Redox-responsive Supramolecular Amphiphilies Constructed via Host-guest Interaction for Photodynamic Therapy

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Redox-responsive Supramolecular Amphiphilies Constructed via Host-guest Interaction for Photodynamic Therapy

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A supramolecular photosensitizer delivery system has been established through the self-assembly of supramolecular amphiphilies constructed by the host-guest interaction between poly(ethylene glycol)-*β*-cyclodextrin (PEG-*β*-CD) and adamantane-terminated porphyrin derivative bearing disulfide bond (TPPC6-SS-Ada). TPPC6-SS-Ada/PEG-*β*-CD supramolecular amphiphilies can self-assemble into spherical micelles in water, and the assembled morphology was respectively characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS). Intracellular uptake and localization of supramolecular photosensitizers were further investigated by flow cytometry and confocal laser scanning microscopy (CLSM), and the result indicated that TPPC6-SS-Ada/PEG-*β*-CD micelles could be effectively up-taken by MCF-7 cells. Furthermore, the phototoxicity evaluated by MTT assay showed TPPC6-SS-Ada/PEG-*β*-CD micelles have very low dark toxicity but greater photo-toxicity compared to free porphyrin. Thus, TPPC6-SS-Ada/PEG-*β*-CD micelles would provide the potential application for photosensitizer delivery.

Introduction

Photodynamic therapy (PDT) is an extraordinary method to treat cancer which employs a photosensitizer (PS) molecule that absorbs light such as laser and visible light,[1-5] and produces reactive oxygen species (ROS), including singlet oxygen ($^1O_2$) and hydroxyl radicals that can kill cancer cells by destroying DNA, RNA, lipids and proteins.[6-9] Porphyrin and their derivatives, as one class of important photosensitizers, usually generate cytotoxic $^1O_2$ under appropriate light irradiation to achieve treatment of diseased tissue.[10-12] However, when the concentration of the porphyrin reached a high level in aqueous solution, they would easily aggregate with each other, since π-π stacking leads to greatly reduce the yield of $^1O_2$, and then decrease the therapeutic efficiency. To address this issue, hydrophilic building blocks are often introduced to improve the solubility and suppress quenching effect of hydrophobic porphyrin molecules.[13] Moreover, there are many other advantages for the introduction of hydrophiphilic blocks such as prolonged circulation time, decreased adverse effects and enhanced accumulation in tumor tissues.[14] Poly(ethylene glycol) (PEG), for example, has been frequently employed to construct amphiphilic photosensitizers as a hydrophilic segment due to its outstanding water solubility and biocompatibility.[15, 16]

In the past few decades, supramolecular amphiphilic systems, especially formed by incorporation of host-guest inclusion complexation, have been developed for drug release.[17] Macrocyclic hosts have widely been used to construct the host-guest complexation including crown ether, cyclodextrin, calixarene, cucurbituril, pillararene and their derivatives. Among them, cyclodextrins (CDs) have been extensively utilized to form inclusion complexes with drugs due to their low toxicity and low immunogenicity.[18] CDs are a series of natural cyclic oligosaccharides composed of *D*-glucose units that are connected by *α*-1, 4-glucosidic linkages and named *α*-, *β*-, *γ*-CD, respectively. The most prominent structural feature of cyclodextrins is the hydrophobic, well-defined cavity and the hydrophilic shell with exterior hydroxyls. The molecular hosts CDs and their hydrophilic derivatives can form inclusion complexes with a variety of guest species such as adamantane, ferrocene and *trans*-azobenzene in their hydrophobic inner cavity in aqueous media.[19-21] The host-guest inclusion interactions are mainly governed by van der Waals forces and hydrophobic interactions,[22] while the exterior hydroxyls of CDs could improve their water solubility and bioavailability of the drugs. During the past few years, CDs and their derivatives have been recognized and frequently investigated as enzyme models and building blocks for the construction of drug

delivery system. Yuan and coworkers employed PEG homopolymer modified with *β*-CD (PEG-*β*-CD) and poly(L-lactide) homopolymer modified with ferrocene (PLLAFc) to build a non-covalent supramolecular block copolymer PLLA-Fc/PEG-*β*-CD through host–guest interaction between the *β*-CD and the Fc moieties, and this amphiphilic supramolecular block copolymer was further used for drug delivery.[23] Ji and coworkers built biocompatible polymer vesicles through inclusion complexation between *α*-cyclodextrins (*α*-CDs) and double-hydrophilic poly(ethylene oxide)-*b*-poly(2-methacryloyloxyethyl phosphorylcholine) (PEO-*b*-PMPC) in aqueous media for drug delivery.[24] Zhang and coworkers reported a kind of polymer micelles formed via the host–guest interaction between *α*-CD and poly(ε-caprolactone) (PCL) for efficiently loading prednisone acetate.[25] To our best knowledge, there are few reports about supramolecular amphiphilic systems used for photodynamic therapy (PDT).

More recently, stimuli-responsive micelles have drawn a lot of attention due to sensitive response of intracellular microenviromental stimuli, including pH,[26-28] temperature,[29, 30] redox,[31, 32] light[33, 34] and so on. This kind of carriers can quickly enter cells and rapidly release the payload, which could be triggered by microenviromental stimuli when they arrived at lesion site, resulting in lethality toward tumor cells and maximal therapeutic efficacy with fewer side effects.[35] Among all kind of possible stimuli-responsive micelles, reduction-responsive micelles containing disulfide linkages may be the most promising nanocarriers on account of the different redox potential between the extracellular milieu and the intracellular microenvironment. The disulfide linkages are sufficiently stable in the circulation and the mildly oxidizing extracellular milieu while cleave rapidly under the reducing intracellular microenvironment, such as high concentration of glutathione (GSH), a thiol-containing tripeptide. The level of GSH in the cytosol is around 2-10 mM, which is significantly higher than that in the blood and extracellular fluids (about 2 μM in blood).[36] Therefore, the difference of GSH concentration provides a unique advantage to reduction-responsive micelles containing disulfide linkages as drug delivery system. Oh and coworkers reported nanocarriers self-assembled by dual disulfide located degradable polylactide (PLA)-based block copolymer for rapid drug release.[37] Hubbell and coworkers reported polymersomes constructed through AB diblock copolymers with an intervening disulfide (PEG_{17}-SS-PPS_{30}) for intracellular drug delivery.[38]

In this paper, a novel type of reduction-responsive supramolecular amphiphilic micelles based on disulfide linkage was designed as a photosensitizer delivery system. The TPPC6-SS-Ada/PEG-*β*-CD supramolecular amphiphile formed through the host-guest interaction between cyclodextrin and adamantine moieties, and then self-assembled into spherical micelles in water. The assembled morphology was respectively characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS). Intracellular uptake and localization of TPPC6-SS-Ada/PEG-*β*-CD micelles were further investigated by flow cytometry and confocal laser scanning microscopy (CLSM), and the phototoxicity of the micelles was evaluated by MTT assay.

Experimental Section

Materials

β-cyclodextrin (Shanghai Seebio Biotechnology, Inc., China) was recrystallized 3 times by deionized water. DMF was dried over calcium hydride and distilled before use. Methoxy poly(ethylene glycol) (PEG, M_n = 2 000), propargyl bromide, 1-adamantanamine, 4′, 6-diamidino-2-phenylindole (DAPI) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Aladdin and used as received. All other reagents and solvents were analytical pure and used as received without further purification.

Characterization

^{1}H NMR, ^{13}C NMR spectra were recorded at 400MHz, using BRUKER AV400 Spectrophotometer in $CDCl_3$ with tetramethylsilane (TMS) as an internal reference. The absorption spectrum was recorded on an AVATAR 360ESP FT-IR spectrometer and the result was collected at 30 scans with a spectral resolution of 1 cm^{-1}. TOF-MS was performed using a AB Sciex 4800 Plus MODI TOF/TOF Analyzer. The UV-Vis spectra of the samples were measured over different irradiation time intervals by using a Thermo Scientific Evolution 220 spectrophotometer. Dynamic light scattering (DLS) measurements were carried out at BECKMAN COULTER Delasa Nano C particle analyzer. Cellular Uptake was measured by BD FACS Calibur flow cytometer and Nikon AIR. All the measurements were carried out at room temperature. Transmission electron microscopy (TEM) analysis was performed on a JEOL JEM1400 electron microscopes operated at 100 kV. Samples for TEM were prepared by dropping the micelle solution onto a carbon-coated copper grid and then dried at room temperature. SEM was performed using a NOVA NanoSEM 230 electron microscope at an acceleration voltage of 1 kV. The micelle solution was directly dropped onto a freshly cleaved mica and then dried at room temperature. Before the measurement, the sample was sputtered by gold.

Synthesis of 5-(4-Hydroxylphenyl)-10, 15, 20-triphenylporphyrin (TPP-OH)

TPP-OH was synthesized according to our previous work.[39] ^{1}H NMR (400 MHz, $CDCl_3$), δ ppm: 8.86 (m, 8H, *β-H*), 8.21 (m, 6H, 10, 15, 20-Ar-*o-H*), 8.08 (m, 2H, 5-Ar-*o-H*), 7.75 (m, 9H, 10, 15, 20-Ar-*m-* and *p-H*), 7.22 (m, 2H, 5-Ar-*m-H*), -2.77 (s, 2H, -N*H*-). ^{13}C NMR(90.5 MHz, $CDCl_3$), δ ppm: 155.8, 142.6, 136.2, 135.0, 132.0-121.0, 128.1, 127.1, 120.5, 114.1. TOF-MS: m/z calcd for $[M+H]^+$: 630.20; found: 630.2487.

Synthesis of 6-(5′-(4′-Phenoxyl)-10′, 15′, 20′-triphenylporphyrin)-1-hexanol (TPPC6-OH)

TPPC6-OH was also synthesized according the previous work.[39] ^{1}H NMR (400 MHz, $CDCl_3$), δ ppm: 8.87 (m, 8H, *β-H*), 8.21 (m, 6H, 10, 15, 20-Ar-*o-H*), 8.11 (m, 2H, 5-Ar-*o-H*), 7.76

(m, 9H, 10, 15, 20-Ar-*m*- and *p*-*H*), 7.28 (m, 2H, 5-Ar-*m*-*H*), 4.26 (t, 2H, -O-CH_2-CH$_2$-), 3.75 (t, 2H, -CH_2-OH), 2.00 (m, 2H, -O-CH$_2$-CH_2-CH$_2$-), 1.76-1.59 (m, 6H, -CH$_2$-(CH_2)$_3$-CH$_2$-OH), -2.77 (s, 2H, -N*H*-). ^{13}C NMR(90.5 MHz, CDCl$_3$), δ ppm: 158.8, 142.7, 136.2, 135.0, 132.0-131.0, 128.2, 127.1, 120.5, 113.1, 66.9, 63.0, 45.0, 32.6, 29.0, 27.5. TOF-MS: m/z calcd for [M+H]$^+$: 730.35; found: 730.3384.

Synthesis of Disulfide-modified Carboxyl Terminal Porphyrin (TPPC6-SS-COOH)

TPPC6-SS-COOH was prepared by the esterification reaction between TPPC6-OH and 3, 3'-thiodipropionic acid. Firstly, 3, 3'-thiodipropionic acid (1 g, 5.6 mmol) was dissolved in 20 mL of DMF at room temperature, then DCC (0.387 g, 1.87 mmol) and DMAP (55 mg, 0.45 mmol) were added in solution in ice bath. TPPC6-OH (0.3 g, 0.41 mmol) were dissolved in 20 mL of DMF and slowly dropped into the solution. Then the reaction was performed at 25 ^{o}C for 24 h. The mixture solution was washed with NaCl solution three times, extracted with dichloromethane, and dried with anhydrous MgSO$_4$. Then the solvent was removed by evaporation. The crude product was purified by column chromatography on silica, eluting with dichloromethane. Yield: 0.34 g (90.1%). ^{1}H NMR (400 MHz, CDCl$_3$), δ ppm: 8.89 (m, 8H, *β*-H), 8.24 (m, 6H, 10, 15, 20-Ar-*o*-H), 8.14 (m, 2H, 5-Ar-*o*-H), 7.78 (m, 9H, 10, 15, 20-Ar-*m*- and *p*-H), 7.30 (m, 2H, 5-Ar-*m*-H), 4.25 (t, 2H, -O-CH_2-CH$_2$-), 4.19 (m, 2H, -O-CH_2-), 2.9 (m, 4H, -S-CH_2-), 2.75 (m, 2H, -CO-CH_2-), 2.60 (m, 2H,-CH_2-COOH), 1.76-1.59 (m, 6H, -CH$_2$-(CH_2)$_3$-CH$_2$-OH), -2.77 (s, 2H, -N*H*-). ^{13}C NMR(90.5 MHz, CDCl$_3$, 20 ^{o}C), δ ppm: ^{13}C NMR(90.5 MHz, CDCl$_3$, 20 ^{o}C), δ ppm: 171.1 159.1, 142.7, 134.9, 131.0-130.0, 128.1, 127.1, 120.0, 112.7, 68.0, 65.1, 34.2, 33.9, 33.4, 32.9, 29.4, 28.7, 26.0, 25.8, 24.9. MALDI-TOF-MS: m/z calcd for: 923.30; found: 923.3286.

Synthesis of TPPC6-SS-Ada

TPPC6-SS-COOH (0.34 g 0.37 mmol), amantadine (0.22 g, 1.47 mmol) and NHS (0.079 g, 0.69 mmol) were dissolved in about 10 mL of DMF. EDC (0.13 g, 0.68 mmol) was dissolved in DMF and slowly dropped into above solution under ice bath. Then the reaction was carried out at 25 ^{o}C for 24 h. The product was extracted with dichloromethane, and dried with anhydrous MgSO$_4$. Then the solvent was removed by evaporation, and the crude product was purified by column chromatography on silica, eluting with petroleum ether/ethyl acetate (2:1, v/v) as the eluent. Yield: (0.34g, 88.1%). ^{1}H NMR (400 MHz, CDCl$_3$), δ ppm: 8.89 (m, 8H, *β*-H), 8.24 (m, 6H, 10, 15, 20-Ar-*o*-H), 8.14 (m, 2H, 5-Ar-*o*-H), 7.78 (m, 9H, 10, 15, 20-Ar-*m*- and *p*-H), 7.30 (m, 2H, 5-Ar-*m*-H), 4.25 (t, 2H, -O-CH_2-CH$_2$-),4.19 (m, 2H,-O-CH$_2$-), 2.9 (m, 4H, -S-CH_2-), 2.75 (m, 2H, -CO-CH_2-), 2.60 (m, 2H,-CH_2-COOH), 2.10 (s, 3H, -CH$_2$-C*H*(CH$_2$)-CH$_2$-), 1.98 (d, 6H, -C(CH_2)$_3$-CH(CH$_2$)$_2$-), 1.78 (q, 6H, -CH(CH$_2$)$_2$-CH_2-CH(CH$_2$)$_2$-), 1.76-1.59 (m, 6H, -CH$_2$-(CH_2)$_3$-CH$_2$-OH), -2.77 (s, 2H, -N*H*-). ^{13}C NMR(90.5 MHz, CDCl$_3$), δ ppm: 172.1, 170.0, 158.8, 142.6, 136.2, 135.0, 132.0-121.0, 128.2, 126.9, 120.0, 113.1, 68.2, 65.0, 52.1, 41.9, 57.0, 56.5, 54.2, 54.0, 53.3, 28.5, 28.3, 26.0. MALDI-TOF-MS: m/z calcd for: 1056.44; found: 1056.4514.

Preparation of TPPC6-SS-Ada/PEG-*β*-CD micelles

TPPC6-SS-Ada and PEG-*β*-CD were dissolved in DMF respectively to get solutions with concentration of 1 mg/mL. Then, the DMF solution of TPPC6-SS-Ada (1 mL) and the DMF solution of PEG-*β*-CD (3 mL) were mixed and stirred at 25 ^{o}C for 24 h, where the molar ratio of porphyrin moiety to cyclodextrin moiety is around 1:1. After that, 1 mL of deionized water was slowly dropped into above 1 mL of mixed solution of TPPC6-SS-Ada and PEG-*β*-CD at a rate of 0.02 mL/min under stirring. After the solution was stirred overnight, the solution was dialyzed against deionized water to remove DMF using dialysis membrane (MWCO = 12 000).

Critical Micellization Concentration (CMC) Measurement

A certain amount of pyrene in acetone was added into a series of volumetric flasks, and the acetone was allowed to evaporate. Then, a predetermined volume of micellar solution and deionized water was added into volumetric flasks to get solutions with different micellar concentrations from 2×10^{-4} to 4×10^{-2} mg/mL. These solutions were gently shaken and then equilibrated at 25 °C for at least 24 h. The excitation spectra of pyrene with different micellar concentrations were measured at the detection emission wavelength (390 nm).

Reduction-triggered Destabilization of TPPC6-SS-Ada/PEG-*β*-CD micelles

The micellar solution (8 mL, 0.5 mg/mL) were divided in three equal parts, and two of them were treated with 10.0 mM GSH under stirring for 4 h and 24 h respectively. The other one was treated without GSH under stirring for 24 h. The changes of size and polydispersity index (PDI) of these samples were measured using DLS at room temperature, and the morphology and average size were measured using TEM and DLS, respectively.

The release of porphyrin from TPPC6-SS-Ada/PEG-*β*-CD micelles was studied by using a dialysis tube (MWCO = 12 000) at 37 °C in 150 mL of PBS (pH = 7.4) with or without 10 mM of GSH. The release media (1 mL) was taken out at regular time intervals and then an equal volume of fresh media were replenished. The amount of released porphyrin was determined by using fluorescence measurements. The release experiments were conducted in triplicate, and the final results are the average data.

Cell Culture

MCF-7 human breast adenocarcinoma line were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (50 units/mL penicillin and 50 units/mL streptomycin) under a humidified atmosphere containing 5% CO_2 at 37 °C.

Cellular Uptake of TPPC6-SS-Ada/PEG-*β*-CD Micelles by MCF-7 cells

For flow cytometry analysis, MCF-7 cells were seeded in 6-well plates at 1×10^6 cells per well and cultured for 24 h. Then MCF-7 cells were treated with micelles for 4 h and 24 h. The porphyrin concentration of each group is 50 μg/mL. At the same time, MCF-7 cells treated with equivalent free porphyrin for 4 h and 24 h were set as the control. Subsequently, the cells were trypsinized and resuspended in PBS. The fluorescence intensity was measured by a BD FACS Calibur flow cytometer. The data was analyzed with Flow Jo.

For confocal laser scanning microscopy (CLSM), MCF-7 cells were seeded in 6-well plates at 5×10^4 cells per well in 1.0 mL of complete DMEM, and cultured for 24 h. A certain amount of TPPC6-SS-Ada/PEG-*β*-CD micelles were added, and then cells were incubated for 4 h and 24 h without irradiation. As a control experiment, a certain amount free porphyrin also was added in control groups, and then cells were incubated for 4 h and 24 h. The medium was removed after cultivating, and MCF-7 cells were washed 3 times with PBS. Then cells were fixed with 4% paraformaldehyde for 30 min and washed 3 times with PBS. Finally, we used 4′, 6-diamidino-2-phenylindole (DAPI) to stain cell nucleus for 3 min and washed with PBS 3 times.

Dark Toxicity of TPPC6-SS-Ada/PEG-*β*-CD Micelles

The MCF-7 cells were seeded at density of 4×10^3 cells/well in 96-well plate. After incubating for 24 h, the cells were treated with different concentrations of TPPC6-SS-Ada/PEG-*β*-CD micelles and free porphyrin in FBS-free DMEM at 37 °C for 24 h in the dark. Subsequently, the cells were washed with sterilized PBS 3 times. Fresh DMEM with 20 μL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) was used to substitute the culture medium. The cells were incubated for another 4 h at 37 °C. After that, MTT-containing solution was removed and 150 μL of DMSO was added with gentle shake to extract the formazan products. Meanwhile, the cells incubated in FBS-free DMEM (without micelles or porphyrin) were used as a control. Then, the absorbance was measured at 492 nm using a spectrophotometric microplate reader (THERMO Multiskan MK3 spectrometer). The percentage of cell viability (%) was calculated according to the equation:

cell viability (%) = $(OD_{test}-OD_{back\ ground})/(OD_{control}-OD_{back\ ground}) \times 100$,

where, OD_{test} was the absorbance at the presence of sample solutions, and $OD_{control}$ was the absorbance of control group. This experiment was conducted in triplicate, and the significant difference was calculated by means of one-way analysis of variance (ANOVA).

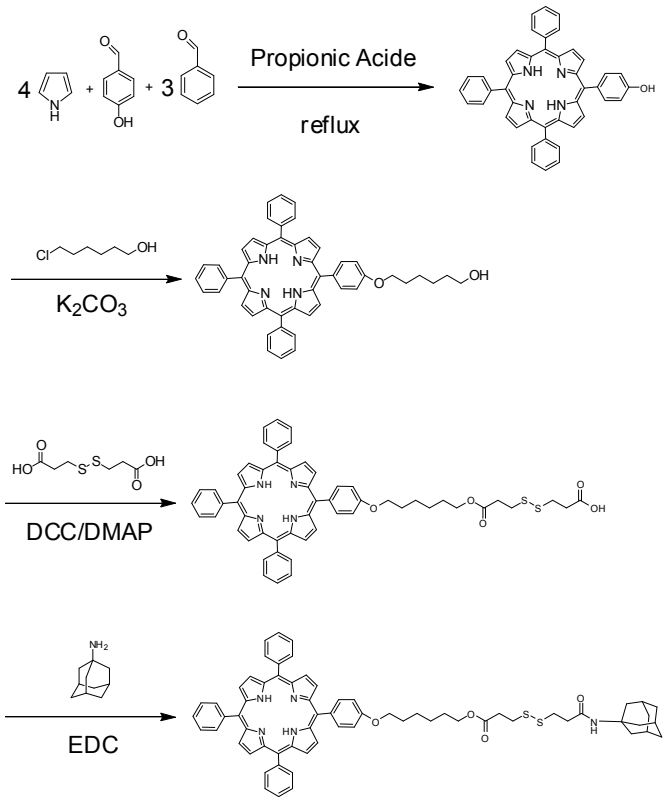
In vitro Phototoxicity of TPPC6-SS-Ada/PEG-*β*-CD micelles

The MCF-7 cells were seeded at density of 4×10^3 cells/well in 96-well plate and incubated for 24 h, then treated with different concentrations of TPPC6-SS-Ada/PEG-*β*-CD micelles and free porphyrin respectively in DMEM at 37 °C for another 24 h in the dark. Subsequently, the cells were washed with sterilized PBS 3 times, and the culture medium was replaced with fresh DMEM with 20 μL of MTT solution (5 mg/mL). The cells were irradiated with visible light (400 mW/cm^2) for 20 min, and then continuously incubated for another 24 h at 37 °C, and finally cell viability was also evaluated using MTT assay. All procedures were carried out in the dark after the addition of micelles or free porphyrin into plates. This experiment was conducted in triplicate, and the significant difference was also calculated by means of one-way analysis of variance (ANOVA).

Results and discussion

Synthesis and Characterization of TPPC6-SS-Ada and PEG-*β*-CD

TPP-OH was first synthesized according to previous literature (**Fig. S1-3**).[39-41] Then it was extended to prepare TPPC6-OH (**Fig. S4-6**) using 6-chloro-1-hexanol.[39] The esterification reaction between TPPC6-OH and 3, 3'-thiodipropionic acid was used to produce TPPC6-SS-COOH (**Fig. S7-9**). The ^{1}H NMR spectrum of TPPC6-SS-COOH is shown in **Fig. S7**. The signals at δ = 8.87-7.28 ppm and -2.77 ppm are separately assigned to aromatic and pyrrole ring protons, and the signals at δ = 2.9 ppm, 2.75 ppm and 2.60 are ascribed to methylene protons adjacent to the disulfide bond, indicating that TPPC6-SS-COOH was successfully synthesized. Finally, the novel porphyrin derivative, namely porphyrin-adamantane dyad (TPPC6-SS-Ada) was prepared by amidation reaction between TPPC6-SS-COOH and amantadine. The ^{1}H NMR spectrum of TPPC6-SS-Ada is shown in **Fig. 1** and the ^{13}C NMR spectrum and MALDI-TOF-MS spectrum of TPPC6-SS-Ada are shown in **Fig. S10** and **Fig. S11**, respectively. Beside the protons' signals of porphyrin moiety in the downfield, the signals at δ = 2.0 ppm, 1.9 ppm and 1.79 ppm are assigned to adamantane protons, which suggests that TPPC6-SS-Ada was successfully prepared. Each step of the synthesis process was shown in **Scheme 1**. Additionally, the UV-Vis absorption of TPPC6-SS-Ada was shown in **Fig. S20**. An intense peak centered at 418 nm (Soret-band, characteristic absorption of electronic spectrum of porphyrin) and a less intense peak at 548 nm (Q band) all could be observed in the absorption spectrum of TPPC6-SS-Ada. To well study the reduction-responsive property, we also synthesized a control sample, TPPC6-Ada without disulfide bond, and the preparation procedure and characterization of TPPC6-Ada were given in Supporting information (**Scheme S1**, **Fig. S12**).



Scheme 1. Synthesis of TPPC6-SS-Ada.

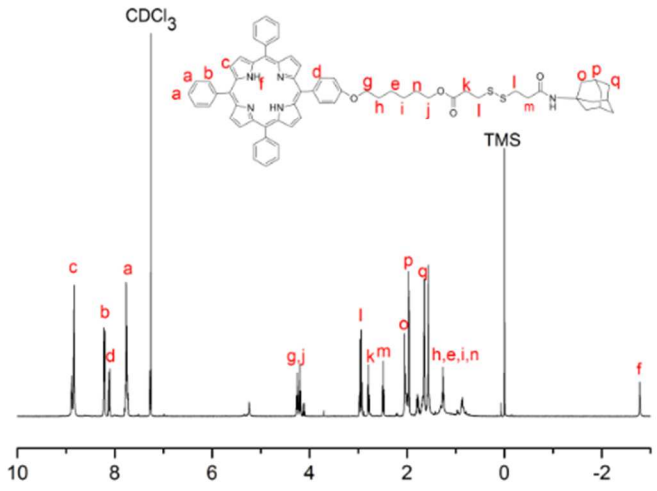
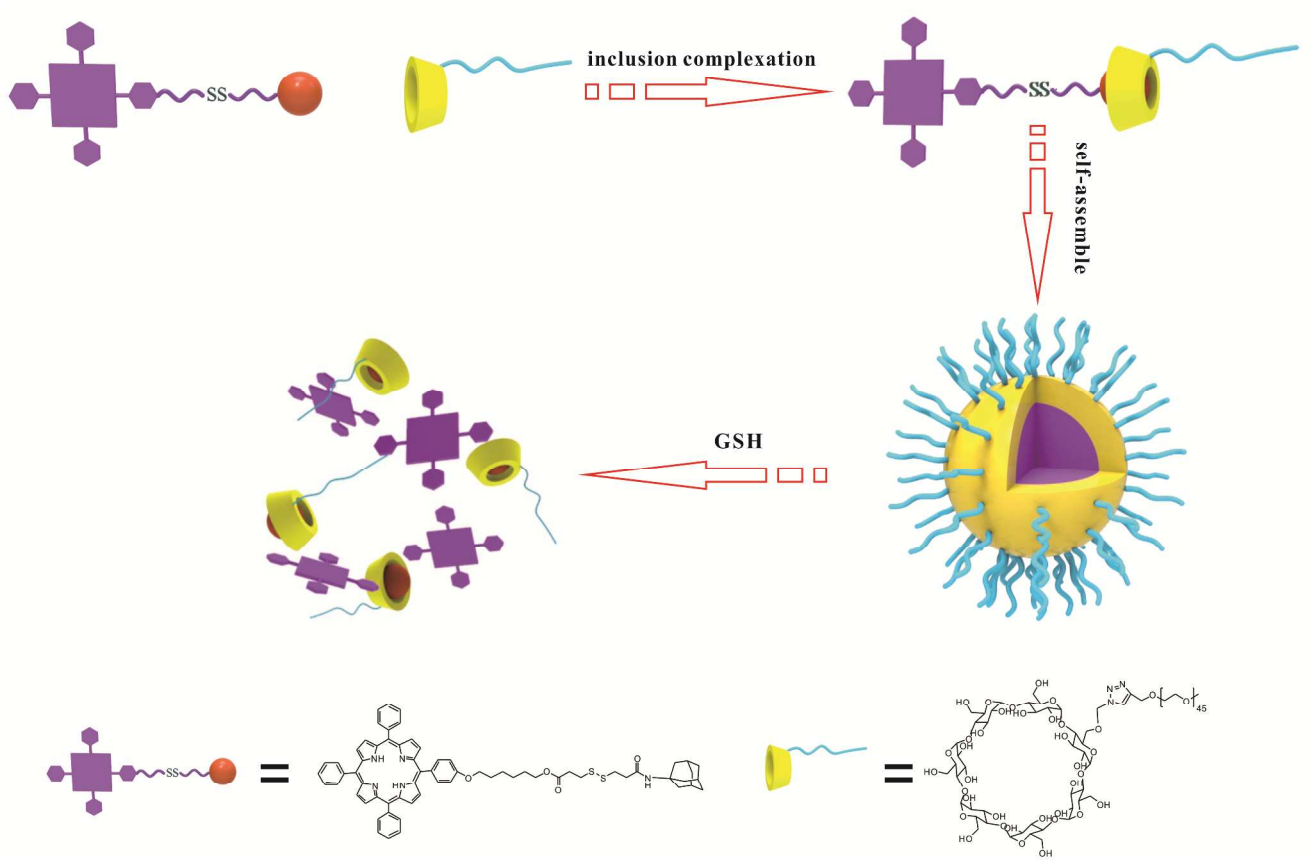


Fig. 1. ^{1}H NMR spectrum of TPPC6-SS-Ada.

The synthesis process of PEG-*β*-CD was synthesized according to the literature.[42, 43] The FT-IR spectrum of azide-modified *β*-CD (*β*-CD-N_3) is shown in **Fig. S13**. A sharp peak at 2 017 cm^{-1} is ascribed to the asymmetric stretching of the azide group of CD-N_3. *Alkynyl*-terminated PEG was prepared through the reaction between PEG-OH and propargyl bromide. PEG-*β*-CD was prepared through click chemistry between *β*-CD-N_3 and *alkynyl*-terminated PEG. After click reaction, the disappearance of azide peak at 2 017cm^{-1} demonstrated PEG-*β*-CD was successfully synthesized (**Fig. S13**). The ^{1}H NMR of PEG-*β*-CD is shown in **Fig. S14**. The signal of δ = 8.02 ppm is assigned to the proton of 1, 2, 3-triazole ring, further indicating the occurrence of click cycloaddition.

Characterization of Micelles

To our knowledge, amphiphilic block copolymers with both hydrophilic and hydrophobic segments could self-assemble into a variety of morphologies in aqueous solution, such as spherical micelles,[44] cylinders[45] and vesicles.[46] The morphologies and sizes of assemblies are mainly dependent on the composition and concentration of the amphiphilic copolymer, and properties of common solvent. In this work, the DMF solution of TPPC6-SS-Ada and PEG-*β*-CD were mixed and stirred at 25 °C for 24 h in order to get orthogonal assembly of host-guest supramolecular complex through host-guest interactions



Scheme 2. Self-assembly and disaggregation process of TPPC6-SS-Ada/PEG-*β*-CD micelles.

between the moiety of CDs and Ada，then the deionized water was slowly dropped into mixed solution under stirring, which induced supramolecular complex to self-assemble into micelles. Finally, the solution of micelles was obtained after dialyzing against deionized water to remove DMF. The formation process of supramolecular photosensitizer and the self-assembly process of micelles were shown in **Scheme 2.**

Critical micelle concentration (CMC) of TPPC6-SS-Ada/PEG-*β*-CD was determined by fluorescence spectroscopy using pyrene as a hydrophobic probe. The excitation spectra of pyrene with different micellar concentrations were measured at the detection emission wavelength (390 nm). The CMC value was obtained from the intersection of two tangent of the line of I_{382}/I_{372}. The value of CMC is about 5.9×10^{-3} mg/mL (**Fig. S15**). The low CMC value of supramolecular amphiphiles could guarantee the stability of micelles in dilute aqueous solution.[47] Similarly, the value of CMC the control, TPPC6-Ada/PEG-*β*-CD was also measured, which is about 4.5×10^{-3} mg/mL (**Fig. S16**). The low CMC value of supramolecular amphiphiles could guarantee the stability of micelles in dilute aqueous solution

The hydrodynamic diameter and polydispersity index (PDI) of TPPC6-SS-Ada/PEG-*β*-CD micelles were measured by dynamic laser light scattering (DLS). As shown in **Fig. 2a**, the average size of micelles is 85.2 nm and polydispersity index is 0.073. In order to intuitively observe the morphology and size of micelles, we used transmission electron microscopy (TEM) and scanning electron microscopy (SEM) morphology analysis. Samples were placed on copper mesh, and dried in air at 37 °C before measurements. As we expected, the supramolecular photosensitizer self-assembled into sphere micelles, as shown in **Fig. 3a** and **Fig. S17**. The average particle size measured by TEM and SEM is about 72 nm, which is apparently smaller than hydrodynamic diameter measured by DLS. This can be attributed to the fact that the hydrodynamic diameter is z-averages and PEG chains are extended in aqueous solution.

The reduction property of supramolecular spherical micelles was further studied using DLS and TEM. The micellar solution was divided into three equal parts, and then 10 mM of GSH was added in two of these samples respectively for 4 h and 24 h under stirring. After adding GSH for 4 h, average micelle size increased from 85.2 nm to about 176 nm with concomitant increase of PDI from 0.073 to 0.158 (**Fig. 2c**). Even some large spherical aggregates with the size of over 1 μm could be observed in **Fig. 3b**. After adding GSH for 24 h, average micelle size further increased to 274 nm and the PDI is 0.137 from DLS result (**Fig. 2d**), but there is no clear morphology form TEM image (**Fig. 3c**). The big aggregates were formed due to reductive cleavage of the disulfide bonds with GSH. Additionally, almost no change in the size and PDI of the micelles were observed after 24 h in the absence of GSH (**Fig. 2b**). For the control, TPPC6-Ada/PEG-*β*-CD micelles without disulfide bonds, it can be seen that there is almost no change in micelle size even with GSH for 24 h (**Fig. S18)** and no big aggregate formation (**Fig. S19**). This further confirms the TPPC6-SS-Ada/PEG-*β*-CD micelle is reduction-responsive to GSH, which is a very important quality for drug delivery,

ensuring micelles are sufficiently stable in the circulation to avoid the side effects to normal cells and tissues.

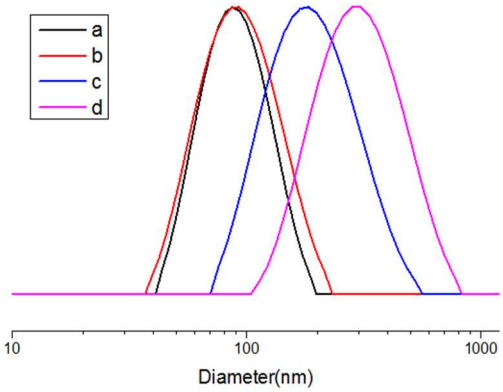


Fig. 2. Hydrodynamic diameter distribution of TPPC6-SS-Ada/PEG-*β*-CD micelles (a), TPPC6-SS-Ada/PEG-*β*-CD micelles treated without GSH for 24 h (b), and with 10 mM of GSH for 4 h (c) and 24 h (d)

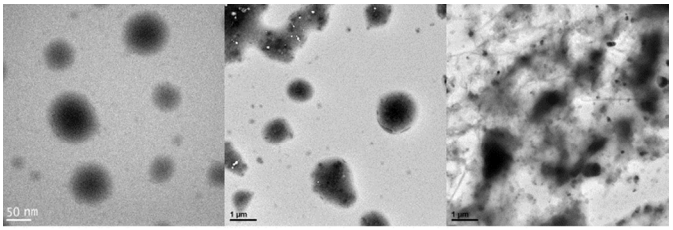


Fig. 3. TEM images of TPPC6-SS-Ada/PEG-*β*-CD micelles (a), and the micelles treated with 10 mM of GSH for 4 h (b) and 24 h (c).

Reduction-Release of Porphyrin from TPPC6-SS-Ada/PEG-*β*-CD Micelles

The release of porphyrin from TPPC6-SS-Ada/PEG-*β*-CD micelles were investigated by using dialysis tube (MWCO = 12 000) in 150 mL PBS (pH = 7.4) with and without 10 mM of GSH at 37 °C in shaking bed. The results were shown in **Fig. 4**. A relatively rapid release during the first 25 h under the treatment with 10 mM of GSH was observed, and nearly 70% porphyrin were released in this period. In comparison with the control group (without GSH), there is nearly no porphyrin released from micelles. The result indicated that TPPC6-SS-Ada/PEG-*β*-CD micelles were reduction-responsive. We also measured the release of porphyrin from the control micelles TPPC6-Ada/PEG-*β*-CD without disulfide bond, in 10 mM GSH solution under the same condition. We found that there is also nearly no porphyrin released from TPPC6-Ada/PEG-*β*-CD micelles, which indicated that disulfide bond is responsive to GSH effectively.

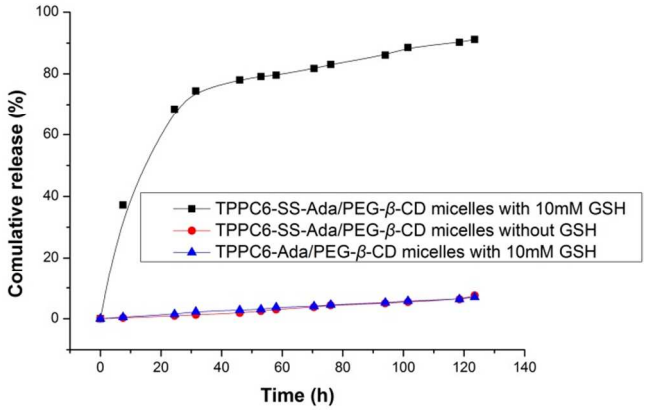


Fig. 4. Reduction-release of porphyrin from TPPC6-SS-Ada/PEG-*β*-CD micelles treated with 10mM GSH, without GSH and TPPC6-Ada/ PEG-*β*-CD micelles treated with 10mM GSH.

Intracellular Drug Uptake and Localization

Fluorescence of porphyrins can be directly tracked by flow cytometry and visualized by confocal laser scanning microscopy (CLSM). TPPC6-SS-Ada/PEG-*β*-CD micelles were added to culture medium with porphyrin concentration of 50 μg/mL and MCF-7 cells were incubated for 4 h and 24 h. The uptake of photosensitizer was measured by flow cytometry as shown in **Fig. 5**. After 4 h incubation with free porphyrin and TPPC6-SS-Ada/PEG-*β*-CD micelles, it can be seen that the fluorescence intensity treated with micelles is clearly stronger than that treated with free porphyrin (**Fig. 5b** and **5d**). The similar result is also found in cells that were incubated for 24 h (**Fig. 5c** and **5e**), which means more TPPC6-SS-Ada/PEG-*β*-CD micelles had been up-taken than free porphyrin. Additionally, the fluorescence intensity of cells incubated for 24 h is much stronger than that for 4 h, indicating the cellular uptake process of porphyrin by TPPC6-SS-Ada/PEG-*β*-CD micelles is time-dependent. This could be attributed to the fact that free porphyrin entered into cells via diffusion, while the TPPC6-SS-Ada/PEG-*β*-CD micelles might be taken up by cells through the endocytosis (**Scheme 3**).[48, 49]

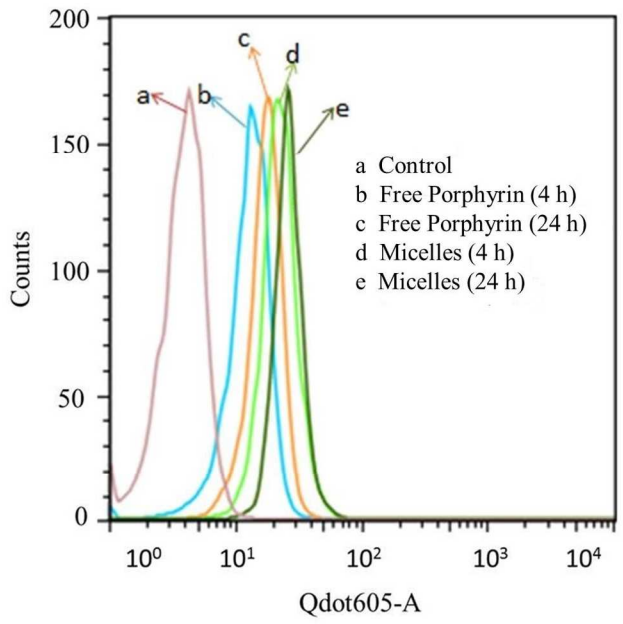
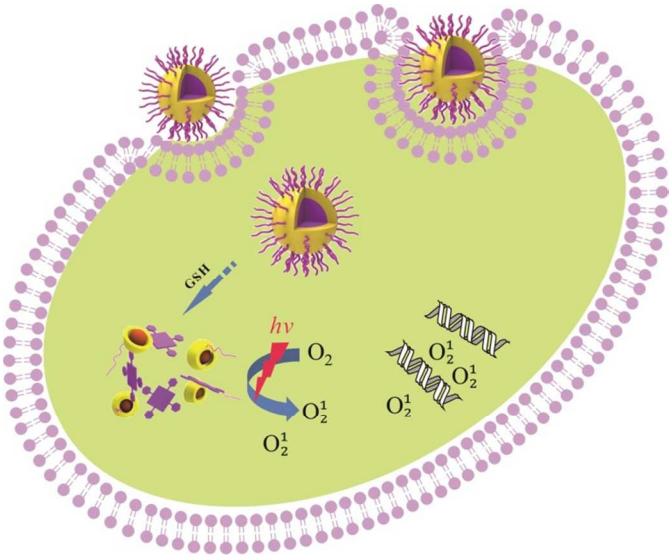


Fig. 5. Flow cytometric demonstration of the control group (a), and MCF-7 cells incubated with free porphyrin for 4 h (b) and 24 h (c), with micelles for 4 h (c) and 24 h (d).



Scheme 3. TPPC6-SS-Ada/PEG-*β*-CD micelles performed PDT in vitro.

The intracellular localization of TPPC6-SS-Ada/PEG-*β*-CD in MCF-7 cells was further investigated by CLSM. MCF-7 cells were cultured with free porphyrin and TPPC6-SS-Ada/PEG-*β*-CD micelles with porphyrin concentration of 50 μg/mL for 4 h and 24 h, respectively. DAPI was used to stain nucleus after the predetermined interval time. As shown in **Fig. 6**, the weak fluorescence image of free porphyrin nearly could not be discerned after incubation for 4 h (**Fig. 6a**), but for treatment with TPPC6-SS-Ada/PEG-*β*-CD micelles, the fluorescence intensity is obviously stronger than that of free porphyrin (**Fig. 6c**). Moreover, the similar result also could be found for incubation for 24 h (**Fig. 6b** and **6d**). The result suggested that TPPC6-SS-Ada/PEG-*β*-CD micelles might be uptaken by a time-dependent process which is well agreeable with the result of flow cytometry.[50, 51]

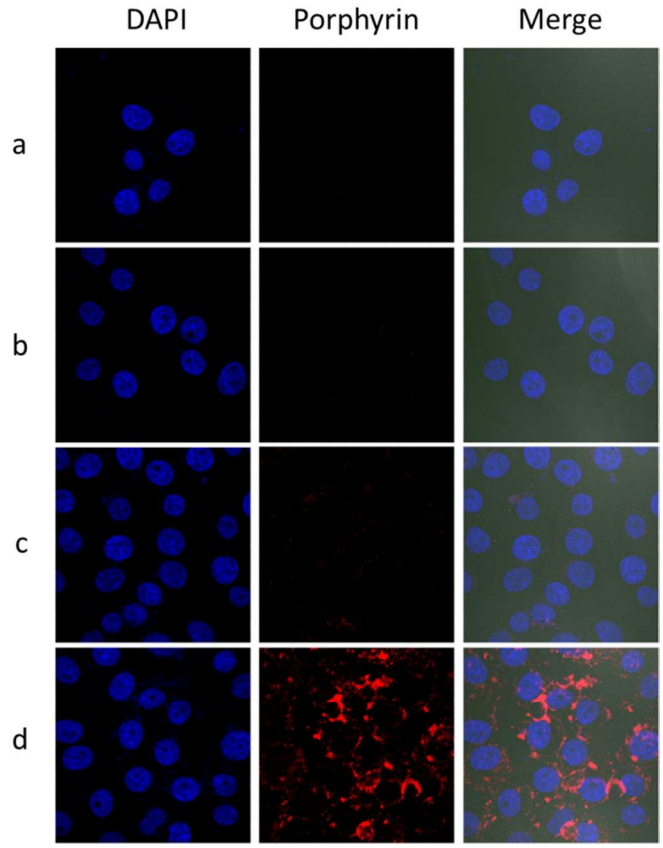


Fig. 6. CLSM images of MCF-7 cells incubated with free porphyrin for 4 h (a) and 24 h (b), and with micelles for 4 h (c) and 24 h (d). The images from left to right were the cells with nuclear staining with DAPI, with porphyrin fluorescence and merge of images.

In vitro Dark Toxicity and Phototoxicity of TPPC6-SS-Ada/PEG-*β*-CD micelles

In vitro dark toxicity and phototoxicity of TPPC6-SS-Ada/PEG-*β*-CD micelles and TPPC6-Ada/PEG-*β*-CD micelles were evaluated by MTT assay, and the MCF-7 cells in dark without treatment were set to the viability of 100% as reference. As shown in **Fig. 7a**, free porphyrin, TPPC6-SS-Ada/PEG-*β*-CD micelles and TPPC6-Ada/PEG-*β*-CD micelles all exhibit no dark cytotoxicity against MCF-7 cells even the porphyrin concentration at 100 μg/ mL. There was no statistically significant difference found in data of **Fig.7a.** The phototoxicity was further studied by irradiation with visible light LED lamps (400 mW/cm^2) for 20 min, and the results showed free porphyrin has low phototoxicity, perhaps due to its low internalization efficiency (**Fig. 7b)**[52]. The phototoxicity of TPPC6-SS-Ada/PEG-*β*-CD micelles increased with the increasing concentration of pophyrin in micelles, which is much higher than that of free porphyrin with the IC50 (calculated for porphyrin concentration) of 31 μg/mL ($p < 0.05$).

However, the phototoxicity of TPPC6-Ada/PEG-*β*-CD micelles was obviously weaker than that of TPPC6-SS-Ada/PEG-*β*-CD micelles. This is because the TPPC6-Ada/PEG-*β*-CD micelle has no disulfide bond, so that the porphyrin can't be well released effectively to induce the high photosensitization efficiency. Thus, reduction-responsive TPPC6-SS-Ada/PEG-*β*-CD micelles exhibit a potential application for PDT.

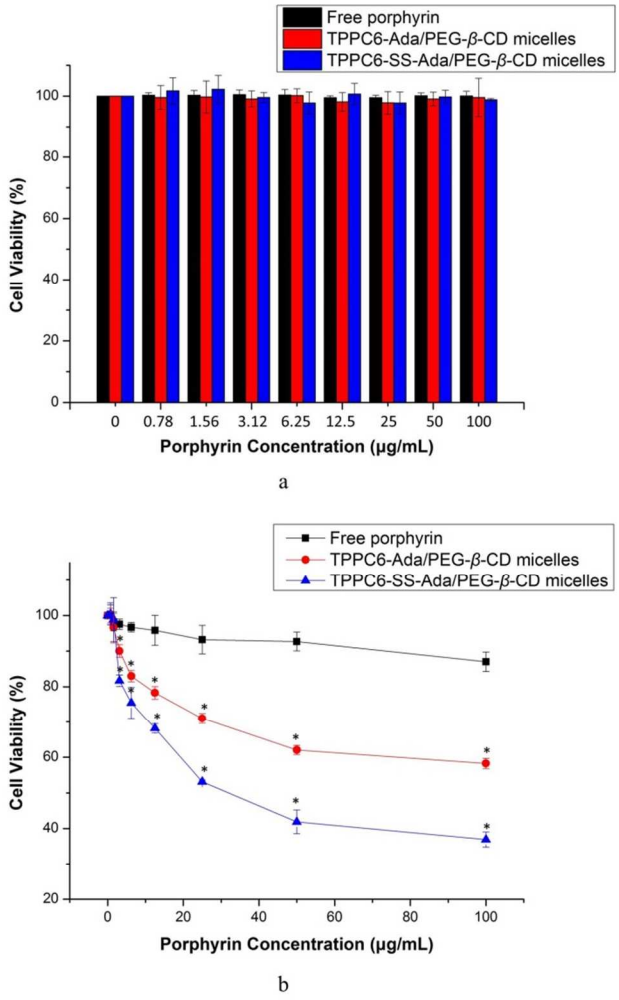


Fig. 7. Viability of MCF-7 cells measured by MTT assay after treating with micelles and free porphyrin without (a) and with light irradiation (b). All values are means ± SD, n = 3. (*) indicates statistically significant differences between the groups under irradiation ($p < 0.05$).

Conclusions

In summary, the amphiphilic supramolecular photosensitizer was fabricated based on the host-guest complexation between TPPC6-SS-Ada and PEG-*β*-CD, and the supramolecular amphiphies could self-assemble into spherical micelles in aqueous solution. The reduction-sensitive release of TPPC6-SS-Ada/PEG-*β*-CD micelles could be well controlled by GSH. Flow cytometry and CLSM results showed that the cellular uptake of porphyrin could be greatly strengthened by the formation of supramolecular amphiphies in comparison with free porphyrin. Furthermore, MTT assay illustrated that micelles almost had no dark toxicity but showed significant phototoxicity, which is a very important property to avoid damaging the normal tissue during drug delivery. Thus, amphiphilic supramolecular photosensitizer based on the host-guest complexation is promising for photodynamic therapy.

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Notes and references

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