Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Visible light-induced singlet oxygen-mediated intracellular disassembly of polymeric micelles co-loaded with photosensitizer and anticancer drug for enhanced photodynamic therapy

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Experimental Section

Materials.

Monomethoxy poly(ethylene glycol) (PEG-OH, M_n = 2000 g mol^{-1}), succinic anhydride, 4-dimethylaminopyridine (DMAP), *N*,*N*′-dicyclohexycarbodiimide (DCC), triethylamine (TEA), *cis*-1,2-dichloroethylene, 2-mercaptoethanol, ε-caprolactone (ε-CL), and sodium hydroxide (NaOH), were purchased from Sigma-Aldrich Co. (St. Louis, MO). Tin (II) 2-ethylhexanoate ($Sn(Oct)_2$) was obtained from Alfa Aesar. ε-CL was dried over CaH_2 and freshly distilled under reduced pressure prior to use. Chlorine e6 (Ce6) and doxorubicin hydrochloride (DOX HCl) were supplied from Frontier Scientific Inc. (Logan, UT, USA), and Wako Pure Chemical Industries (Osaka, Japan), respectively. Carboxylic acid end-functionalized PEG (PEG-COOH) was synthesized according to the method reported in the literature.[1] Dulbecco Modified Eagle's medium (DMEM) and Roswell Park Memorial Institute medium (RPMI 1640), penicillin-streptomycin, fetal bovine serum (FBS) and Dulbecco's phosphate buffered saline (DPBS) were obtained from Corning (Manassas, VA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, USA). All other chemicals were of analytical grade and used as received unless otherwise specifically mentioned.

Characterizations

^1H NMR spectra were recorded on a FT-300 MHz Bruker Aspect Spectrometer using the residual proton resonance of the solvent as the internal standard. The chemical shifts are reported in parts per million (ppm). Gel permeation chromatography (GPC) measurements

were performed on a Waters Alliance e2695 GPC system, equipped with three consecutive Styragel® columns (HR1, HR2 and HR4). The detection was performed on a 2414 refractometer. THF was used as the eluent at a flow rate of 1 mL/min. A series of polystyrene standards were used for calibration. The molecular weight and polydispersity index of the polymers were analysed using Empower 2 software. The dynamic light scattering (DLS) measurements were conducted using a Zetasizer Nano S90 system (Malvern Instruments, Worcestershire, UK). All the measurements were carried out at 25 °C with a 90° detection angle. The morphology of the micelles was examined using a HITACHI-7066 (Japan) transmission electron microscope (TEM). For sample preparation, a drop of the micellar suspension was placed on a copper grid, followed by blotting of excess water and air-dried before negative staining with 1% uranyl acetate. UV-visible spectra were recorded on a UV 2550 spectrophotometer (Shimadzu), and fluorescence spectra were recorded on a RF-5301 PC spectrofluorophotometer (Shimadzu). Confocal laser scanning microscope (CLSM) image was obtained from Olympus FV-1000 and analysed with OLYMPUS FLUOVIEW ver. 1.5 Viewer software.

Synthesis of $^1O_2$-responsive PEG-OH ($^1O_2$-PEG-OH) macroinitiator

First, a vinyldithioether-based $^1O_2$-sensitive linker was synthesized by employing a procedure reported by Baugh et al as follow.[2] A mixture of 2-mercaptoethanol (3 g; 38.39 mmol) and NaOH (1.535 g; 38.39 mmol) in 15 mL of ethanol was stirred at 0 °C for 30 min. After the drop wise addition of *cis*-1,2-dichloroethylene (1.86 g; 19.19 mmol) in 2 mL of ethanol, the resulting solution was heated at 80 °C for 18 h. Then, the solution was cooled to ambient temperature, diluted with water (20 mL) and washed with diethyl ether (3 × 10 mL). The

combined organic layers were washed with water (2 × 20 mL), dried over $MgSO_4$, and concentrated to give the crude product. The product was purified by column chromatography on silica gel using the mixture of ethylacetate/hexane (70:30) to obtain the desired pure (Z)-2,2'-(ethene-1,2-diylbis(sulfanediyl))diethanol (Yield: 70%).

Second, the as-synthesized linker was conjugated to the PEG-COOH by Steglich esterification reaction. PEG-COOH (2 g; 0.952 mmol) and excess of (Z)-2,2'-(ethene-1,2-diylbis(sulfanediyl))diethanol (0.605 g; 3.355 mmol) were dissolved in 30 mL of anhydrous CH_2Cl_2. DCC (0.393 g; 1.904 mmol) and DMAP (0.116 g; 0.952 mmol) were added to the solution under nitrogen. The solution was stirred at room temperature for 48 h. The precipitated *N*,*N*′-dicyclohexyl urea was removed by filtration. The filtrate was concentrated under vacuum and precipitated in ether to obtain $^1O_2$-PEG-OH macroinitiator, which was further purified by redissolving it in THF and reprecipitating in ether. The final product was filtered and dried under vacuum at room temperature (Yield: 76%).

Synthesis of $^1O_2$-responsive PEG-*b*-poly(caprolactone) ($^1O_2$-PEG-*b*-PCL) copolymer

$^1O_2$-PEG-*b*-PCL diblock copolymer was synthesized via ring opening polymerization of ε-CL using $^1O_2$-PEG-OH as a macroinitiator and $Sn(Oct)_2$ as catalyst. In brief, $^1O_2$-PEG-OH (0.5 g; 0.229 mmol), ε-CL (0.784 g; 6.87 mmol), $Sn(Oct)_2$ (0.046 g; 0.114 mmol), and 3.4 mL of anhydrous toluene were charged into a flame dried Schlenk flask under dry nitrogen. The mixture was degassed and polymerization was carried out at 100 °C for 24 h with constant stirring. After cooling to room temperature, the polymer was precipitated in diethyl ether. The precipitated diblock copolymer was filtered and dried in a vacuum at room temperature for 48 h, affording a yield of about 80%. M_n(NMR) = 5033 g mol^{-1}, M_n(GPC) = 6099 g mol^{-1},

M_w/M_n = 1.60. A control PEG-*b*-PCL diblock copolymer with a similar molecular weight (M_n(NMR) = 4853 g mol^{-1}, M_n(GPC) = 6041 g mol^{-1}, M_w/M_n = 1.65) was synthesized using PEG-OH (M_n = 2000 g mol^{-1}) as a macroinitiator.

Preparation of Ce6-loaded, and Ce6 and DOX co-loaded micelles

Ce6-loaded and Ce6 and DOX co-loaded micelles were prepared by a simple dialysis method.[3] In brief, block copolymer (100 mg) was dissolved in 4 mL of DMSO. To this appropriate feed amount of Ce6 or Ce6 and DOX with TEA (3 equivalent of DOX) dissolved in each 1 mL of DMSO was added and mixed well. Under vigorous stirring, 20 mL of distilled water added drop wise. After stirring for further 30 min at room temperature, the solution was transferred to a dialysis tube of molecular weight cut-off 3500 Da and dialyzed for 24 h against water to remove the organic solvent and free drug. The micellar solution was further filtered through 0.80 μm syringe filter to remove the residual aggregates. The drug loaded micelles were lyophilized.

Drug loading content and loading efficiency

The amount of Ce6 in the micelles was determined using UV-vis spectrophotometer at 405 nm for Ce6 loaded micelles and 664 nm for Ce6 and DOX co-loaded micelles. The amount of DOX in the micelles was determined by spectrofluorophotometer at 587 nm. For this experiment, Ce6 loaded and Ce6 and DOX co-loaded micelles were dissolved in DMSO. Calibration curves were made by dissolving different concentrations of Ce6 in DMSO and DOX in DMSO using UV-Vis spectrophotometer and spectrofluorophotometer, respectively. The drug loading content and loading efficiency were calculated using the following

formulae:

$$Loading\ content\ (wt.\%) = \frac{weight\ of\ loaded\ drug}{weight\ of\ polymer} \times 100\%$$

$$Loading\ efficiency\ (\%) = \frac{weight\ of\ loaded\ drug}{weight\ of\ drug\ in\ feed} \times 100\%$$

***In vitro* $^1O_2$-Generation of the micelles**

The generation of singlet oxygen from the micelles was evaluated using *N*,*N*-dimethyl-4-nitrosoaniline (RNO)-histidine colorimetric assay[4,5]. In this assay, the generated $^1O_2$ reacts with the imidazole moiety and forms a transient complex, which bleaches the RNO molecules. Thus the amount of $^1O_2$ produced can be directly correlated with a decrease in the RNO absorbance in the UV-vis spectrum. Briefly, 200 μL of RNO (250 mM) and 600 μL of histidine (0.03 M) were mixed with 1 mL of free Ce6, $^1O_2$-PM-Ce6-DOX or C-PM-Ce6-DOX (each containing 50 μg of Ce6) dissolved in distilled water containing 1% DMSO. The resulting solution was bubbled with oxygen for 15 min and then irradiated with a 660 nm laser (30 mW/cm^2). The absorbance of RNO was monitored at 440 nm using a UV-Vis spectrophotometer as a function of time.

***In vitro* drug release**

The *in vitro* release profile of DOX from the micelles was evaluated using dialysis method. For this experiment, micelles were dispersed in PBS solution (pH 7.4) at a concentration of 1 mg/mL and placed in a dialysis membrane bags (MW cutoff = 3500). The dialysis bags were

immersed in release media (PBS, pH 7.4), and each samples were gently shaken in a 37 °C water bath at 100 rpm. At predetermined time intervals, the release medium was refreshed and the DOX concentration was determined using spectrofluorophotometer at 588 nm. For the light-triggered release of DOX, the micelles were irradiated with red light laser (50 mW/cm^2; 660nm, 2 h) before transferring to the dialysis bag.

Cell Culture

Human prostate carcinoma cell line was cultured in RPMI164 medium and human cervix epithelial carcinoma (HeLa) cell line was cultured in DMEM medium. Both medium contains 10 % FBS and 1% penicillin/streptomycin and cells were grown and maintained in a humidified atmosphere with 5 % CO_2 at 37 ^oC. The cells were subcultured whenever the confluency becomes 80% by using 0.25 % trypsin/EDTA.

Intracellular release of DOX observed by confocal laser scanning microscope (CLSM)

Intracellular release of DOX by red laser irradiation was evaluated by confocal microscopy image. Briefly, cells (5×10^4 cells/well) were seeded on the glass cover slips in 12-well culture plate and incubated for overnight. Then, the medium was replaced by fresh serum-free medium containing 1 μg/mL of $^1O_2$-PM-Ce6-DOX or C-PM-Ce6-DOX. After 4 h of incubation, cells were washed carefully and irradiated by red laser (50 mW/cm^2) for 10 min under fresh medium. Cells were washed with PBS and fixed with 10 % neutrally buffered formalin (NBF) immediately after irradiation or after 4 h of incubation. Cells on the coverslip were mounted in Vectashield anti-fade mounting medium with DAPI (Vector Laboratories). Intracellular fluorescence of DOX was observed by CLSM with excitation at 470 nm and

emission at 556 nm.

***In vitro* cellular cytotoxicity**

The dose-dependent cytotoxicity of drug-free micelles ($^1O_2$-PM and C-PM) with and without irradiation was evaluated. Cells (8×10^3 cells/well) were seeded on 96-well culture plates and incubated for overnight. Medium was replaced by fresh serum-free medium containing 0 to 10 μg/mL of polymers and incubated for 4 h. After washing with PBS, the cells were irradiated by red laser (660 nm diode laser, Shanghai dream lasers, China) with power density of 50 mW/cm^2 or incubated at dark for 10 min. Cytotoxicity was measured by MTT assay after further incubation for 24 h. Briefly, the medium was replaced with 200 μL of fresh medium and 20 μL of 5 mg/mL MTT solution were added. After 4 h, the medium was removed and 200 μL of DMSO was added to each well to dissolve the internalized purple formazan crystals. An aliquot of 100 μL was taken from each well and transferred to a fresh 96-well plate. The absorption was measured at 570 nm using a microplate spectrofluorometer (VICTOR3 V Multilabel Counter, Perkin-Elmer-Wellesley, MA, USA). The control cells which were not exposed to the polymers and laser, were used to represent 100 % cell viability. The results are presented as mean and standard deviation (n = 3).

Cytotoxicity of Ce6, and Ce6-DOX co-loaded micelles with and without laser irradiation was evaluated under various concentrations. Cells (8×10^3 cells/well) were seeded on 96-well culture plates and incubated for overnight. Micelles at final concentration of 0 - 5 μg/mL were treated under serum-free medium. After 4 h of incubation, cells were washed with PBS and irradiated by red laser with power density of 50 mW/cm^2 or incubated at dark for 10 min under fresh medium. Cytotoxicity was measured by MTT assay after further incubation for

24 h. The detailed procedures for MTT assay are identical to those already described above.

References

1. S. Zalipsky, C. Gilon and A. Zilkha, *Eur. Polym. J.,* 1983, **19,** 1177.

2. S. D. P. Baugh, Z. Yang, D. K. Leung, D. M. Wilson and R. Breslow, *J. Am. Chem. Soc.,* 2001, **123,** 12488.

3. H. Sun, B. Guo, R. Cheng, F. Meng, H. Liu and Z. Zhong, *Biomaterials,* 2009, **30**, 6358.

4. I. Kraljic and S. E. A. Mohsni, *photochem. Photobiol.,* 1978, **28,** 577.

5. G. Pasparakis, *Small,* 2013, **9,** 4130.

Supplementary Figures

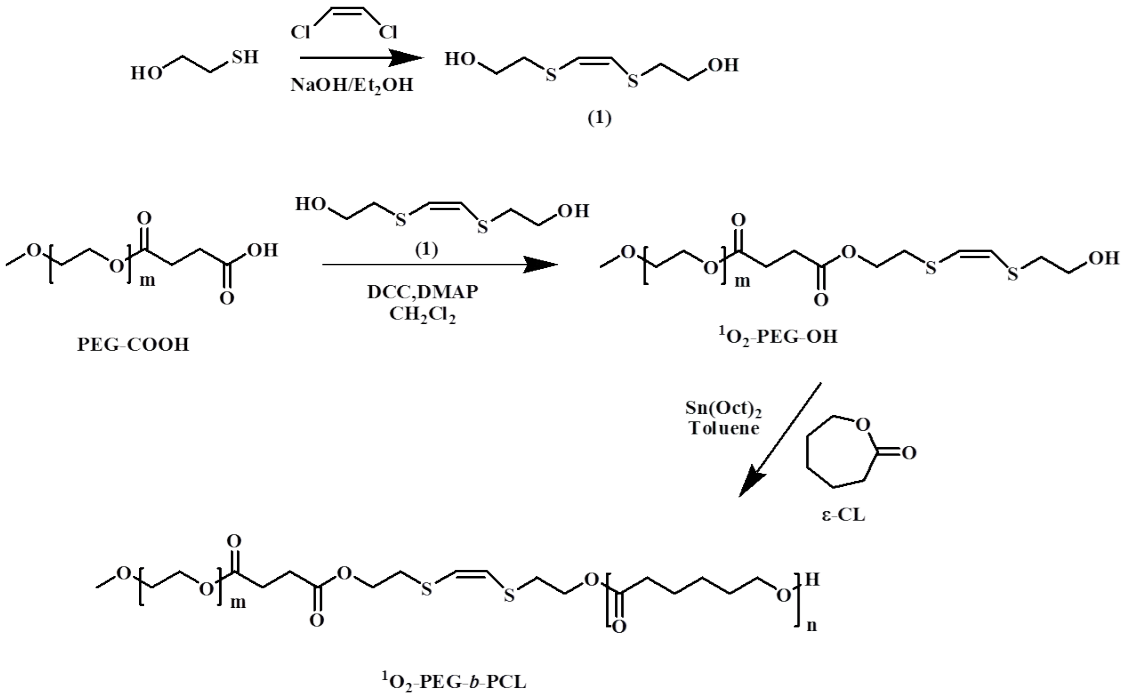

Fig. S1. Reaction scheme for the synthesis of $^1O_2$-PEG-*b*-PCL copolymer.

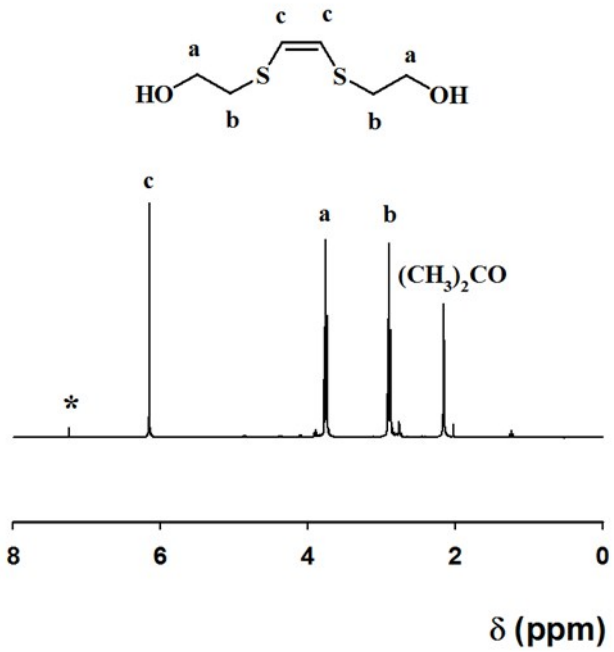

Fig. S2. ^{1}H-NMR spectrum of $^1O_2$-Linker in C*D*Cl$_3$.

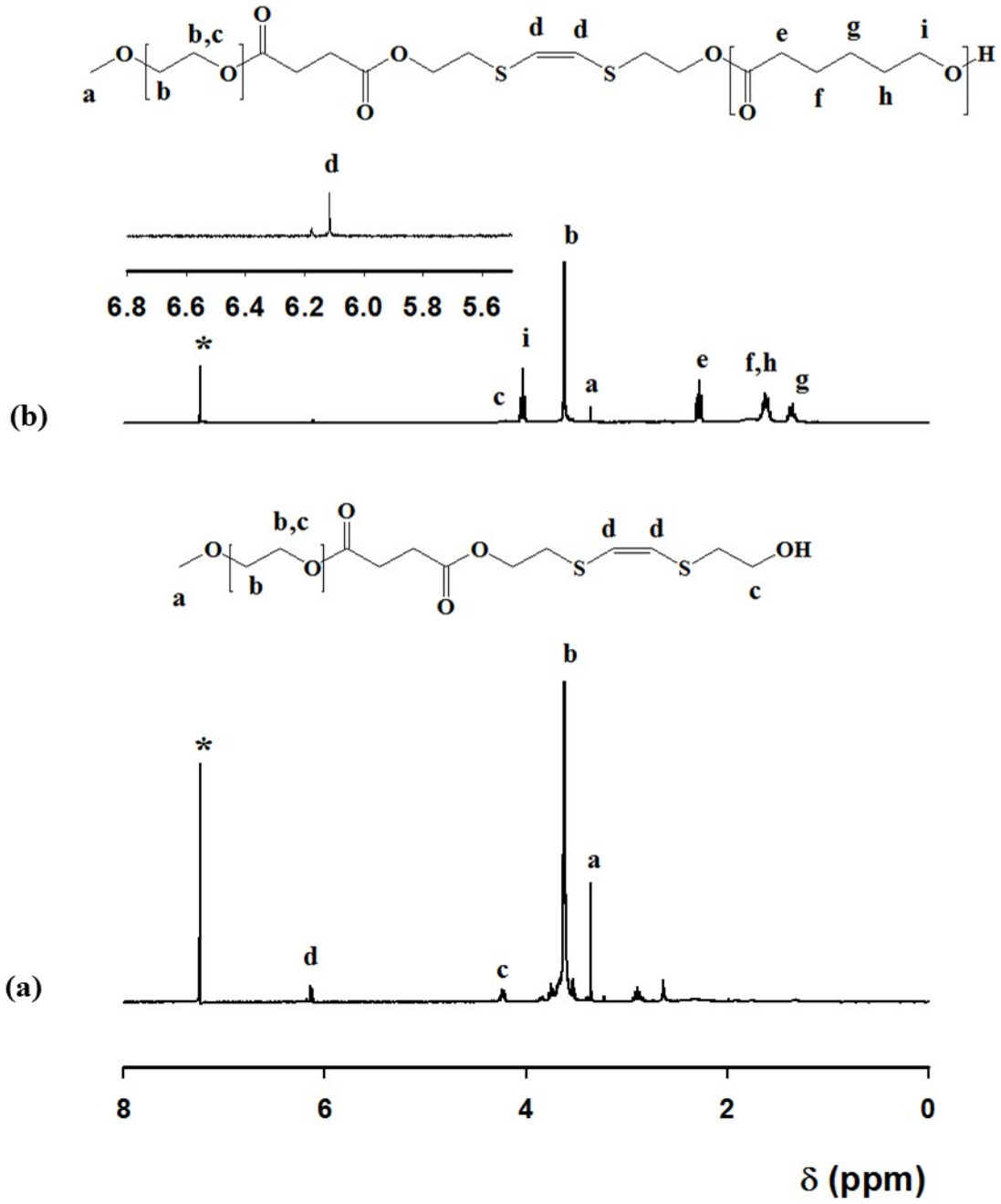

Fig. S3. ^{1}H-NMR spectra of (a) $^{1}O_2$-PEG-OH macroinitiator and (b) $^{1}O_2$-PEG-*b*-PCL copolymer in C*D*Cl$_3$.

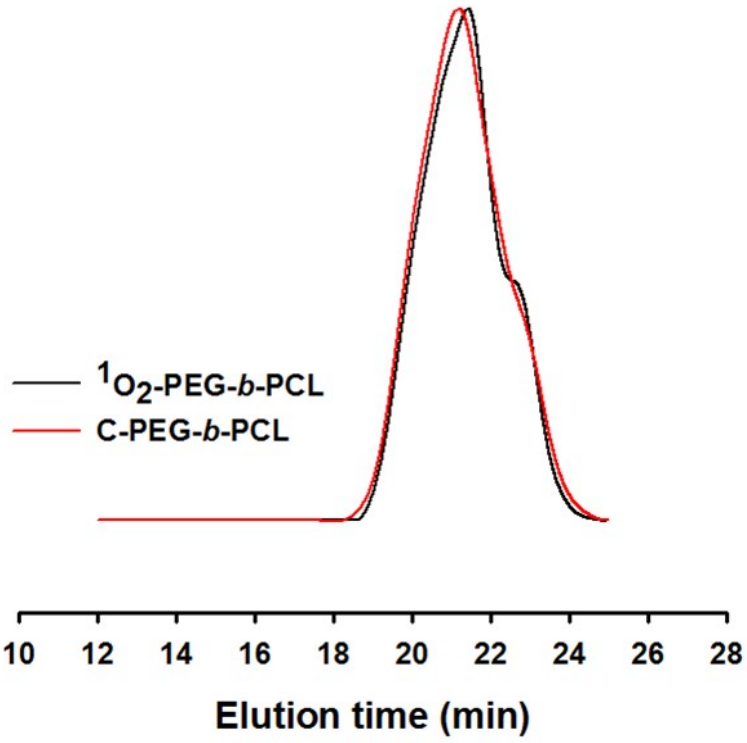

Fig. S4. GPC profile of $^1O_2$-PEG-*b*-PCL and C-PEG-*b*-PCL.

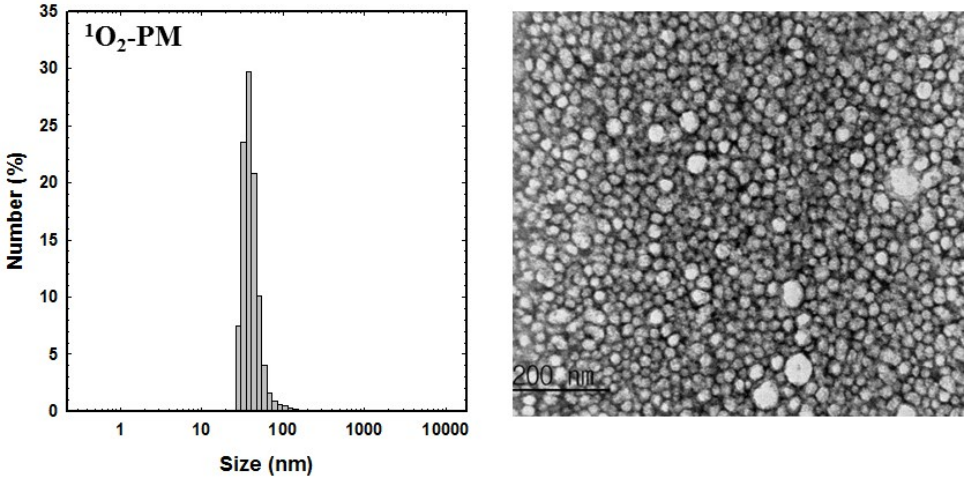

Fig. S5. DLS size distribution and TEM images of $^1O_2$-PM (scale bar = 200 nm).

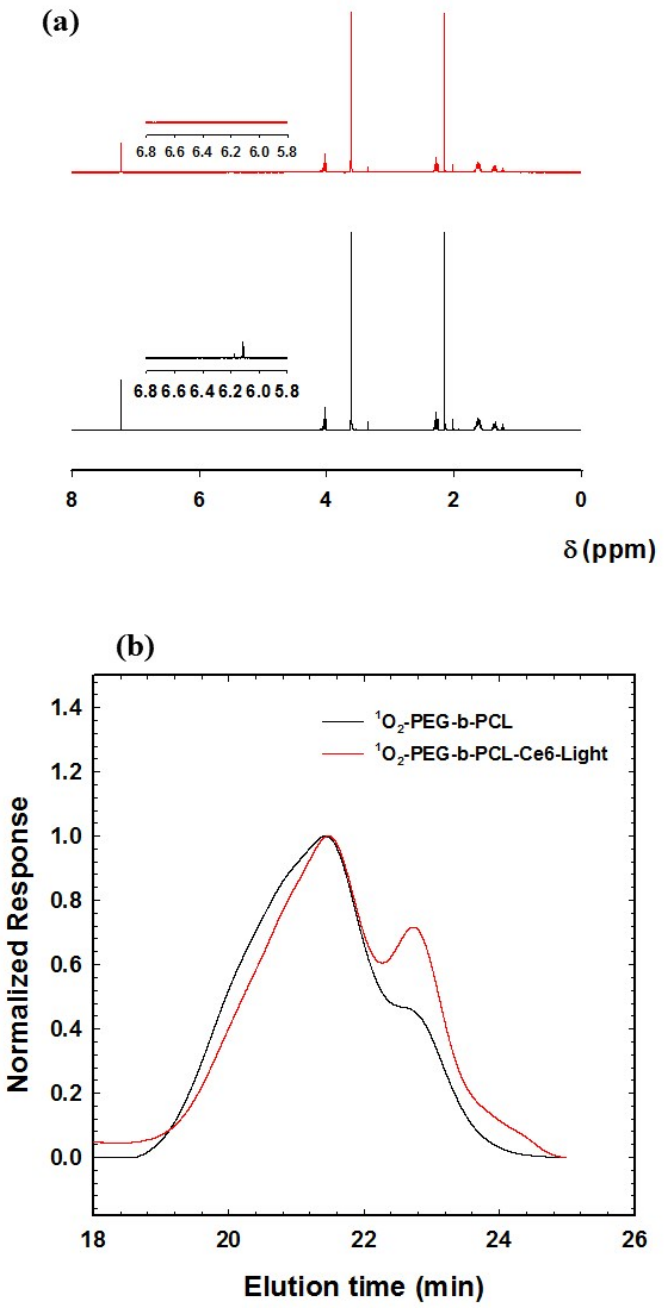

Fig. S6. (a) ^{1}H-NMR spectra of $^1O_2$-PEG-b-PCL with Ce6 (~5 wt%) in the dark and after exposing laser irradiation (30 mW/cm^2, 3 h), where the solvent is C*D*Cl$_3$, and (b) their GPC profiles.

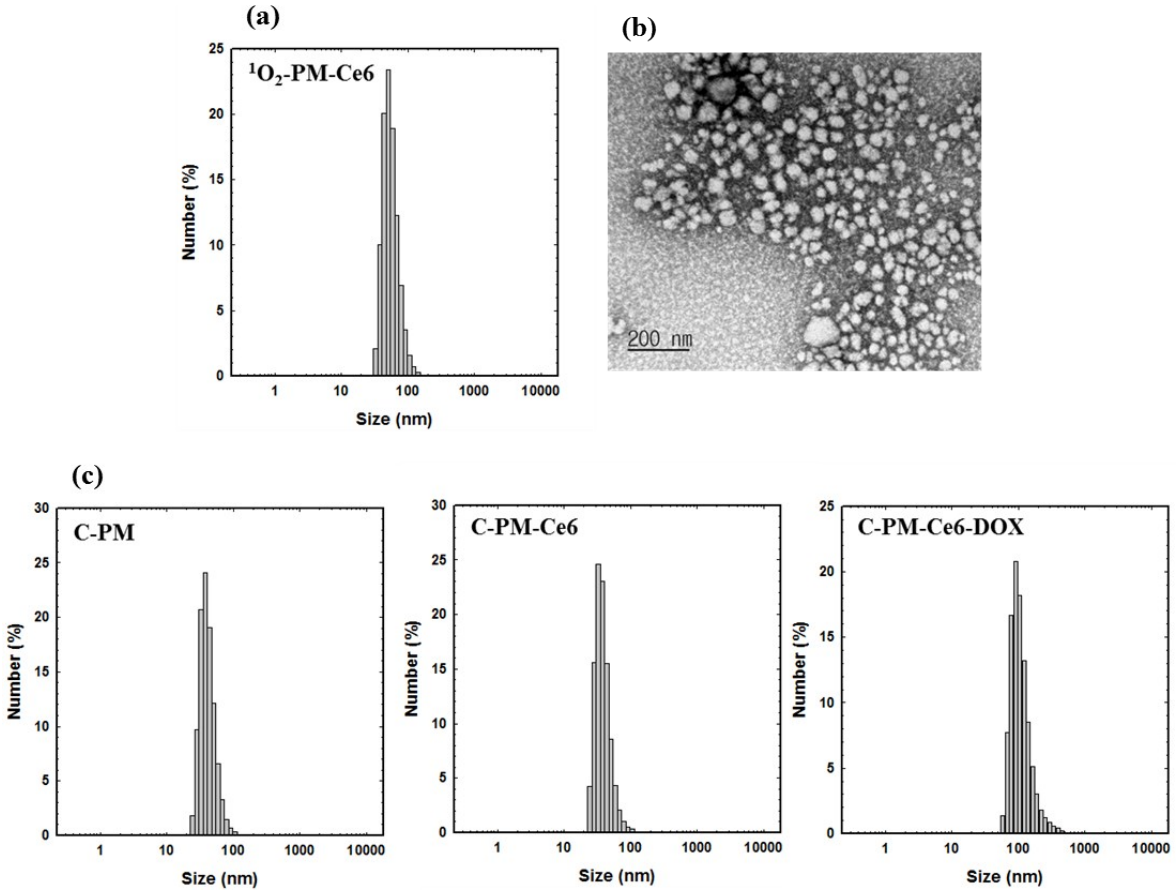

Fig. S7. (a) DLS size distribution and (b) TEM image of $^1O_2$-PM-Ce6. (c) DLS size distributions of C-PM, C-PM-Ce6, and C-PM-Ce6-DOX micelles.

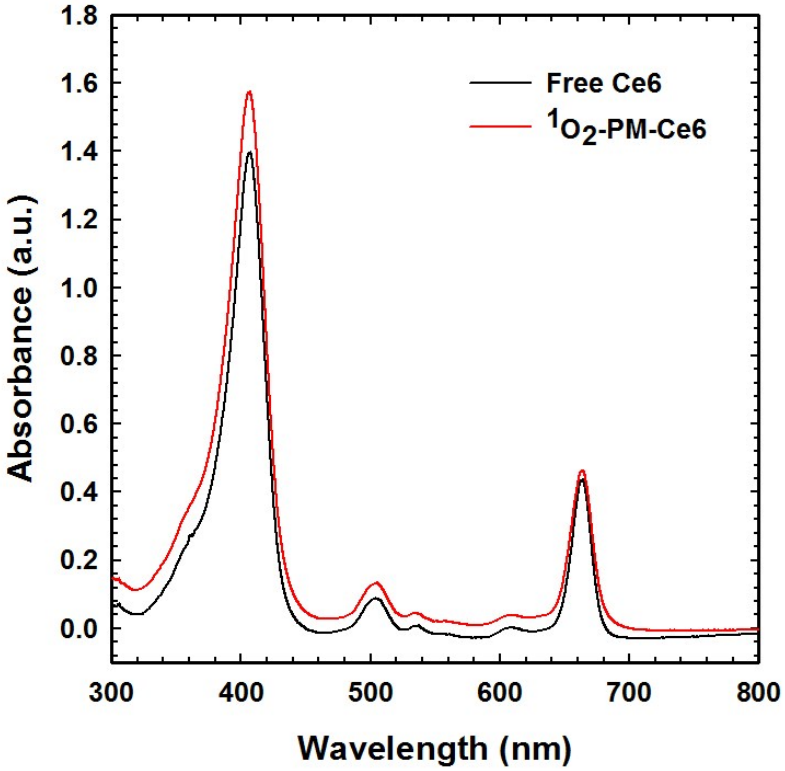

Fig. S8. Absorbance and fluorescence spectra of free Ce6 in DMSO and $^1O_2$-PM-Ce6 micelles.

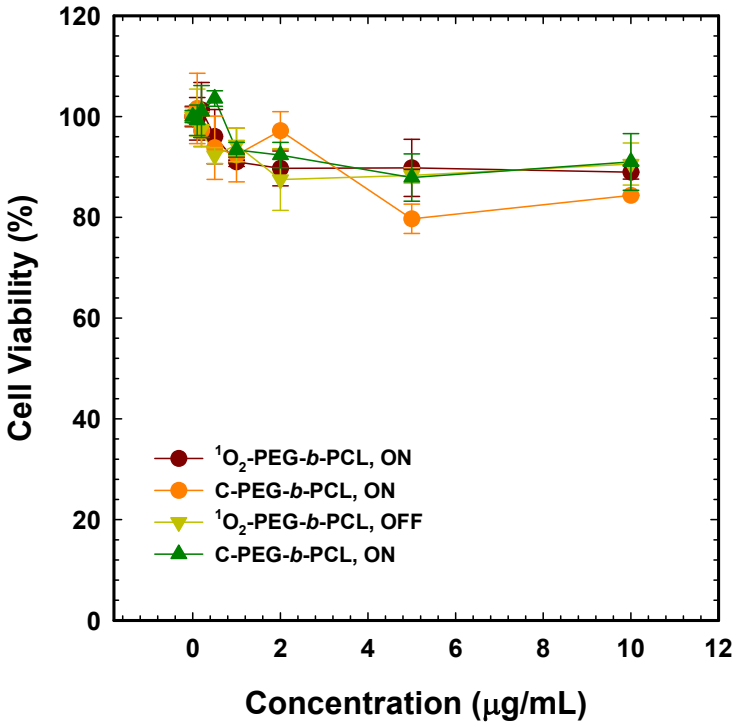

Fig. S9. *In vitro* cytotoxicity of $^1O_2$-PM and C-PM with and without laser irradiation in HeLa Cells.

Table S1. Characteristics of block copolymers.

Sample	$[\varepsilon\text{-CL}]_0/[I]_0$	M_n[a] (NMR)	M_n[b] (GPC)	M_w/M_n[b]
$^1O_2$-PEG_{44}-*b*-PCL_{25}	30	5033	6099	1.60
PEG_{44}-*b*-PCL_{25}	30	4853	6041	1.65

[a] determined by ^{1}H-NMR using C*D*Cl_3 as solvent.

[b] determined by GPC using polystyrene as standard and THF as eluent at a flow rate of 1 mg/mL.

Table S2. Physicochemical characteristics of Ce6/DOX loaded polymeric micelles.

Sample	Feed amount		Loading Content (LC)[a] (wt.%)		Loading Efficiency (LE)[a] (%)		Size[b] (nm)
	Ce6	DOX	Ce6	DOX	Ce6	DOX	
$^1O_2$-PM-Ce6	10	-	5.7 ± 0.1	-	57.4 ± 0.8	-	66 ± 3.3
$^1O_2$-PM-Ce6-DOX	6	6	5.2 ± 0.1	3.4 ± 0.1	85.9 ± 2.0	56.1 ± 1.9	124 ± 58.4
C-PM-Ce6	10	-	5.6 ± 0.2	-	55.8 ± 1.8	-	40 ± 15.0
C-PM-Ce6-DOX	6	6	5.0 ± 0.2	3.4 ± 0.1	82.7 ± 3.8	56.7 ± 0.9	100 ± 17.0

[a] Ce6 LC and LE determined using UV visible spectroscopy, and DOX LC and LE were determined using fluorescence spectroscopy.

[b] determined using particle size analyzer.