTT/PDT treatments.

2. Materials and methods

2.1. Materials

β -Benzyl-L-aspartate N-carboxyanhydride (BLA-NCA) was obtained from Enlai Biological Technology Company (Chengdu, China). mPEG-NH₂ (12000 MW) was purchased from Jenkem Technology Company (Beijing, China). mPEG-*b*-PAsp was synthesized as mentioned method in the reference (The degree of polymerization of PAsp segment was calculated to be 64 according to ¹H NMR spectrum, Figs. S1 and S2) [32,33]. AO was purchased from Amresco. Decylamine were obtained from SigmaAldrich. Cypate was synthesized as reported in Refs. [34,35].

2.2. Synthesis

mPEG-*b*-PAsp was reacted with decylamine in anhydrous N-methyl pyrrolidone (NMP) at 45 °C overnight. Then, the solutions were dialyzed in the mixture of ethanol and water for removing excess amines and then were dialyzed in water. Finally, mPEG-*b*-PAsp(DA) was obtained by lyophilizing the dialyzed solutions, and their degree of polymerization was calculated to be 54 according to ¹H NMR spectrum (Fig. S2) [32].

2.3. Preparation of micelles

2 mg Cypate, 2 mg Ce6 and 6 mg mPEG-*b*-PAsp(DA) were mixed at the ratio of 1:1:3 in the mixture (0.5 mL) of DMSO, and then the solution was dispersed into 4 mL distilled water. Subsequently, Cy/Ce6-Micelles were prepared by dispersing the mixture into de-ionized water and purified using dialysis (Cut-off 3.5 K MW). The mixture of free Cypate and Ce6 (free Cypate/Ce6) was prepared by dissolving Cypate and Ce6 in 5% DMSO.

2.4. Characterization

The hydrodynamic diameter of micelles was measured using DLS (Malvern, UK) at 25 °C. The morphology of the micelles was observed using TEM (Tecnai-G20). The absorbance and fluorescent spectrums of Cypate or Ce6 were measured using UV-vis Spectrophotometer (UV2600, Shimadzu) and Fluorescence Spectrophotometer (LS 55, Perkin Elmer), respectively. The photoacoustic intensity of Cy/Ce6-Micelles was measured at the wavelengths of 680, 780 and 880 nm using PA system (Endra Nexus 128, Ann Arbor, MI).

2.5. Monitoring of singlet oxygen

Free Cypate/Ce6 and Cy/Ce6-Micelles containing various concentrations of Cypate and Ce6 were dispersed in PBS solutions containing 32.0 $\mu\text{g/mL}$ DPBF (1.0 mL), and were further irradiated for 3 min at 1.5 W/cm^2 (785 nm) or 10 min at 1.0 W/cm^2 (660 nm) for singlet oxygen measurement. The fluorescent spectrum was scanned from 430 to 550 nm using an excitation wavelength of 403 nm. Then, the fluorescence intensity at 485 nm was recorded. The solution of DPBF without Cypate or Ce6 in darkness was used as the control.

2.6. Photostability

Cy/Ce6-Micelles and free Cypate/Ce6 (10 $\mu\text{g/mL}$ Cypate or Ce6, each 0.5 mL) were irradiated at 1.5 W/cm^2 (785 nm) or at 1.0 W/cm^2 (660 nm) for 0, 0.5, 1, 2, 3, and 10 min, respectively. The absorbances of Cypate or Ce6 were measured using UV-vis Spectrophotometer (UV2600, Shimadzu).

2.7. Photothermal effect

The solutions of Cy/Ce6-Micelles and free Cypate/Ce6 (each 0.5 mL) at the concentrations of 1, 2, 5, 10, 25, 50, and 100 $\mu\text{g/mL}$ Cypate were stored in the glass vials with the volume of 0.5 mL, respectively, and then the solutions were irradiated at the excitation wavelength of 785 nm *via* a optical fiber (1.5 W/cm^2 , FS-Optics, China). Simultaneously, the temperature of the solutions was measured during 300 s using a thermocouple thermometer. PBS was used as the negative control in this experiment.

2.8. Release behavior

The drug release behaviors of Cypate and Ce6 from Cy/Ce6-Micelles containing 0.1 mg Cypate and 0.1 mg Ce6 were evaluated using dialysis method respectively, and free Cypate/Ce6 in 5% DMSO was used as the control. The drug release was performed in air Constant Temperature Oscillator shaker at 37 °C. 100 mL PBS (0.2 M, pH 7.4) was used as the release medium, respectively. The samples (1.0 mL) were taken after 2, 4, 8, 12, and 24 h with the replacement of an equal volume of fresh medium. UV-vis spectrometer was used to measure the concentrations of Cypate and Ce6 in the samples, respectively.

2.9. Cellular uptake

4T1 cells were seeded on 6-well culture plates (1.2×10^6 cells/well) and incubated overnight in RPMI 1640 containing 10% FBS. Cy/Ce6-Micelles and free Cypate/Ce6 (10 $\mu\text{g/mL}$ Cypate/Ce6) were respectively applied to the wells. After 24 h incubation, the cells were washed 3 times using PBS. The cells were incubated with 0.5 mL trypsin (Sigma-Aldrich, USA) for 3 min at 37 °C, and then the cells were collected *via* centrifuge for cell counting. For the cellular uptake of Cypate, the counted cells suffered from ultrasonication, and then were treated with methanol for the extraction of Cypate. Finally, the concentrations of the extracted Cypate were measured using UV-vis assay. For the cellular uptake of Ce6, the cells were washed 3 times using PBS, treated with trypsin, centrifuged at 4 °C, and finally suspended in 0.5 mL of PBS. The fluorescent intensity of Ce6 in cells was analyzed using flow cytometry (BD LSR II, NJ) after filtration. The results are presented as a mean and standard deviation obtained from 6 samples.

2.10. Endocytotic pathways using flow cytometry

4T1 cells (1.2×10^6 cells/well) were seeded in 6-well plates. The cells were treated with PBS (control) and the different inhibitors including chlorpromazine (inhibitor of clathrin-mediated uptake, 10 $\mu\text{g/mL}$), filipin (inhibitor of caveolae-mediated uptake, 5 $\mu\text{g/mL}$), and amiloride (inhibitor of macropinocytosis, 100 $\mu\text{g/mL}$) in serum-free RPMI 1640 medium for 1 h at 37 °C or 4 °C, respectively. Then, Cy/Ce6-Micelles (10 $\mu\text{g/mL}$ Cypate or Ce6) were further added to the medium for 1 h incubation. Subsequently, the cells were washed 3 times using PBS, treated with trypsin, centrifuged at 4 °C, and finally suspended in 0.5 mL of PBS. The fluorescent intensity of Ce6 in cells was analyzed by flow cytometry (BD LSR II, NJ) after filtration.

2.11. Disruption of lysosomal membranes

To evaluate the disruption of lysosomal membranes, AO was utilized as an intracellular indicator of acidic organelle integrity in 4T1 cells. AO is a meta-chromatic fluorophore that becomes charged and is retained by proton trapping within acidic compartments. When normal cells were excited with blue light, AO in lysosomes emits an intense red fluorescence, while AO in nuclei and cytosol displays green fluorescence. Upon the disruption of acidic compartments such as the lysosomes, the red fluorescence from AO disappears and only green fluorescence can be observed. In this study, 4T1 cells were seeded overnight on glass slides in 35-mm dishes, and treated with PBS and Cy/Ce6-Micelles at the concentrations of 0.1, 0.2, 0.5 and 2.0 $\mu\text{g/mL}$ Cypate for 6 h, respectively. Then, the cells were incubated in fresh medium, followed by 3 min photoirradiation at 785 nm, 1.5 W/cm^2 . After 1 h, the cells were washed using PBS and further incubated with 6 μM AO (1.0 mL) for 15 min. The cells were washed 3 times with PBS before subjected to fluorescent microscopy observation (IX 51, Olympus) with the excitation wavelength of 488 nm, and emission wavelengths from 515 to 545 nm (green) and from 610 to 640 nm (red).

2.12. MTT assay

4T1 cells were incubated with different formulations including free Cypate/Ce6 and Cy/Ce6-Micelles at various concentrations of Cypate or Ce6 including 0.005, 0.05, 0.25, 0.5, 1.0, 2.0, 3.0, and 4.0 $\mu\text{g/mL}$ for 24 h, and then the cells were washed using PBS, followed by 3 min PTT or 10 min PDT photoirradiation (PTT: 785 nm, 1.5 W/cm^2 ; PDT: 660 nm, 1.0 W/cm^2). After 24 h, the cell viability was evaluated using MTT assay. For photothermal injury of Cy/Ce6-Micelles, the cells were incubated with Cy/Ce6-Micelles containing various concentrations of Cypate for 24 h, and then suffered from 3 min photoirradiation (785 nm) at 1.0, 1.5 and 2.0 W/cm^2 , respectively. Subsequently, the cell viability was measured using MTT assay.

2.13. Biodistribution

4T1 cells (2×10^6 cells/each mouse) were subcutaneously injected into the flanks of female BALB/c mice (16–18 g) for constructing the tumor-bearing mice. Then, the tumor-bearing mice were injected intravenously with Cy/Ce6-Micelles and free Cypate/Ce6 (5% dimethylsulfoxide (DMSO)) at the dose of 7.5 mg/kg Cypate/Ce6, respectively. Then, the various tissues including heart, liver, spleen, lung, kidney and tumor were extracted from the

mice at 24 h post-injection, respectively. Finally, the tissues were imaged using IVIS Lumina II with the excitation wavelength of 745 nm. The NIRF signals from Cy/Ce6-Micelles in the tissues were calculated to describe the *ex vivo* biodistribution of Cypate at 24 h.

2.14. In vivo NIRF and PA imaging

4T1 cells (2×10^6 cells/each mouse) were subcutaneously injected into the flanks of female BALB/c nude mice (16–18 g) for constructing the mice bearing 4T1 tumors. Cy/Ce6-Micelles and free Cypate/Ce6 (5% DMSO) were intravenously injected into these mice at the dose of 7.5 mg/kg Cypate and Ce6, respectively. Then, the mice were imaged using IVIS Lumina II with the excitation wavelength of 745 nm at 24, 48, 72, and 96 h post-injection. The average NIRF intensity at tumor was calculated at different time.

Additionally, for *In vivo* PA imaging of Cy/Ce6-Micelles, PBS, Cy/Ce6-Micelles and free Cypate/Ce6 (5% DMSO) at the dose of 7.5 mg/kg were injected into the mice bearing 4T1 tumor through intravenous administration, respectively. Then, the mice were observed using Endra Nexus 128 with the excitation wavelength of 680 nm at 0, 6, 24, and 48 h post-injection. The average PA imaging intensity at tumor was analyzed at different time. The statistic difference was analyzed using *t*-test where *P* value of <0.05 is considered significant.

2.15. In vivo efficacy

4T1 cells (2×10^6 cells/each mouse) were subcutaneously transplanted into the flanks of female mice. When the tumors reached a size of $\sim 100 \text{ mm}^3$ (about 6 days after transplantation), various formulations including PBS and Cy/Ce6-Micelles were injected intravenously into the mice at the dose of 7.5 mg/kg Cypate/Ce6 on day 0, 2, and 4, respectively. Subsequently, the tumors suffered from various photoirradiation treatments including PTT treatment (5 min, 785 nm, 1.0 W/cm^2), PDT treatment (10 min, 660 nm, 1.0 W/cm^2), sequence of initial PTT and subsequent PDT treatment (PTT/PDT), and reverse sequence of initial PDT and subsequent PTT treatment (PDT/PTT) at 24 h post-injection, respectively. The tumor volumes were measured to evaluate the anticancer efficacy of various groups. The tumor volume (*V*) was calculated as follows: $V = L \times W^2/2$, where *W* is the tumor measurement at the widest point, and *L* is the tumor dimension at the longest point. The tumor volumes are normalized against the original volumes at 0 day for monitoring the tumor growth. The mice were sacrificed by cervical dislocation under an anesthetic status after the experiments (16 days post-injection). The statistic difference was analyzed using *t*-test where *P* value of <0.05 is considered significant.

2.16. Ex vivo histological staining

4T1 cells (2×10^6 cells/each mouse) were subcutaneously transplanted into the flanks of female mice. When the tumors reached a size of $\sim 60 \text{ mm}^3$ at 4 d post-transplantation, various formulations including PBS and Cy/Ce6-Micelles were injected intravenously into the mice at the single dose of 7.5 mg/kg Cypate. Subsequently, the tumors suffered from PTT treatment (3 min, 785 nm, 1.0 W cm^{-2}) or PDT (10 min, 660 nm, 10 W/cm^2) at 24 h post-injection. Tumors were dissected from mice at 6 h after PTT treatment, fixed in a 4%

formaldehyde solution for 24 h at room temperature. The tumors were frozen and sections (10 μm in thickness) were cut on a cryostat. Hematoxylin and eosin (H&E) staining (BBC Biochemical, Mount Vernon, WA) was performed following manufacturer's instructions and observed by an IX73 bright field microscopy (Olympus).

3. Results and discussion

3.1. Synthesis, preparation and characterization

Polymeric micelles with the corona of poly(ethylene glycol) (PEG) were used as a highly effective nanocarrier for intracellular delivery of anticancer drugs owing to their multiple properties such as high loading capacity, enhanced stability, sustained release, enhanced permeability and retention (EPR) effect and superior cell internalization [36–40]. Here, we synthesized the copolymer composed of monomethoxy poly(ethylene glycol) and decylamine-grafted poly(L-aspartic acid) (mPEG-*b*-PAsp(DA)) *via* the ring-opening polymerization of β -benzyl L-aspartate N-carboxy-anhydride (BLA-NCA) with mPEG (12 kDa) and subsequent aminolysis of decylamine (Fig. S1) [32,33,41]. ^1H NMR spectra validated the chemical structures of the copolymers (Fig. S2). In order to prepare Cy/Ce6-Micelles, mPEG-*b*-PAsp(DA) was mixed with Ce6 and Cypate in dimethylsulfoxide, which was further dispersed into water, followed by dialysis against de-ionized water to purify the micelles. Cy/Ce6-Micelles exhibited high loading capacity to encapsulate Ce6 (20% (w/w)) and Cypate (20% (w/w)), which might be attributed to the good compatibility between the micellar cores with alkyl chains with the hydrophobic segments of Ce6 and Cypate [42,43]. Transmission electron microscopy (TEM) imaging shows that Cy/Ce6-Micelles had a spherical morphology with the average size of about 55.0 nm (Fig. 1A). Dynamic light scattering (DLS) measurement of Cy/Ce6-Micelles exhibited a slightly larger diameter of 63.6 nm with the polydispersity index of 0.164 (Fig. 1B) and also maintained a good size stability during storage (Fig. S3). We further found that Cy/Ce6-Micelles resulted in much better chemical stability of Ce6 at pH 7.4 and serum during as compared to free Ce6 (Fig. 1C and Fig. S4), implying that the micelles may maintain a stable micellar structure in aqueous solution and physiological environment, and then protect Ce6 from chemical degradation at physiological pH owing to its effective encapsulation within micellar cores. The poor stability of free Ce6 might result from the destruction of double bonds of Ce6 triggered by some radicals in aqueous solution. UV-vis spectroscopy analysis shows that Cy/Ce6-Micelles exhibited two characteristic sorbet peaks at 660 nm and 785 nm, which were originated from Ce6 and Cypate within micellar cores, respectively (Fig. S5).

3.2. Photothermal effect, generation of singlet oxygen, photostability, release behavior and PA properties

To demonstrate the photothermal effect of Cy/Ce6-Micelles, we evaluated their photothermal capacities at various concentrations of Cypate in aqueous solutions. Cy/Ce6-Micelles (0.5 mL) exhibited a quick increase of temperature from 28 $^{\circ}\text{C}$ to 42 $^{\circ}\text{C}$ in 200 s at the concentration of 2.0 $\mu\text{g/mL}$ Cypate upon 1.5 W/cm^2 photo-irradiation (Fig. 1D). PBS as a negative control did not trigger significant increase of temperature. It indicates that Cy/Ce6-Micelles at a relatively low concentration of Cypate can quickly trigger the increase of

temperature and display a concentration-dependent hyperthermia (above 42 °C) during a short photoirradiation, which is a key prerequisite to trigger cell damage [29,44].

To explore the ability of Cy/Ce6-Micelles to generate singlet oxygen *via* photodynamic effect, we irradiated an aqueous solution using 1,3-diphenyliso-benzofuran (DPBF) as a probe, which can be specifically quenched in the presence of singlet oxygen. Fig. 1E shows that Cy/Ce6-Micelles began to generate detectable amount of singlet oxygen from Ce6 at a concentration of as low as 0.01 µg/mL upon 660 nm photoirradiation (1.0 W/cm²), and exhibited a concentration-dependent production of singlet oxygen in aqueous solution, indicating that Ce6 is highly capable of inducing singlet oxygen even at a low concentration. In addition, Cy/Ce6-Micelles were also found to generate singlet oxygen upon photoirradiation at 785 nm which was possibly originated from Cypate (Fig. 1E) [19], even though its production efficiency of singlet oxygen is lower than that from Ce6.

Given that photostability may greatly affect the efficiency of both PTT and PDT treatments, we further studied the photostability of Cypate and Ce6 within Cy/Ce6-Micelles upon photoirradiation (Fig. S6). UV-vis spectra show that free Cypate exhibited quick decrease of absorbance upon 785 nm photoirradiation within 2 min, presumably due to the rapid photobleaching (Fig. 1F) [6]. However, Cypate in Cy/Ce6-Micelles had much slower decrease of the absorbance under the same condition (Fig. 1F). Moreover, the photoirradiation at 785 nm for several minutes had a negligible influence on the photostability of Ce6, owing to the absence of absorbance at 785 nm, implying that 785 nm photoirradiation mainly causes PTT and does not lead to the photobleaching of Ce6 prior to PDT treatment (Fig. S7). Additionally, the micelles can also reduce the photobleaching of Ce6 under photoirradiation at 660 nm as compared to free Ce6 (Fig. 1F) [6]. In particular, PDT treatment generally requires relatively long photoirradiation, thus Cy/Ce6-Micelles with enhanced photostability are expected to improve PDT efficacy over free PS.

We studied the release behavior of Cypate and Ce6 from Cy/Ce6-Micelles at pH 7.4 and 37 °C. The micelles exhibited the sustained releases of both Cypate and Ce6 as compared to free Cypate/Ce6 owing to the encapsulation within the micelles (Fig. 1G and Fig. S8), indicating that Cy/Ce6-Micelles are able to decrease undesirable release before their access to tumor.

Generally, imaging agent with photothermal effect has great potential as contrast agent for PA imaging. To demonstrate the PA properties of Cy/Ce6-Micelles, *in vitro* PA imaging was performed in vials using a photoacoustic instrument at the wavelengths of 680, 780, and 880 nm, respectively (Fig. 1H). It indicates that PA signals from Cy/Ce6-Micelles exhibited a dose-dependent behavior at all these wavelengths (Fig. 1I). Although the PA imaging intensity at 780 or 880 nm displays an unsatisfied contrast enhancement, Cy/Ce6-Micelles can act as a good contrast agent for PA imaging at the wavelength of 680 nm (Fig. 1I).

3.3. Cellular uptake and endocytotic pathways

To demonstrate whether Cy/Ce6-Micelles can be internalized by cancer cells, we evaluated their cellular uptake on 4T1 murine breast cancer cells. Fig. 2A and B shows that Cy/Ce6-Micelles significantly improved the cellular uptakes of Cypate and Ce6 by 3.7-fold and 3.4-

fold after 24 h incubation compared to free Cypate/ Ce6, respectively, indicating that the micelles can easily improve their cellular uptakes [45]. Possibly, free Cypate/Ce6 can easily aggregate into the clusters in aqueous environment, which result in poor cellular uptake, and the encapsulation of Cypate and Ce6 within the micelles can overcome this obstacle. The enhanced uptakes are expected to reach sufficient accumulation to trigger hyperthermia and intracellular singlet oxygen production. Subsequently, the endocytotic pathways of Cy/Ce6-Micelles were further elucidated using flow cytometry, and several inhibitors of endocytosis including chlorpromazine, filipin, and amiloride were used to inhibit various endocytotic pathways [46]. Fig. 2C shows that chlorpromazine as an inhibitor of clathrin-mediated endocytosis resulted in the decrease of 48% cellular uptake of Ce6, suggesting that Cy/Ce6-Micelles were mainly internalized *via* a clathrin-mediated endocytotic pathway, which plays a major role for the internalization of the micelles into cancer cells [46]. The treatment with filipin also decreased about 20.7% in cellular uptake of Ce6, suggesting that caveolae-mediated pathway might also contribute in part to cellular uptake of the micelles. In addition, low temperature (4 °C) also led to a significant decrease of cellular uptake, indicating that the endocytotic cellular uptake is also energy-dependent [47]. The clathrin-mediated pathway allows the micelles to undergo the endolysosomal transport for their intracellular delivery [45].

3.4. Photothermal cytotoxicity, destabilization of subcellular structure and synergistic photodynamic activity

To examine the photothermal injury of Cy/Ce6-Micelles on cancer cells, we incubated 4T1 cells with Cy/Ce6-Micelles for 24 h, followed by photoirradiation with various irradiation intensities (785 nm, 3 min). Cy/Ce6-Micelles could act as a non-toxic agent without photoirradiation owing to their negligible cytotoxicity (Fig. 2D). However, Cy/Ce6-Micelles induced severe photothermal damage on 4T1 cells under PTT treatment ($IC_{50} \sim 10.0 \mu\text{g/mL}$ at 1.5 W/cm^2), which was also correlative to the irradiation intensity and concentrations of Cypate (Fig. 2D) [15,31]. It indicates that Cy/Ce6-Micelles can generate severe photothermal cell damage owing to sufficient intracellular accumulation of Cypate and therefore hyperthermia.

In order to demonstrate the influence of Cy/Ce6-Micelles on the subcellular structure under 785 nm PTT treatment, we further monitored the photoirradiation-induced disruption of lysosomal membranes using acridine orange (AO) as an intracellular indicator, which can emit red fluorescence in intact acidic lysosomes, and generate green fluorescence in neutralized cytosol and nuclei. Fluorescence microscopy imaging shows that the acidic lysosomes in 4T1 cells treated with PBS exhibited red fluorescence without photoirradiation, which was similar to that suffered from PTT treatment (Fig. 3). It indicates that PBS did not change the lysosomal membranes under 785 nm irradiation. However, the red fluorescence from AO in the lysosomes was remarkably decreased in the presence of Cy/Ce6-Micelles ($0.2 \mu\text{g/mL}$ Cypate) upon PTT treatment (Fig. 3). It indicates that Cy/Ce6-Micelles can effectively disrupt the lysosomal membranes upon PTT treatment at a relatively low dose of Cypate. The disruption of lysosomal membranes is possibly attributed to the hyperthermia triggered by photo-thermal effect [23]. Additionally, the generation of singlet oxygen from Cypate might also contribute to the disruption of lysosomal membranes

owing to the photochemical internalization effect of the reactive oxygen species (ROS) on the lysosomal membranes [48,49]. Because the endolysosomal compartments are not highly susceptible to oxidative damage of ROS, the cytoplasmic distribution of PS is highly desirable to maximize their photodynamic activity [3]. In contrast, free Cypate/Ce6 did not result in the effective disruption of lysosomes at same dose of Cypate, possibly owing to their low cellular uptake (data were not shown). Obviously, Cy/Ce6-Micelles might escape from lysosomes into cytosol upon 785 nm PTT treatment and then facilitate the cytoplasmic delivery of Ce6 owing to the disruption of lysosomes, which may potentially contribute to the accessibility of Ce6 to target organelles such as nucleus and subsequent synergistic photodynamic damage of Cy/Ce6-Micelles.

To further demonstrate the synergistic photodynamic cytotoxicity of Cy/Ce6-Micelles on cancer cells, we incubated 4T1 cells with various formulations for 24 h, followed by various photoirradiation modalities of PTT and PDT. Cy/Ce6-Micelles exhibited no cytotoxicity in the absence of photoirradiation, and only exhibited a slight cytotoxicity under 785 nm PTT treatment (3 min, 1.5 W/cm²) in the relatively low concentration range of less than 3.0 µg/mL Cypate (Fig. 4). However, Cy/Ce6-Micelles exhibited much stronger cytotoxicity upon 660 nm PDT treatment with IC₅₀ value of about 1.5 µg/mL (10 min, 1.0 W/cm²). Importantly, Cy/Ce6-Micelles displayed the IC₅₀ value of 0.5 µg/mL when first treated with 785 nm PTT and then 660 nm PDT treatment, showing significantly stronger cytotoxicity than Cy/Ce6-Micelles under PTT or PDT treatment alone, and free Cypate/Ce6 under PTT/PDT treatments [5]. In contrast, Cy/Ce6-Micelles exhibited a higher IC₅₀ value (about 1.0 µg/mL) when first exposed to 660 nm PDT and then 785 nm PTT treatment. Obviously, the sequential PTT/PDT treatments resulted in a superior synergistic cytotoxicity of Cy/Ce6-Micelles compared to the reverse sequence of PDT/PTT treatments. The synergy of PTT/PDT is possibly due to the PTT-triggered disruption of lysosomal membranes and thus enhanced accessibility of PS to target organelles [48,49]. The enhanced accessibility is critical to the therapeutic index of PS since singlet oxygen has very short half-life and diffusion range in biological system [3].

3.5. In vivo biodistribution and NIRF imaging

To demonstrate the *In vivo* biodistribution behavior of Cy/Ce6-Micelles, we injected Cy/Ce6-Micelles intravenously into mice bearing 4T1 murine tumors and evaluated the *ex vivo* NIRF intensities of Cypate in various tissues at 24 h post-injection, which can semi-quantitatively reflect the biodistribution of Cypate. Cypate from Cy/Ce6-Micelles were mainly accumulated in the tumor, liver, and kidneys at 24 h post-injection (Fig. 5A). Cy/Ce6-Micelles exhibited the high accumulation of Cypate in the tumor area (Fig. 5B), which is much higher than that of free Cypate/Ce6 owing to the EPR effect of the micelles (Fig. 5C and D) [37,42]. However, free Cypate/Ce6 as the control exhibited a high accumulation at liver and had a poor ability to accumulate at tumor owing to the absence of the targeting effect (Fig. 5C and D). The targeting capacity of Cy/Ce6-Micelles to tumor provides a prerequisite for guiding subsequent PTT and PDT treatments.

To further demonstrate the capacity of Cy/Ce6-Micelles for cancer imaging, we subsequently evaluated the *In vivo* NIRF imaging of Cy/Ce6-Micelles on the mice bearing

4T1 tumors. Cy/Ce6-Micelles exhibited a significant tumor accumulation of NIRF signals from Cypate at 24 h post-injection (Fig. 5E and F), which is in accordance with its biodistribution as shown in Fig. 5B. In particular, Cy/Ce6-Micelles exhibited a relatively prolonged retention of NIRF signals in the tumor during the imaging period of 4 days when compared with free Cypate/Ce6 (Fig. S9), accompanying with low NIRF noise from some major normal tissues such as lung, kidneys and spleen at 24 h after post-injection. The enhanced accumulation of Cypate in tumor and its rapid clearance from normal tissues account for the high signal-to-noise ratio (SNR) at the tumor [14], which is highly key to achieve the ultrasensitive detection of tumor.

3.6. PA imaging

PA imaging can visualize microscopic biological tissues such as solid tumor and inner vasculatures [20,21]. To further demonstrate the capacity of Cy/Ce6-Micelles for cancer imaging with high spatial resolution and anatomical localization, we performed the *In vivo* PA imaging of Cy/Ce6-Micelles on 4T1 tumor xenograft model. Fig. 6A shows that the tumor displayed weak photoacoustic signals before injection owing to the low intrinsic absorption of oxyhemoglobin and deoxyhemoglobin in the NIR region. Only large tumor vessels were observed. However, PA imaging exhibited a clear three-dimensional tumor microstructure with high contrast and spatial resolution at 6 h post-injection of Cy/Ce6-Micelles as shown in Supplemental Video S1, and the PA signals at tumor were further enhanced at 24 h and 48 h post-injection (Fig. 6A), while PBS as the control showed no significant change of PA signals during 48 h post-injection, and free Cypate/Ce6 only had a negligible increase of PA signals as compared to Cy/Ce6-Micelles (Fig. S10). The increase of PA signals is highly advantageous to improve PA imaging contrast and spatial resolution, and also agrees with the *In vivo* NIRF imaging behavior. In particular, the enhanced signals after 24 h post-injection also revealed uniform distribution of Cypate within and outside the microvessels in the tumor, implying that Cy/Ce6-Micelles are capable of triggering effective photothermal and photodynamic damages on both tumor cells and vasculature upon photoirradiation at 24 h post-injection. Our results show that Cy/Ce6-Micelles exhibit a powerful capacity to visualize the tumor microstructure with high spatial resolution and imaging contrast for subsequent PTT/PDT treatments [21].

Supplementary video related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2014.02.018>

3.7. In vivo anticancer efficacy

To elucidate the *In vivo* synergistic efficacy of Cy/Ce6-Micelles by integrating PTT and enhanced PDT, Cy/Ce6-Micelles were injected into the mice bearing 4T1 tumor at the dose of 7.5 mg/kg Cypate and Ce6, and then the tumors were treated with various photoirradiation modalities at 24 h post-injection. Subsequently, tumor volumes were monitored for up to 16 days. The tumor volumes were normalized against their original volumes (0 day) for monitoring the tumor growth behavior (Fig. 7A). The control group (PBS) exhibited 9.0-fold increase of average tumor volume compared to their original volumes regardless of photoirradiation. Cy/Ce6-Micelles exhibited a significant photothermal injury on tumors under 785 nm PTT treatment, and triggered the tumor

necrosis 2 days post-injection, which resulted in a 5.4-fold increase of tumor volumes 16 days post-injection. Obviously, Cy/Ce6-Micelles might exhibit a significant hyperthermia and tumor inhibition effect owing to the sufficient tumor accumulation of Cypate *via* EPR effect, although there was no free Cypate/Ce6 as the control owing to their strong hydrophobicity. Especially, PTT does not require subcellular delivery of Cypate for triggering subcellular damage, so the enhanced tumor accumulation of Cypate can directly lead to preferable anticancer efficacy. Fig. 7A also shows that Cy/Ce6-Micelles triggered 7-fold increase of tumor volumes under PDT treatment, indicating that Cy/Ce6-Micelles exhibited photodynamic damage on tumors as well. Obviously, photodynamic activity of Cy/Ce6-Micelles exhibited less tumor inhibition effect as compared to their photothermal damage, which is possibly due to insufficient cytoplasmic distribution of PS and shallower tissue penetration depth of 660 nm light. Most interestingly, Cy/Ce6-Micelles caused the obvious tumor necrosis at 2 days post-injection and did not show significant tumor growth during 16 days under sequential PTT/PDT treatments, and achieved superior tumor inhibition effect (Fig. 7A), which is superior to those of Cy/Ce6-Micelles under PTT or PDT treatment alone owing to the synergy of PTT and PDT ($P < 0.01$). In contrast, Cy/Ce6-Micelles exposed to PDT/PTT treatments exhibited less tumor inhibition efficacy than those under the sequential PTT/PDT treatments, which is in accordance with their cytotoxic difference in cell culture as shown in Fig. 4. Obviously, the sequential PTT/PDT treatments result in a synergistic efficacy rather than a combinational effect. Possibly, the PTT-triggered disruption of lysosomal membranes contributes to the synergistic efficacy *via* the enhanced cytoplasmic delivery of Ce6, which provides an effective pathway to transport more PS to cytosol and targeted organelles, improves the susceptibility of cancer cells to PS, and finally facilitates the synergistic effect of PTT and PDT [48]. Because NIR light with good tissue transparency can effectively penetrate into inaccessible tumors, the micelles are highly potential to precisely treat the tumor with small size after precise localization. In particular, the theranostic micelles can provide the ultrasensitive detection of tumor with spatial resolution during a prolonged imaging period, which allow us to precisely treat tumor with minimized adverse side effect at normal tissues.

3.8. H&E staining

To further elucidate the *In vivo* synergistic effect of Cy/Ce6-Micelles under PTT treatment, Cy/Ce6-Micelles were injected into the mice bearing 4T1 tumor at the single dose of 7.5 mg/kg Cypate, and then the tumors were treated with or without photoirradiation at 24 h post-injection, followed by H&E staining of tumor sections at 6 h post-photoirradiation. Fig. 7B shows that Cy/Ce6-Micelles resulted in more severe cancer necrosis with less cancer cells and extensive hemorrhagic inflammation under PTT/PDT treatment as compared to those under PDT/PTT treatments, while those under PTT or PDT treatment only exhibited hemorrhagic inflammation and sporadic necrotic region surrounded by malignant cells with nuclear atypia. In contrast, PBS as the control did not exhibit obvious tumor necrosis with or without PTT/PDT treatments. The results suggest that Cy/Ce6-Micelles exhibit superior synergistic effect on tumor under sequential PTT/PDT treatments.

4. Conclusions

In summary, we have developed PS-loaded micelles integrating cyanine dye for precise anatomical tumor localization and superior synergistic therapy effect. The micelles possess enhanced stability, cell internalization and tumor accumulation, which result in high imaging contrast and spatial resolution for tumor localization. Moreover, the destabilization of the lysosomes under PTT treatment facilitates synergistic photodynamic activity of PS under subsequent PDT treatment *via* enhanced cytoplasmic delivery. Our strategy provides a dual-modal cancer imaging and subsequent synergistic regimen of PS, and has a great potential for clinical translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary material

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Scheme 1.

Schematic illustration of PS-loaded micelles integrating cyanine dye for dual-modal cancer imaging and synergistic therapy of PTT and PDT *via* an enhanced cytoplasmic delivery of PS.

Fig. 1.

(A) TEM image of Cy/Ce6-Micelles, (B) Size distribution of Cy/Ce6-Micelles, (C) Normalized absorbances of Ce6 in Cy/Ce6-Micelles in aqueous solution (pH 7.4) at different time, (D) Change of temperature of 0.5 mL Cy/Ce6-Micelles at various concentrations of Cypate as the function of photoirradiation time (1.5 W/cm^2), (E) Normalized fluorescent intensity of DPBF after trapping singlet oxygen from Cy/Ce6-Micelles at various concentrations of Cypate under 785 nm (1.5 W/cm^2) and 660 nm (1.0 W/cm^2) photoirradiation, (F) Normalized absorbances of Cypate and Ce6 in Cy/Ce6-Micelles under 785 nm and 660 nm photoirradiation, respectively, (G) Accumulative release profiles of Cypate from Cy/Ce6-Micelles and Free Cypate/Ce6, (H) *In vitro* PA imaging and (I) PA intensity (ROI) of Cy/Ce6-Micelles at 680, 780, and 880 nm wavelengths, respectively.

Fig. 2.

(A) Amounts of internalized Cypate by 4T1 cells after 24 h incubation with Cy/Ce6-Micelles and free Cypate/Ce6 containing 10.0 $\mu\text{g}/\text{mL}$ Cypate/Ce6, (B) Relative amounts of Ce6 by 4T1 cells after 24 h incubation with Cy/Ce6-Micelles and free Cypate/Ce6 containing 10.0 $\mu\text{g}/\text{mL}$ Cypate/Ce6, respectively, (C) Relative fluorescent intensity of Cy/Ce6-Micelles internalized by 4T1 cells treated with PBS (control), chlorpromazine (10.0 $\mu\text{g}/\text{mL}$), filipin (5.0 $\mu\text{g}/\text{mL}$), amiloride (100.0 $\mu\text{g}/\text{mL}$) at 37 °C, and PBS at 4 °C using *in vitro* flow cytometry analysis, (D) Cell viability of 4T1 cells treated with Cy/Ce6-Micelles at various concentrations under darkness, 1.0 W/cm², 1.5 W/cm², and 2.0 W/cm² photoirradiation (3 min), respectively.

Fig. 3.

Observation of lysosomal disruption of 4T1 cells treated with Cy/Ce6-Micelles with 3 min photoirradiation (1.5 W/cm^2) using AO staining. The cells were respectively treated with PBS under 785 nm photoirradiation (A), PBS without photoirradiation (B), Cy/Ce6-Micelles containing 0.2 µg/mL Cypate under 785 nm photoirradiation (C), Cy/Ce6-Micelles containing 0.2 µg/mL Cypate without photoirradiation (D), Cy/Ce6-Micelles containing 0.5 µg/mL Cypate under 785 nm photoirradiation (E), Cy/Ce6-Micelles containing 0.5 µg/mL Cypate without photoirradiation (F), Cy/Ce6-Micelles containing 2.0 µg/mL Cypate under 785 nm photoirradiation (G), Cy/Ce6-Micelles containing 2.0 µg/mL Cypate without photoirradiation (H), followed by AO staining. AO as an intracellular indicator emits red fluorescence in acidic lysosomes ($610\text{--}640 \text{ nm}$, red), and displays green fluorescence in neutralized cytosol and nuclei ($515\text{--}545 \text{ nm}$, green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Cell viability of 4T1 cells treated with Cy/Ce6-Micelles and free Cypate/Ce6 at various concentrations under various photoirradiation modalities including 785 nm PTT (3 min, 1.5 W/cm²) and 660 nm PDT (10 min, 1.0 W/cm²) treatments, respectively.

Fig. 5.

(A) *Ex vivo* imaging and (B) NIRF intensities of Cypate from Cy/Ce6-Micelles in heart, liver, spleen, lung, kidney, and tumor of the mice bearing 4T1 tumor at 24 h post-injection at the dose of 7.5 mg/kg Cypate/Ce6, respectively, (C) *Ex vivo* imaging and (D) NIRF intensities of Cypate from Free Cypate/Ce6 in heart, liver, spleen, lung, kidney, and tumor of the mice bearing 4T1 tumor at 24 h post-injection at the dose of 7.5 mg/kg Cypate/Ce6, respectively, (E) *In vivo* NIRF imaging and (F) Its NIRF intensities of the mice bearing 4T1 tumor injected with Cy/Ce6-Micelles at the dose of 7.5 mg/kg Cypate/Ce6 at 24, 48, 72 h and 96 h post-injection, respectively.

Fig. 6. (A) *In vivo* PA imaging and (B) Calculated PA intensity (ROI) of the mice bearing 4T1 tumor injected with PBS and Cy/Ce6-Micelles at the dose of 7.5 mg/kg Cypate/Ce6 at pre-injection, and 6, 24, and 48 h post-injection, respectively ($n = 3$).

Fig. 7.

(A) Tumor growth inhibition profiles of the mice bearing 4T1 tumor injected with Cy/Ce6-Micelles at the dose of 7.5 mg/kg Cypate/Ce6 on day 0, 2, and 4, followed by various photoirradiation treatments at 24 h post-injection including 785 nm PTT (5 min, 1.0 W/cm²) and 660 nm PDT (10 min, 1.0 W/cm²) treatments, respectively ($n = 4$). (B) Images of H&E-stained tumor sections harvested from the mice suffered from the various treatments including PDT alone, PTT alone, PDT/PTT treatments and PTT/PDT treatments after intravenous administration of PBS and Cy/Ce6-Micelles at the dose of 7.5 mg/kg Cypate/Ce6.