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Supporting Information

Light-controlled reactive oxygen species (ROS)-producible polymeric micelles with simultaneous drug-release triggering and endo/lysosomal escape

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Materials and methods

Materials

Propylene sulfide, thioglycolic acid (TGA), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), dimethyl sulfoxide (DMSO), triethylamine (TEA), 1,3-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP) , poly(ethylene glycol) (PEG, MW 6,000), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and doxorubicin hydrochloride (DOX·HCl) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Chloroform-D (D, 99.8%) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, USA). Chlorin e6 (Ce6) was purchased from Frontier Scientific, Inc. (Utah, USA). Singlet Oxygen Sensor Green (SOSG), 4',6-diamidino-2-phenylindole (DAPI) and LysoTracker Green DND-26 were purchased from Molecular Probes, Inc. (Eugene, USA). The dialysis membranes were obtained from Spectrum Laboratories, Inc. (California, USA). RPMI1640 medium, fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin) were obtained from Gibco BRL, Inc. (Carlsbad, USA).

Synthesis of carboxy-end functional poly(propylene sulfide) (PPS-COOH)

Carboxy-end functional poly(propylene sulfide) (PPS-COOH) was synthesized by ring-opening polymerization using thioglycolic acid. Briefly, propylene sulfide (160 mg) was ring-opening polymerized using thioglycolic acid (10 mg) as an initiator in the presence of DBU (23 mg) as a base in DMSO (5 mL) using an adjacent flask in a Schlenk line under an N_2 atmosphere. The next day, the polymer solution was filtered to remove the precipitated salt and further purified by three precipitations into cold methanol before vacuum drying to yield a colorless viscous polymer. The ^{1}H-NMR spectra were recorded in $CDCl_3$-d_1 at room temperature in a Bruker 300 MHz NMR Spectrometer (Karlsruhe, Germany).

Synthesis of HO-PEG-Ce6

The conjugation of HO-PEG-Ce6 via DCC and DMAP-mediated ester formation was performed. Briefly, PEG (1 g) was dissolved in DMSO (30 mL) and Ce6 (0.13 g, corresponding to a mole ratio of Ce6 to PEG of 1.3) was dissolved in DMSO (10 mL), followed by the addition of 1.3 molar equivalents DCC and DMAP by stirring at room temperature for 4 h. The PEG solution was then added dropwise, and the reaction was gently stirred for 24 h at room temperature. The reacted solution was dialyzed (Spectra/Por; molecular weight cut-off (MWCO) = 1,000 Da) for 3 days against distilled water to remove unconjugated Ce6 and DMSO, and the final solution was lyophilized.

The purification of HO-PEG-Ce6 and Ce6-PEG-Ce6 in the following hydrophobic chromatographic columns was previously reported by our group.[1] The unrefined polymer was purified using an open column filled with Sephadex LH-20 (Sigma-Aldrich Korea, Seoul, Korea) as the stationary phase, and methanol served as the mobile phase. The Sephadex LH-20 (4 g) was dissolved in methanol (10 mL) for activation, applied to a chromatographic column (2 × 60 cm) packed with LH-20, and eluted with 95% (v/v) methanol by gravity. After loading the unrefined polymers, the flow rate was 0.5 mL/min, with the mobile phase starting from 50% methanol (5:5 MeOH in water) (0–60 min), and proceeding to 80% methanol (8:2 MeOH in water) (61–110 min). Each fraction was collected, and pure products (HO-PEG-Ce6 and Ce6-PEG-Ce6) were obtained. The ^{1}H-NMR spectra were recorded in deuterated chloroform ($CDCl_3$-d_1) at room temperature using a Bruker 300 MHz NMR Spectrometer (Karlsruhe, Germany).

Synthesis of PPS-PEG-Ce6

The conjugated PPS-PEG-Ce6 was synthesized via DCC and DMAP-mediated ester

formation. The HO-PEG-Ce6 (100 mg) was dissolved in DMSO (8 mL). PPS-COOH (20 mg, corresponding to a mole ratio of PPS-COOH to HO-PEG-Ce6 of 1.5) was dissolved in DMSO (4 mL), followed by the addition of 1.3 molar equivalents DCC and DMAP by stirring at room temperature for 4 h. The HO-PEG-Ce6 solution was then added dropwise, and the reaction was gently stirred for 24 h at room temperature. The resulting solution was dialyzed against distilled water using a dialysis membrane (Spectra/Por; molecular weight cut-off (MWCO) = 12,000 Da) to remove the unreacted HO-PEG-Ce6 for 3 days. The ^{1}H-NMR spectra were recorded in deuterated chloroform ($CDCl_3$-d_1) at room temperature in a Bruker 300 MHz NMR Spectrometer (Mn_{NMR} = 8120, Karlsruhe, Germany). ^{1}H-NMR ($CDCl_3$-d_1): δ1.3-1.4 (a, CH_3 in PPS chain), δ2.5-2.7 (b, CH_2 in PPS chain), δ2.8-3.0 (c, CH in PPS chain), δ3.5-3.7 ppm (d, CH_2 in PEG chain).

Gel permeation chromatography (GPC)

The samples were analyzed using an HPLC system (Waters) equipped with a 515 HPLC pump, a pump control module, a 717plus autosampler, a column oven, and a GPC KF-804 or GPC KF-805 column (Shodex, Tokyo, Japan) at a flow rate of 1.0 mL/min with N,N-dimethylformamide as the mobile phase at room temperature. Detection was performed using a 410 differential refractometer. The average molecular weight of the sample was calibrated against narrow molecular weight poly(ethylene glycol) standards.

Preparation of D-LRPMs and un-loaded LRPMs

DOX·HCl (5 mg) was dissolved in 10 mL of anhydrous DMSO containing 6.2 μL of triethylamine (TEA) for 12 h at room temperature. PPS-PEG-Ce6 (10 mg) and DOX (0.5 mg) were dissolved in DMSO (2 mL); un-loaded LRPMs were prepared with PPS-PEG-Ce6 only. The solution was stirred at room temperature for 4 h. The D-LRPMs and un-loaded LRPMs

were dialyzed against PBS buffer (1 L) using a dialysis membrane (Spectra/Por; molecular weight cut-off (MWCO) = 1,000 Da) for 24 h. The final product was filtered with a syringe filter (0.45 μm, Millipore®) and then used for in vitro and in vivo studies. The doxorubicin (DOX) encapsulation efficiency was calculated by following equation:

$$\text{DOX encapsulation efficiency (\%)} = (A_0 - A_1) / A_0 \times 100$$

Where A_0 is the feeding amount of DOX, and A_1 is the encapsulated amount of DOX.

Particle size and zeta-potential measurement

The particle size and zeta-potential of the resulting D-LRPMs and LRPMs were determined using dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Instruments Ltd., UK). DLS was performed at 25 °C in distilled water, 10 mM phosphate buffered saline solution and RPMI1640 medium with the sampling time and analysis set to automatic. The sample concentration was maintained at 0.2 g/L.

Field emission scanning electron microscopy (FE-SEM) and energy-dispersive X-ray spectroscopy (EDX)

The morphology of the D-LRPM was observed using field emission scanning electron microscopy (FE-SEM; S-4700; Hitachi, Japan). To observe the nanoparticles in a dried state, a drop of D-LRPMs in distilled water was dried in an oven at 60 °C for 1 day and then placed on a graphite surface and coated with platinum by sputtering for 45 s at 15 mA. EDX (EX-250 X-stream, HORIBA, Co. Ltd., Japan) was used to analyze the oxygen contents of PPS-PEG-Ce6 with and without irradiation.

Evaluation of singlet oxygen generation (SOG)

To evaluate the SOG of PPS-PEG-Ce6, a chemical method using SOSG was employed with

fluorescent spectroscopy. The SOSG probe works via intramolecular electron transfer, which quenches the fluorescence from the light-emitting chromophore prior to reaction with the singlet oxygen. Reaction with the singlet oxygen results in the formation of the endoperoxide, preventing electron transfer and thus leading to the recovery of fluorescence.[2] PPS-PEG-Ce6 derivatives dissolved in distilled water were blended with a SOSG solution (2 mM) and then irradiated with 20 mW/cm^2 of a 670 nm light source (fiber-coupled laser system, LaserLab®, Korea). The fluorescence intensity of the SOSG was detected at an excitation wavelength and maximum wavelength of 504 nm and 525 nm, respectively using fluorescence spectroscopy (RF-5301, Shimadzu, Japan).

Measurement of critical micelle concentration (CMC)

The CMC was measured by fluorescence spectroscopy using pyrene as a probe. Typically, 60 μL of an acetone solution of pyrene (2.5×10^{-5} M) was added to a 5 mL plastic centrifuge tube, and then the acetone was evaporated in air. A total of 2 mL of the polymer aqueous solution at the prescribed concentrations was introduced into the tubes, and the pyrene concentration in all the samples was approximately 6×10^{-7} M, slightly lower than the saturation solubility of pyrene in water. These solutions were vigorously shaken and then allowed to equilibrate at room temperature for at least 24 h. The excitation spectra of pyrene with the different PPS-PEG-Ce6 concentrations were measured at the detection emission wavelength (λ_{ex} = 390 nm). The CMC value was obtained from the intersection of the tangent to the linear concentration-dependent section with the concentration-dependent section of I_{373}/I_{383}.

In vitro drug release test

In vitro drug release profiles of D-LRPM solutions were investigated in 10 mM phosphate

buffered saline solution (PBS, pH 7.4) containing 0.1% Tween 20 using a dialysis membrane (Spectra/Por; molecular weight cut-off (MWCO) = 1,000 Da). D-LRPMs were dissolved in the release medium and introduced into the dialysis membrane. The dialysis membrane was then placed in 20 mL of release medium and incubated in a shaking water bath (50 rpm, 37 °C). After the designated time interval, all of the outer solution was exchanged for fresh PBS containing 0.1% Tween 20 solution, and the solution containing the released DOX was used for quantitative analysis.

In vitro cell culture and incubation conditions

HCT-116 (human colon cancer) cells and K-1735 (murine melanoma cell) were obtained from the Korean Cell Line Bank (HCT-116=KCLB No. 10247, K-1735=KCLB No. 80013) and cultured in RPMI1640 supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 and subcultured in new medium every 2-3 days.

In vitro cellular uptake test

To verify the cellular uptake of D-LRPMs, HCT-116 cells were seeded in 6-well cell culture plate at a density of 2.5×10^5 cells per well and incubated for 12 h at 37 °C in 5% CO_2. The medium was then exchanged for serum-free (SF) medium containing D-LRPMs and the cells were incubated for 4 h before being rinsed, harvested and resuspended with DPBS. The cellular uptake was quantitatively analyzed using flow cytometry with a Becton-Dickinson FACS Canto II (San Jose, CA, USA). For each sample, 10,000 cells (gated events) were counted, and the DOX fluorescence was detected with logarithmic settings (FL4; Em = 670 nm). Each experiment was analyzed statistically using the FACS Diva software (BD).

Light-controlled endo/lysosomal escape

HCT-116 cells were seeded in a 35 mm cell culture plate, and the medium was removed after 12 h and replaced with serum-free (SF) medium containing D-LRPMs (10 μg mL^{-1} of DOX; 0.5 μg mL^{-1} of Ce6), following which the cells were incubated for 4 h. During incubation, the endo/lysosomes were labeled with LysoTracker Green DND-26 for 1 h. After incubation, the medium was removed, and the cells were rinsed twice with DPBS. Complement medium (100 μL) was added to the wells, and each well was irradiated using a 670 nm light source (0 or 1.2 J/cm^2; 6.0 mW/cm^2, 0 or 200 sec, fiber coupled laser system, LaserLab®, Korea). The cells were then washed twice with DPBS, fixed with 4% paraformaldehyde and stained with DAPI. The cells were mounted in mounting medium (Dako, Glostrup, Denmark) and visualized using a confocal laser scanning microscope (LSM 710 Meta; Carl Zeiss, Germany). Fluorescence images were analyzed using the LSM image browser software (Zen series, Carl Zeiss, Germany).

In vitro cell cytotoxicity study

HCT-116 cells were seeded in 35 mm cell culture plates at a density of 2.0×10^5 cells per well and incubated for 12 h. Free DOX, LRPM or D-LRPM (10 μg mL^{-1} of DOX; 0.5 μg mL^{-1} of Ce6) was added to each well in medium (1 mL), and the dishes were returned to the incubator for 4 h. After incubation, the wells were rinsed twice with DPBS to remove samples that had not been internalized into the cells. One hundred microliters of complement medium was added to the wells, and each well was irradiated using the pre-determined 670 nm light source (0 or 1.2 J/cm^2; 6.0 mW/cm^2, 0 or 200 sec, fiber coupled laser system, LaserLab®, Korea). The cells were then incubated for a further 48 h. The cell viability was assessed using the MTT colorimetric assay. The absorbance intensity was measured at 540 nm using a microplate reader (Bio-Tek, VT, USA).

Live/dead assay

The Live/dead viability/cytotoxicity assay kit (Molecular Probes, USA) was used as a two-color fluorescence cell viability assay. The dyes in this kit are 2 μM calcein AM, which stains live cells green, and 4 μM EthD-1, which stains dead cells red. HCT-116 cells were seeded in 35 mm cell culture dishes at a density of 2.0×10^5 per well. The cells were incubated for 12 h at 37 °C in 5% CO_2. After incubation, the medium was removed, and the cells were incubated in medium containing free DOX, LRPM or D-LRPM (10 μg mL^{-1} of DOX; 0.5 μg mL^{-1} of Ce6) for 4 h. The medium was removed, and the cells were rinsed twice with DPBS. Then, irradiation (1.2 J/cm^2; 6.0 mW/cm^2, 200 sec) was performed using a 670 nm light source (fiber coupled laser system, LaserLab®, Korea). The cells were then incubated in complement medium for 48 h, and the cell viability was observed using fluorescence microscopy (Zeiss, Germany).

In vivo test

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Catholic University of Korea (Republic of Korea) in accordance with the "Principles of Laboratory Animal Care", NIH publication no. 85-23, revised in 1985. K-1735 cells (2.0×10^5) were subcutaneous tumor implanted into the right flanks of mice. When the tumors grew to 75 – 100 mm^3 in volume, solutions of PBS, free DOX, LRPM (4 mg kg^{-1} of DOX; 0.2 mg kg^{-1} of Ce6) with or without laser irradiation, or D-LRPM with or without laser irradiation were injected into the mice via the tail vein (n = 3 per each group). 24 h post-injection, the tumor tissues were irradiated with a 670 nm light source. The optimal laser dose was determined to be 100 J/cm^2. The tumor volume was calculated using the equation Volume = $0.5 \times L \times W^2$, where "W" and "L" are the width and length of the tumor, respectively.

For histological analysis, mice were sacrificed, and tumors were collected and fixed for 24 h in 4% paraformaldehyde and then paraffin embedded to obtain 5 μm thick tumor sections. After deparaffinisation, the tissue sections (5 μm) were stained with hematoxylin/eosin (H&E) and observed by optical microscopy (Axio Imager D2, Carl Zeiss, Thornwood, U.S.A.).

The immunohistochemical study was performed using a terminal deoxynucelotidyl transferase dUTP nick end labelling (TUNEL) assay kit (EMD Chemicals Inc., Darmstadt, Germany). The stained slides were observed by optical microscope (Axio Imager D2, Carl Zeiss, Thornwood, U.S.A.).

Statistical Analysis

Data are expressed as the means ± standard deviation (SD). Differences between the values were assessed using Student's t test.

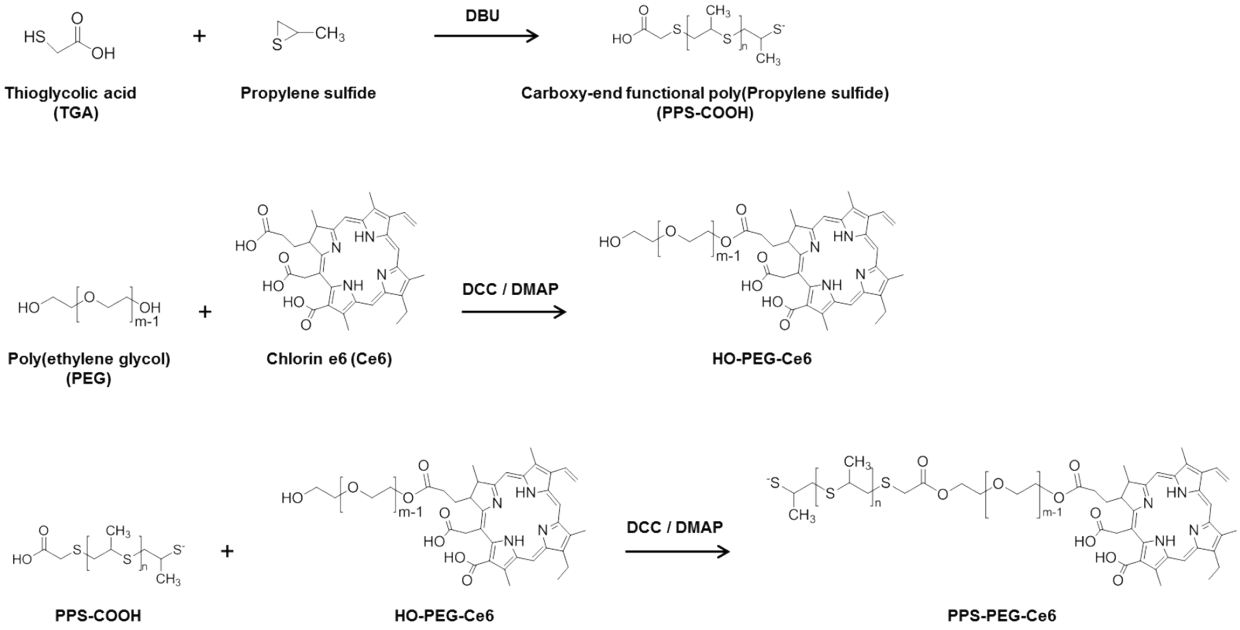
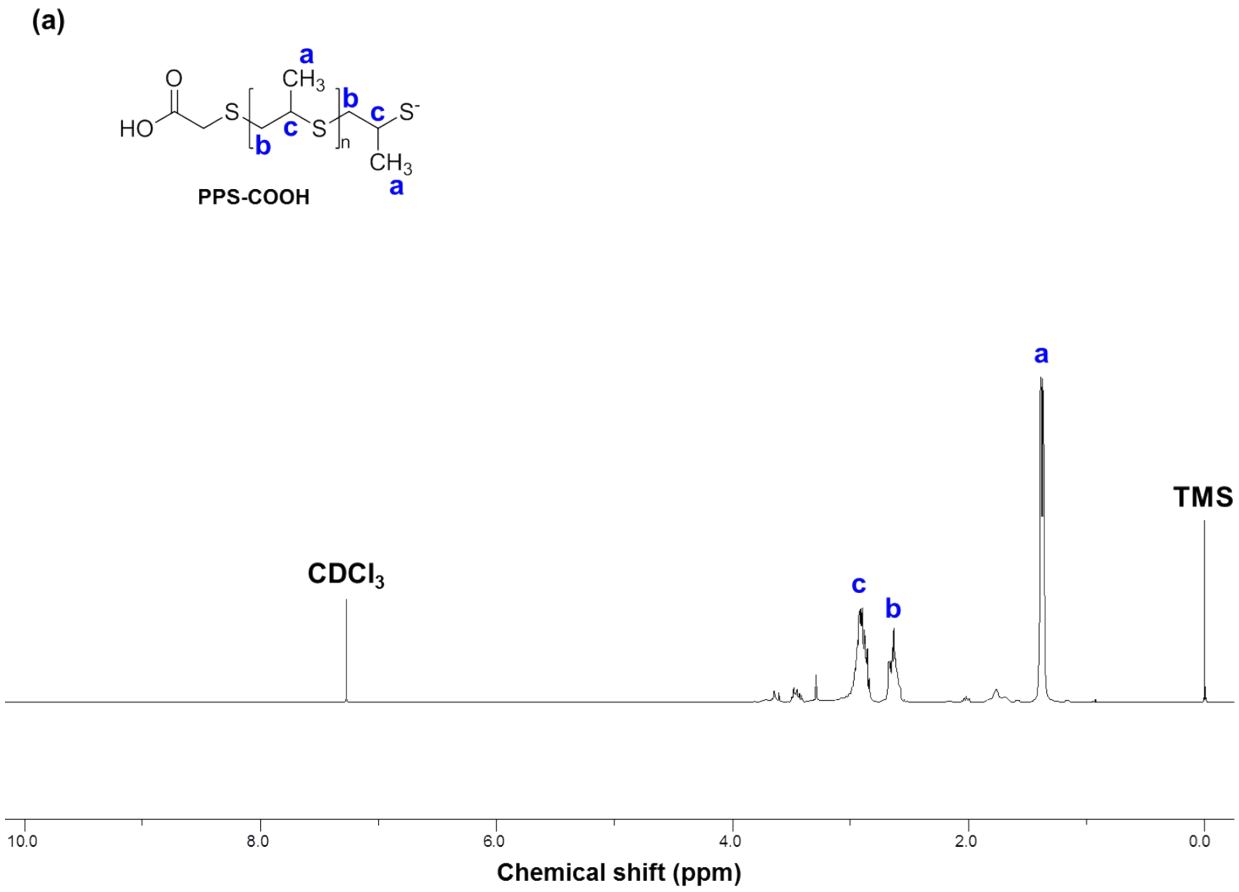
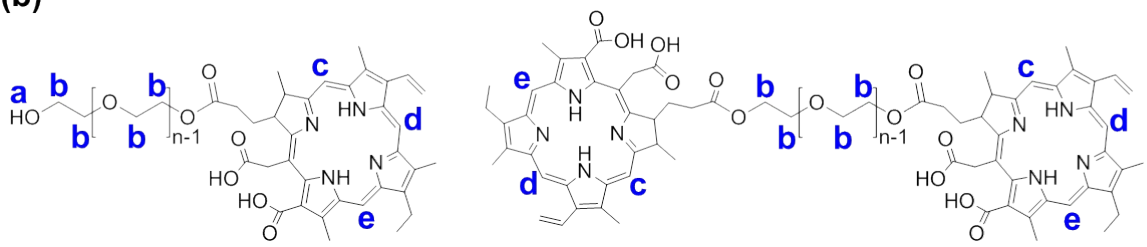


Figure S1. Overall scheme for the chemical synthesis of PPS-PEG-Ce6.

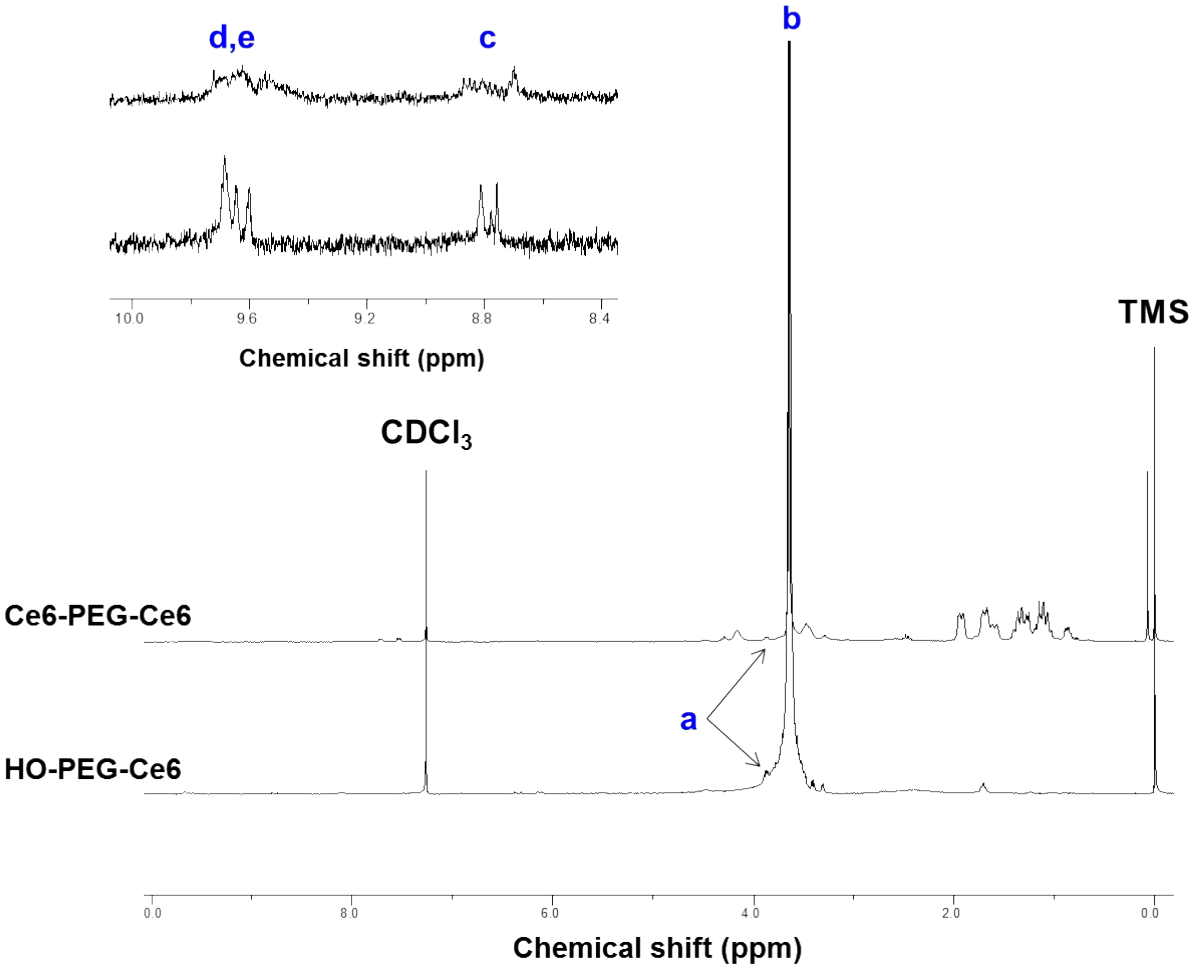
Figure S2. The characterization of the PPS-PEG-Ce6 conjugate was confirmed by the ^{1}H-NMR spectra of (a) PPS-COOH, (b) HO-PEG-Ce6 and Ce6-PEG-Ce6, and (c) PPS-PEG-Ce6 in $CDCl_3$.



(b)



HO-PEG-Ce6 Ce6-PEG-Ce6



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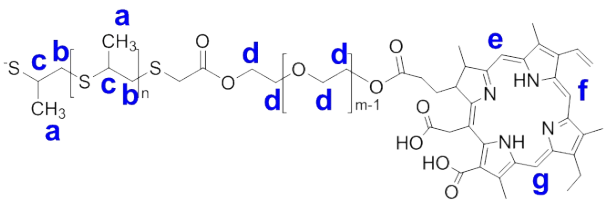
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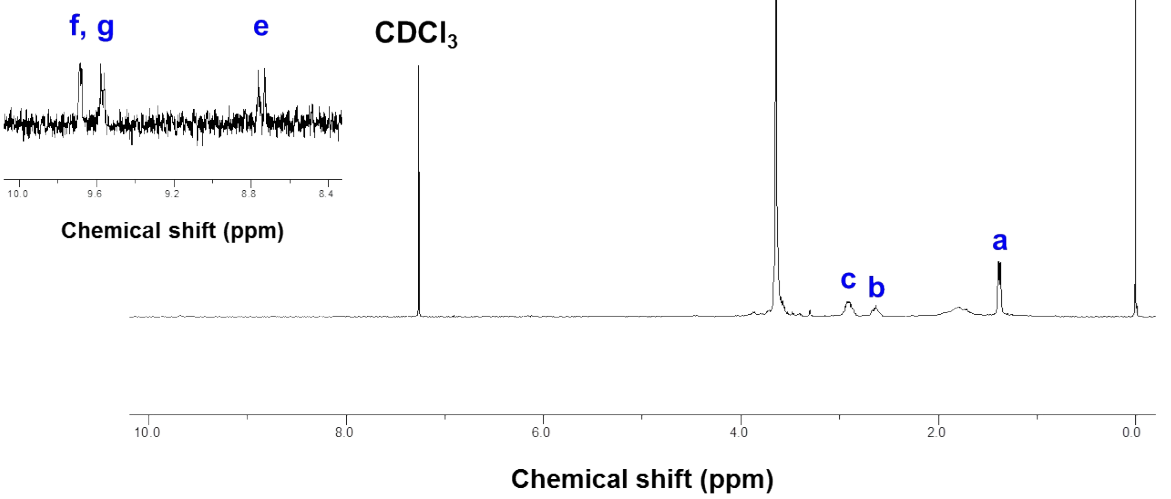
PPS-PEG-Ce6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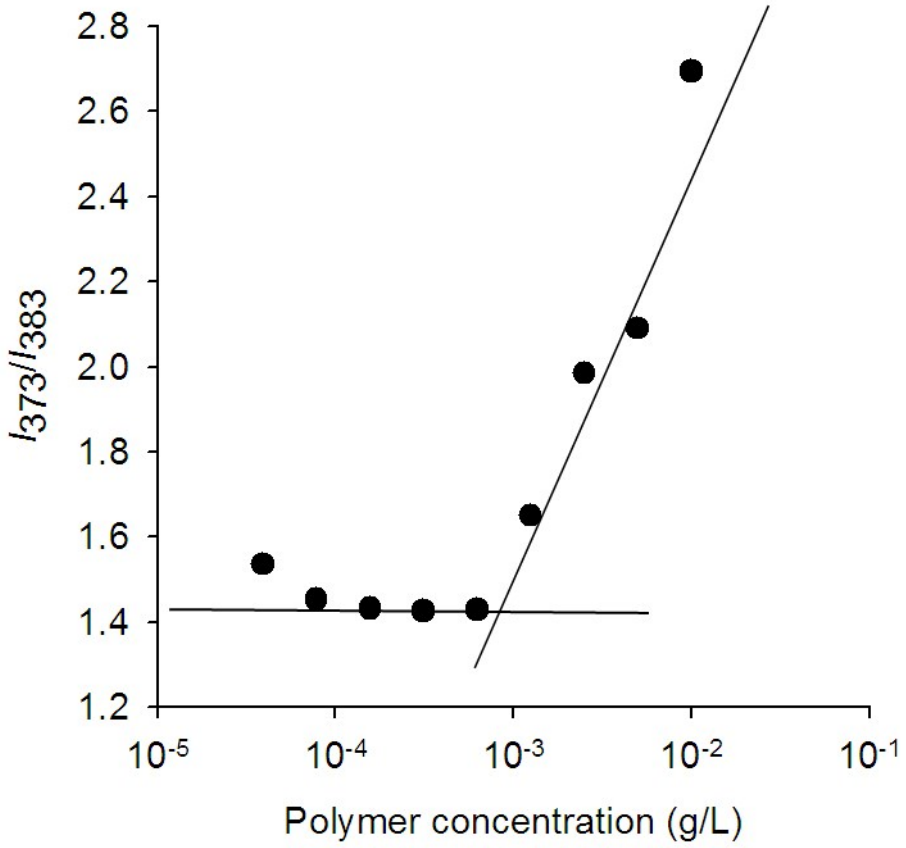

Figure S3. Critical micelle concentration (CMC) of LRPMs.

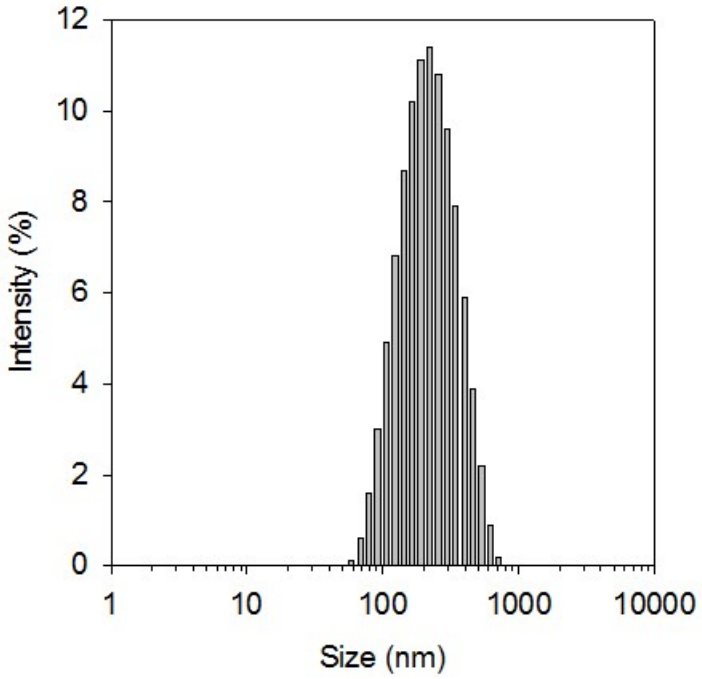

Figure S4. Hydrodynamic size distribution from dynamic light scattering (DLS) of LRPMs.

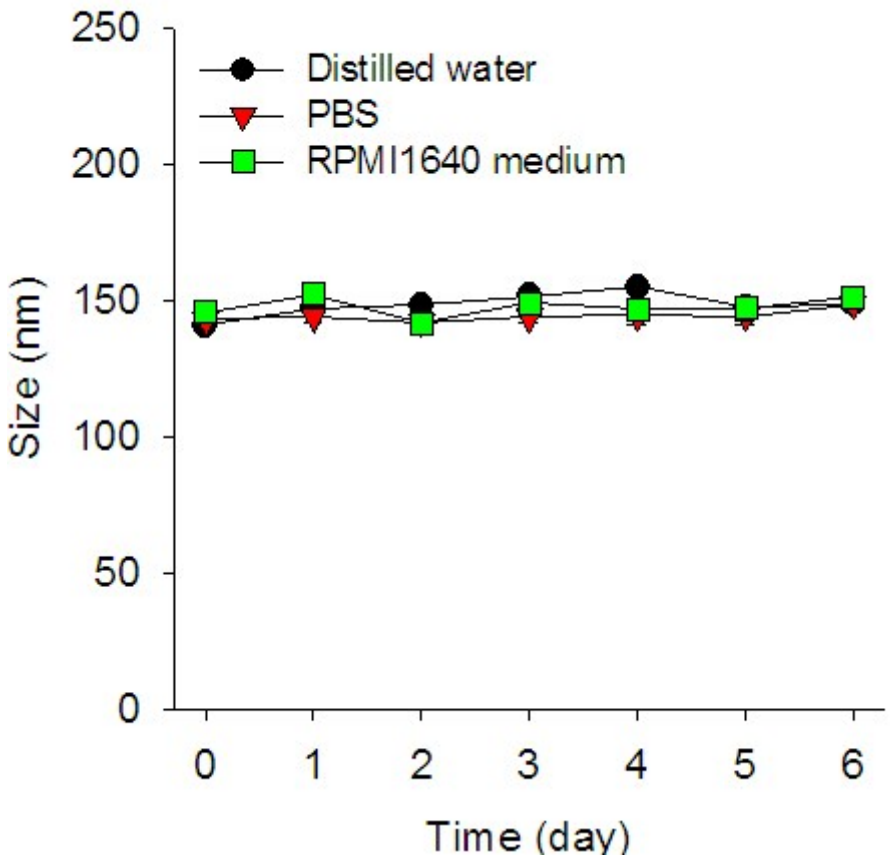

Figure S5. Hydrodynamic size changes of D-LRPMs in distilled water, PBS, or RPMI1640 medium for 6 days.

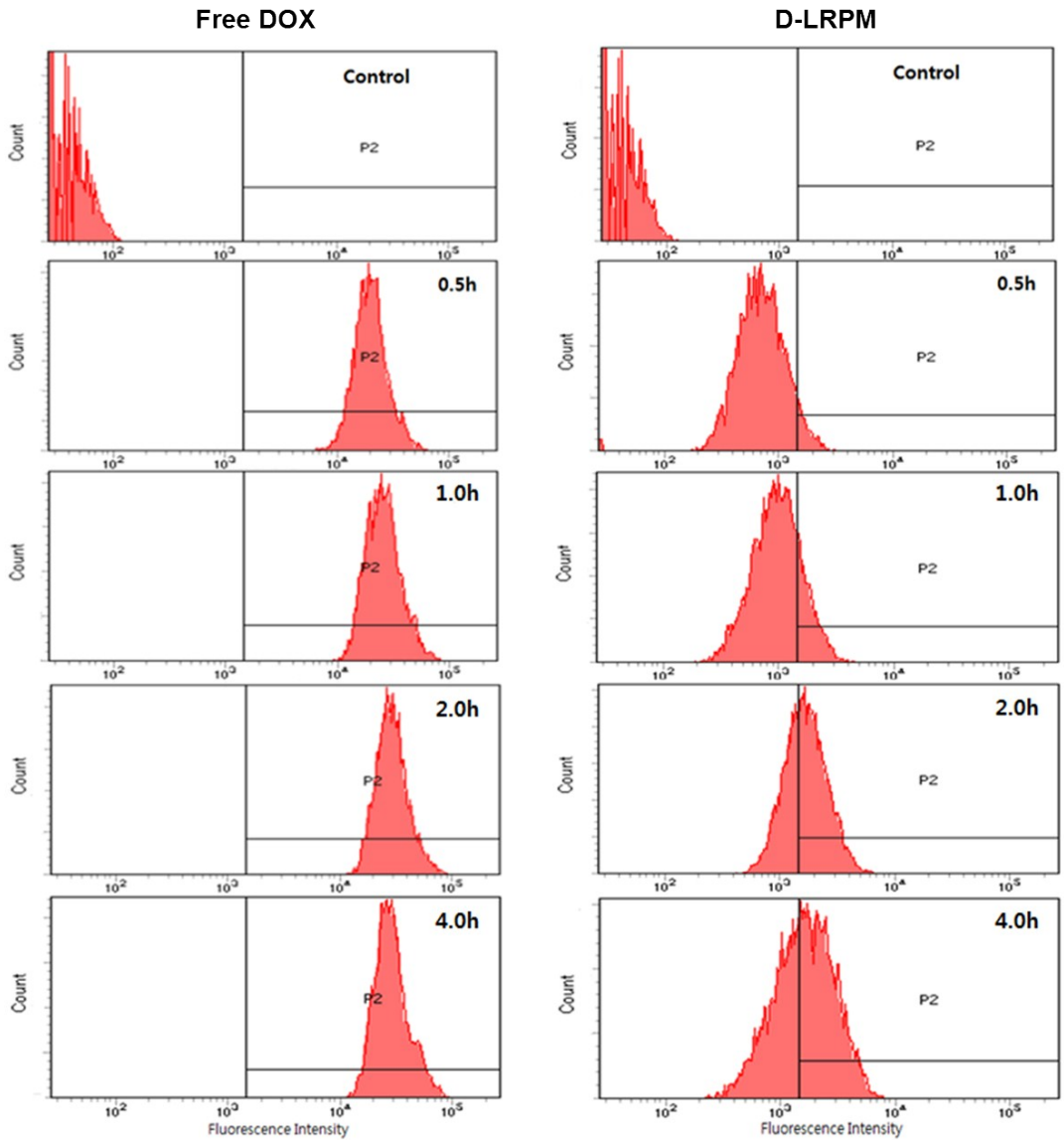

Figure S6. Cellular uptake. (a) Flow cytometric quantification of HCT-116 cancer cells. The cells were treated with free DOX and D-LRPMs.

References

1 H. Park and K. Na, *Biomaterials*, 2013, **34**, 6992-7000.

2 S. Jeong, W. Park, C. S. Lee and K. Na, *Macromol Biosci*, 2014, **14**, 1688-1695.