Supporting Information

Sono-Polymerization of Poly(ethylene glycol)-Based Nanoparticles for Targeted Drug Delivery

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Contents of Supporting Information

Experimental Section

Materials

The acrylate-terminal methoxy poly(ethylene glycol) (ACLT-$PEG_{2k/5k}$, Mn = 1 kDa, 2 kDa and 5 kDa), succinimidyl ester-terminal acrylate poly(ethylene glycol) (ACLT-PEG_{5k}-NHS, Mn = 5 kDa) were purchased from Jenkem Technology Co. Ltd. (Beijing, China). Succinic anhydride, 2-aminoethyl methacrylate hydrochloride (AEMA) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmpholinium hydrochloride (DMTMM) were obtained from J&K (Shanghai, China). Cisplatin was bought from Shandong Boyuan Pharmaceutical Co., Ltd. (Shandong, China). Cyclic peptides of RGDfK (RGD) was from GL Biochem Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM, High Glucose) and Dulbecco's Phosphate-Buffered Saline (DPBS) were bought from Beijing Neuronbc Laborataries Co., Ltd. (Beijing, China). Fetal bovine serum (FBS) was obtained from Gibco (Germany). Alexa Fluor 647 succinimidyl ester (AF647-NHS), Wheat germ agglutinin Alexa Fluor 488 conjugate and Hoechst 33342 were obtained from Thermo Fisher Scientific (Shanghai, China). Methyl-thiazolyldiphenyl-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Shanghai, China). Other chemicals and solvents such as *N,N*-dimethylformamide (DMF), ether, acetone sodium hydroxide (NaOH) and hydrochloric acid (HCl) were from local suppliers with the quality of analytical grade. Water was obtained using Milli-Q ultrapure water apparatus (Integral 5) with a resistivity of 18.2 MΩ cm.

Instruments and Characterizations

All the polymerization processes were performed on a RF generator (AG series amplifier LVG 60-10 produced by T&C Power Conversion Inc.) in combination with a 400 kHz plate transducer (Model 6G12 by Honda Electronics Co. Ltd.) The concentration of hydroxyl radical was determined by iodimetry on a UV-2600 UV-Vis spectrometer using a 1 cm path length quartz cuvette at 353 nm. [1,2] Proton nuclear magnetic resonance (^{1}H NMR) spectra were operated on Bruker Avance 400 spectrometer (USA). Zeta potential of NPs was measured on a Malvern Zetasizer Nano ZS90 (England) instrument. The morphology of NPs was characterized by transmission electron microscope (TEM) (JEOL JEM-1400 TEM, 120 kV) and atomic force microscope (AFM) (Asylum Research, Cypher ES). The concentration of Pt was detected by a Thermo Scientific iCAP RQ ICP-MS (USA). Molecular weight of PEG-based NPs was measured on DAWN HELEOS II (USA).

Determination of the Concentration of Hydroxyl Radicals

The concentration of hydroxyl radicals was determined by spectroscopy as reported by Qiao and Hochanadel[1,2]. Briefly, 1 mL of stock solution A (0.4 M KI, 0.1 M NaOH and 0.02 mM $(NH_4)_6Mo_7O_{24}$), 1 mL of stock solution B (0.1 M $C_8H_5KO_4$), and 1 mL of freshly sonicated sample was mixed together and then analyzed by UV/Vis spectroscopy at 353 nm. The detection mechanism is shown as follows:

$$H_2O \longrightarrow H\cdot + OH\cdot \quad (1)$$

$$2OH\cdot \longrightarrow H_2O_2 \quad (2)$$

$$H_2O_2 + 2I^- \longrightarrow 2OH^- + I_2 \quad (3)$$

$$I_2 + I^- \longrightarrow I_3^- \quad (4)$$

Synthesis of *c,c,t*-[Pt(NH_3)$_2$$Cl_2$(OH)($O_2CCH_2CH_2CO_2H$)] (Pt Prodrug)

The Pt prodrug of *c,c,t*-[Pt(NH_3)$_2$$Cl_2$(OH)($O_2CCH_2CH_2CO_2$ H)] was synthesized according to a modified protocol reported before.[3,4] Briefly, *c,c,t*-[Pt(NH_3)$_2$$Cl_2(OH)_2$] (0.60 g, 1.8 mmol) was dissolved in 40 mL of DMSO in a 150 mL round-bottom flask under vigorous stirring. Succinic anhydride (0.18 g, 1.8 mmol) dissolved in DMSO (10 mL) was added into the mixture solution and stirred at 40 ºC for 12 h. DMSO was lyophilized and 30 mL of acetone was added to precipitate the product. The product was washed with acetone and diethyl ether three times and then dried. The product yield was 63% (0.49 g). ESI-MS: Calc. = 434, Found = 433. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm) = 5.7-6.2 (br, 6H, NH_3), 2.35-2.45 (m, 4H, CH_2C$H_2$$CO_2$H) (**Figure S6**).

Synthesis of ACLT-PEG$_{5k}$-RGD

ACLT-PEG$_{5k}$-RGD was synthesized by amidation reaction. In brief, RGD (36.22 mg, 0.6 mmol) and ACLT-PEG$_{5k}$-NHS (36.22 mg, 0.2 mmol) were dissolved in 2 mL of anhydrous DMSO and reacted at 25 ºC for 12 h. The mixture was dialyzed for 2 days to remove the unreacted RGD. The product was lyophilized to obtain white powder (yield 90%). ^{1}H NMR (400 MHz, D_2O), δ (ppm) = 6.1-6.2 (1H, CH_2=CH), 7.1-7.3 (1.8H, -C_6H_5) (**Figure S7**). 36% ACLT-PEG$_{5k}$-NHS have been modified with RGD.

Preparation of NPs

Ultrasound was used to initiate the polymerization to prepare PEG-based NPs. Taking the sample of ACLT-PEG$_{2k}$/AEMA/ ACLT-PEG$_{5k}$-RGD (molar ratio 6/3/1) as an example, ACLT-PEG$_{2k}$ (138 mg, 0.069 mmol), AEMA (4.5 mg, 0.0345 mmol) and ACLT-PEG$_{5k}$-RGD (57.5 mg, 0.0115 mmol) were dissolved in 1 mL of water and

subsequently removed oxygen via nitrogen bubbling for 30 min. The mixture was then sonicated at 40 ºC for 20 min (412 kHz, 40 W). Subsequently, the reaction was terminated by adding 9 mL of water. The obtained NPs were purified by dialysis for 3 days (molecular weight cutoff 8-14 kDa) and freeze-dried to obtain white powder, which was named RGD-PEG NPs (yield: 76%). The synthesis process of NPs composed of ACLT-PEG_{2k}/AEMA/ACLT-PEG_{5k} (molar ratio 6/3/1) was same as above and named as PEG NPs.

Conjugation of Pt Prodrugs and Fluorescent Dyes

For drug loading, the Pt prodrug (20 mg, 0.046 mmol), DMTMM (19.09 mg, 0.069 mmol) and PEG-based NPs (30 mg) were mixed in 1 mL of phosphate buffer saline (PBS, 10 mM, pH 7.4) and stirred for 24 h at 25 ºC, followed by dialysis for 2 days to remove the excess Pt prodrugs and DMTMM and lyophilization to obtain drug-loaded PEG NPs (Pt@PEG-RGD NPs or Pt@PEG NPs). For fluorescent dye labeling, PEG-based NPs were dispersed in 1 mL of PBS buffer (10 mM, pH 7.4), followed by adding 20 μL of AF647-NHS (1 mg mL^{-1} in DMSO). After stirring for 12 h, the mixture was dialyzed for 2 days and then lyophilized (AF647-labeld PEG-RGD NPs or AF647-labeld PEG NPs).

***In Vitro* Pt Release Kinetics**

To monitor the release of Pt, 2 mL of Pt@PEG NPs (1 mg mL^{-1}) was added in a dialysis bag and dialyzed against 5 mM ascorbic acid in 48 mL of PBS buffer (10 mM, pH 6.5). 1 mL of PBS was collected at different time intervals and then an equal volume

of fresh PBS was added. The concentration of Pt was quantified by ICP-MS. For the control group without ascorbic acid, the Pt@PEG NPs were dialyzed against PBS.

In Vitro Cell Association

Cell association was investigated by incubating U87 MG cells with AF647-labeld PEG-RGD and PEG NPs. U87 MG cells were seeded into 24-well plates at the density of 5×10^4 cells per well in DMEM media with the addition of 10% (v/v) FBS and allowed to attach for 12 h. These two kinds of PEG-based NPs with different concentration (50, 100, 200 μg mL^{-1}) were incubated with cells for 2, 4, 8, and 12 h at 37 ºC, respectively. After incubation, the culture medium was removed and then the cells were washed twice by DPBS. Subsequently, the cells were harvested by trypsinization and then resuspended in 500 μL DPBS. The cells were analyzed with flow cytometer to determine the cell association and mean fluorescence intensity of cells.

For cells imaging, U87 MG cells were seeded in 14 mm confocal dishes with the density of 3×10^4 cells per well in DMEM media with the addition of 10% (v/v) FBS and cultured at 37 ºC for 12 h. AF647-labeld PEG-RGD and PEG NPs (100 μg mL^{-1}) were incubated with U87 MG cells for 2, 4, 8, and 12 h, respectively. Subsequently, the cells were washed twice with DPBS and then fixed with 4% paraformaldehyde (PFA) for 15 min at 25 ℃. After washing the cells with DPBS twice, the resulting cells were stained with Hoechst 33342 and WGA AF488 conjugate. To investigate the receptor-mediated cell binding of targeted PEG-based NPS, AF647-labeld PEG-RGD NPs were incubated with U87 MG cells at 4 and 37 ℃ for 1 h (PEG-RGD NP concentration 200 μg mL^{-1}).

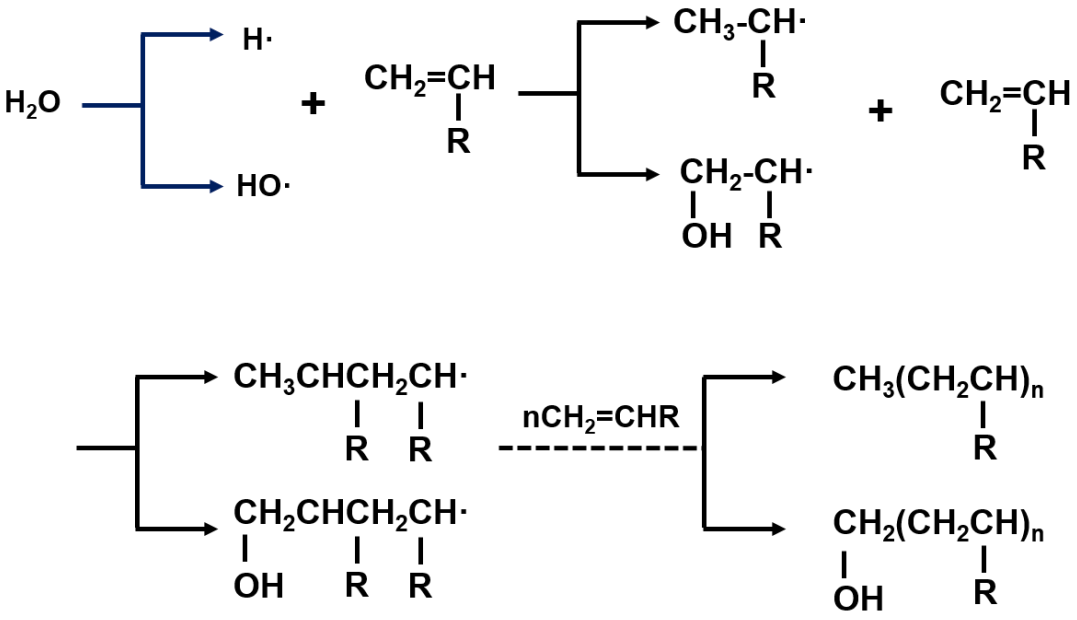
Fluorescence images were observed by confocal laser scanning microscopy (CLSM, Leica TCS SP8 STED 3X). (Cy5 channel for AF647 excited at 647 nm, FITC channel for AF488 excited at 495 nm, and DAPI channel for Hoechst 33342 excited at 358 nm).

In Vitro Cytotoxicity

The cytotoxicity of drug-loaded PEG-based NPs was measured by MTT assay. U87 MG cells were seeded in 96-well plates at the density of 8×10^3 cells per well in DMEM media with the addition of 10% (v/v) FBS and then cultured 12 h for cell attachment. The cells were then incubated with different concentrations of cisplatin or Pt-loaded PEG NPs (equivalent Pt of 0.3, 0.6, 1.3, 2.5, 5.0 and 10.0 μg mL^{-1}) for 48 h or 72 h. Next, 10 μL of MTT (5 mg mL^{-1}) solution was added to each well and then incubated for another 4 h. The cell medium was removed and 100 μL DMSO was added to dissolve the formazan crystals. The plates were shaken for 10 min before absorbance measurements (570 nm) with plate reader (Tecan Austria GmbH).

Cell Apoptosis

U87 MG cells were seeded in 24-well plates at a density of 5×10^4 cells per well in DMEM media with the addition of 10% (v/v) FBS and cultured for 12 h. The medium was removed and the cells were incubated with a predetermined amount of cisplatin or Pt-loaded PEG NPs (equivalent Pt of 5 μg mL^{-1}) for 48 h. Then, the medium was replaced with 500 μL of dye solution containing 2 μmol L^{-1} of Calcein-Am and 4 μmol L^{-1} PI). After 10 min incubation, representative fluorescence images were acquired by an inverted fluorescence microscope (ZEISS Axio Observer 3).



Scheme S1. Illustration of the sono-polymerization mechanism.

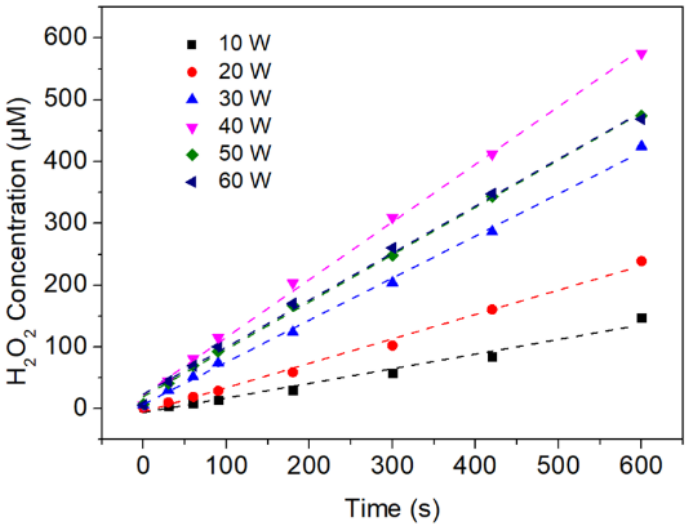


Figure S1. Influence of power on the generation of hydroxyl radicals (412 kHz, 40 ºC).

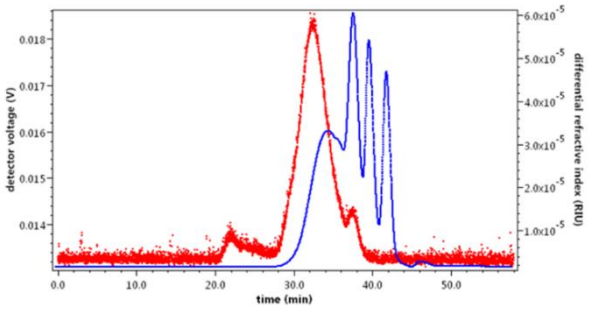


Figure S2. GPC profile of ACLT-PEG$_{2k}$-10%-2 h (red: LS 11, blue: dRI).

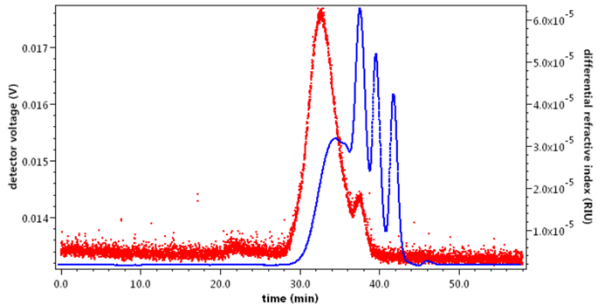


Figure S3. GPC profile of ACLT-PEG$_{5k}$-10%-2 h (red: LS 11, blue: dRI).

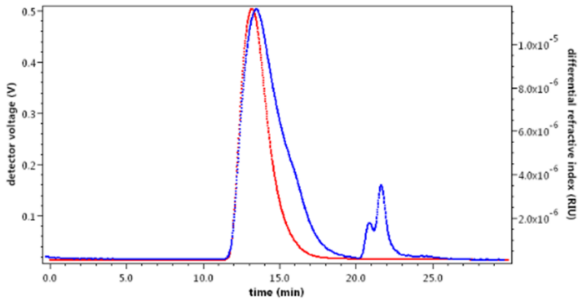


Figure S4. GPC profile of ACLT-PEG$_{2k}$/AEMA/ACLT-PEG$_{5k}$-RGD-20%-20 min (red: LS 11, blue: dRI).

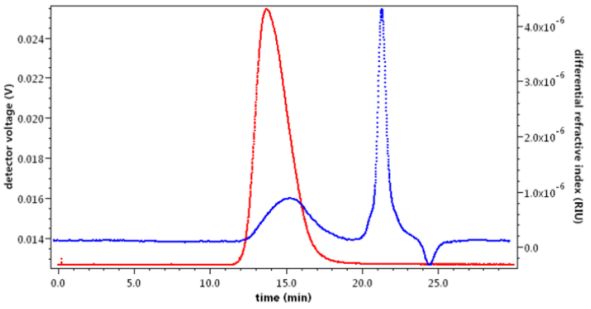


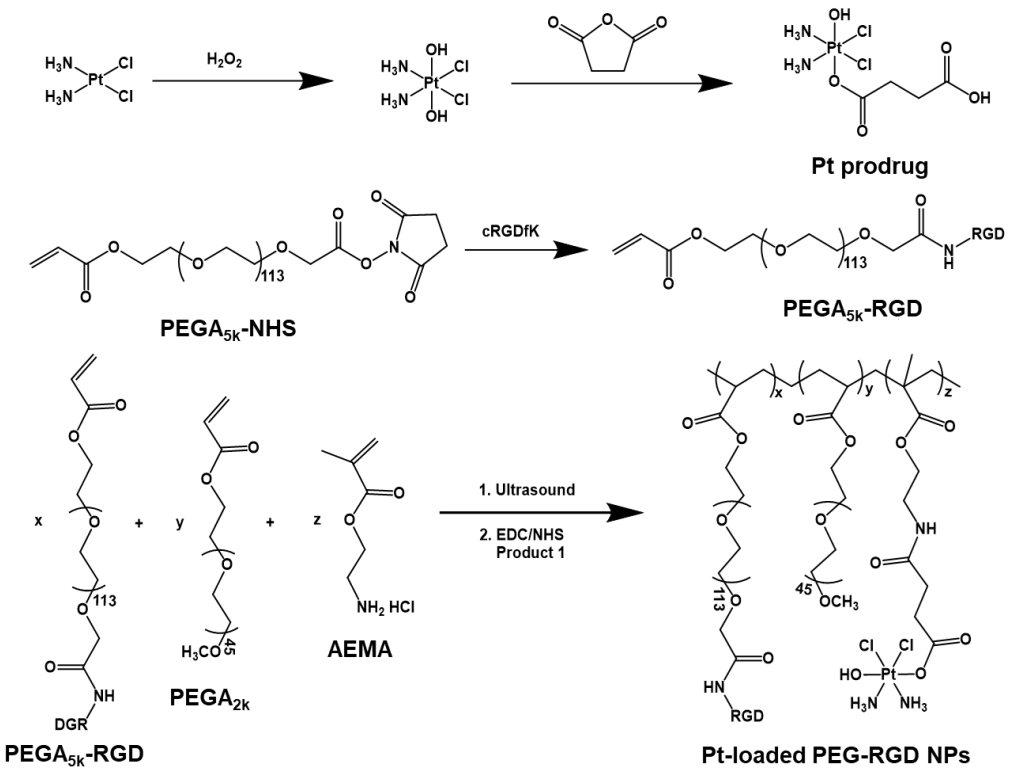
Figure S5. GPC profile of ACLT-PEG$_{5k}$/AEMA/ACLT-PEG$_{5k}$-RGD-20%-7 min (red: LS 11, blue: dRI).

Table S1. Molecular weight of PEG-based NPs from GPC.

Sample	Mn kDa	Mw kDa	PDI
ACLT-PEG_{2k}-10%-2 h	28.8[1] 7.4[2]	32.6[1] 7.7[2]	1.1[1] 1.1[2]
ACLT-PEG_{5k}-10%-2 h	18.7[1] 7.9[2]	20.3[1] 8.5[2]	1.2[1] 1.1[2]
ACLT-PEG_{2k}/AEMA/ACLT-PEG_{5k}-RGD-20%-20 min	1117	1377	1.2
ACLT-PEG_{5k}/AEMA/ACLT-PEG_{5k}-RGD-20%-7 min	1008	1233	1.2

[1,2] There are two peaks for the GPC results when the monomer concentration was 10%.

Molecular weights and PDI were calculated from peaks 1 and 2, respectively.



Scheme S2. Synthesis of Pt prodrug, ACLT-PEG$_{5k}$-RGD, and Pt@PEG-RGD NPs.

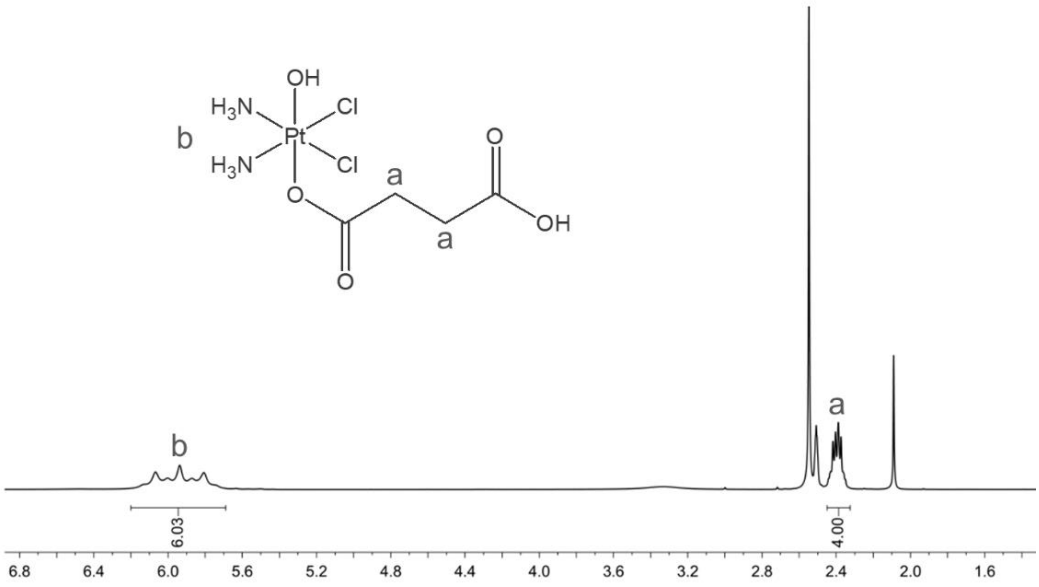


Figure S6. ^{1}H NMR spectrum of *c,c,t*-[$Pt(NH_3)_2Cl_2(OH)(O_2CCH_2CH_2CO_2H)$], (400 MHz, DMSO-d6).

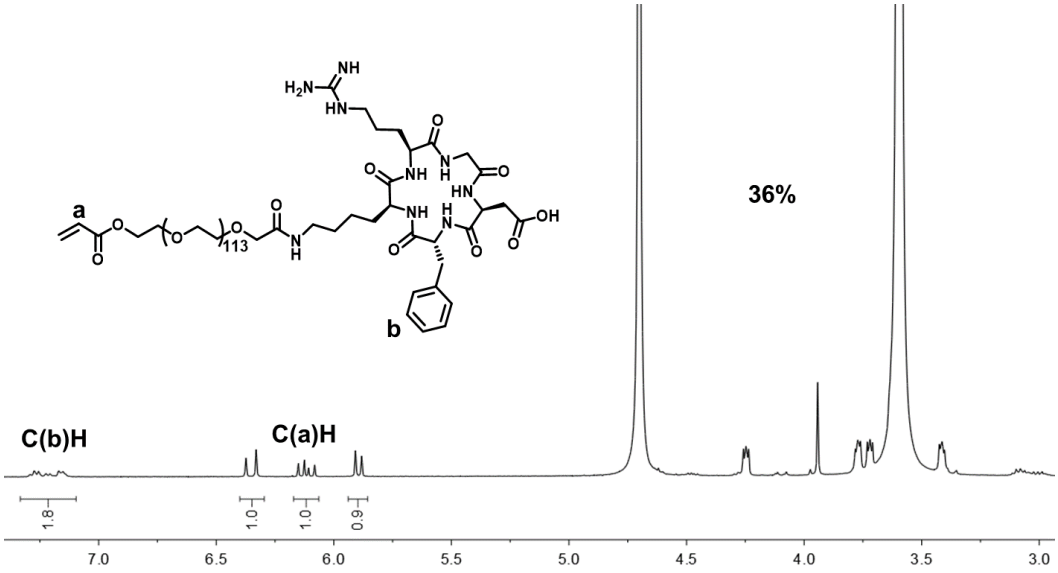


Figure S7. ^{1}H NMR spectrum of ACLT-PEG$_{5k}$-RGD, (400MHz, D_2O).

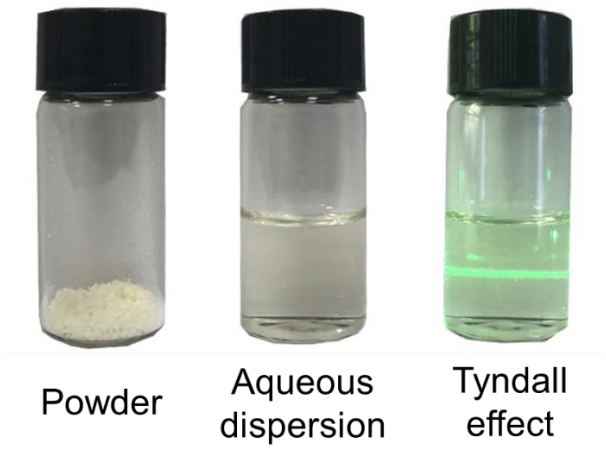


Figure S8. Photographs of the powder and aqueous dispersion of PEG-based NPs with Tyndall effect.

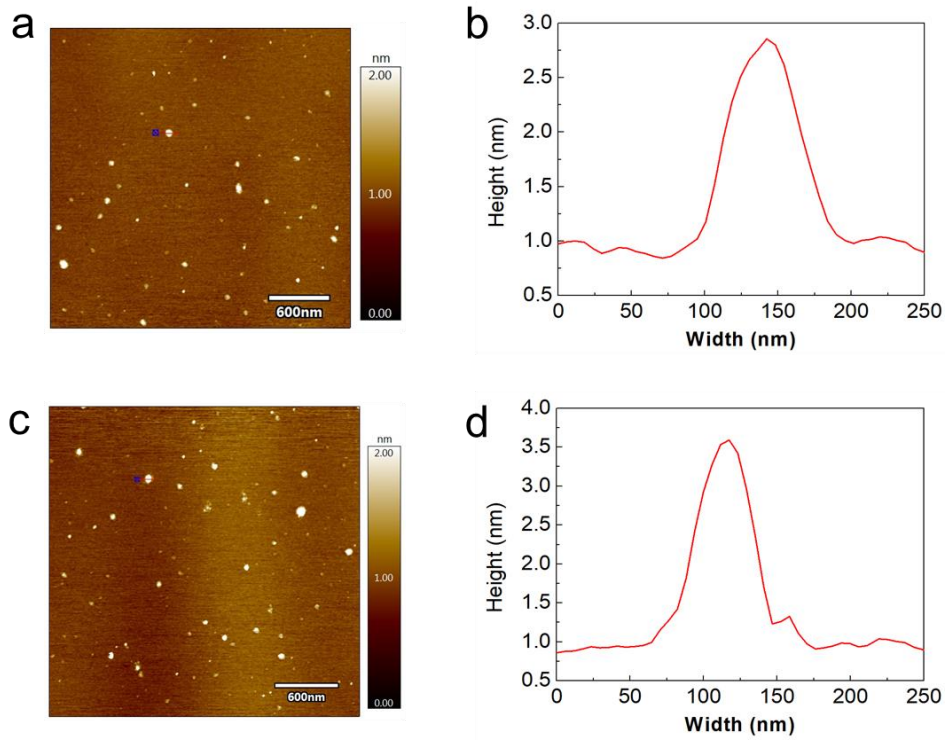


Figure S9. (a,c) AFM images and (b,d) height analysis of (a) PEG and (c) PEG-RGD NPs.

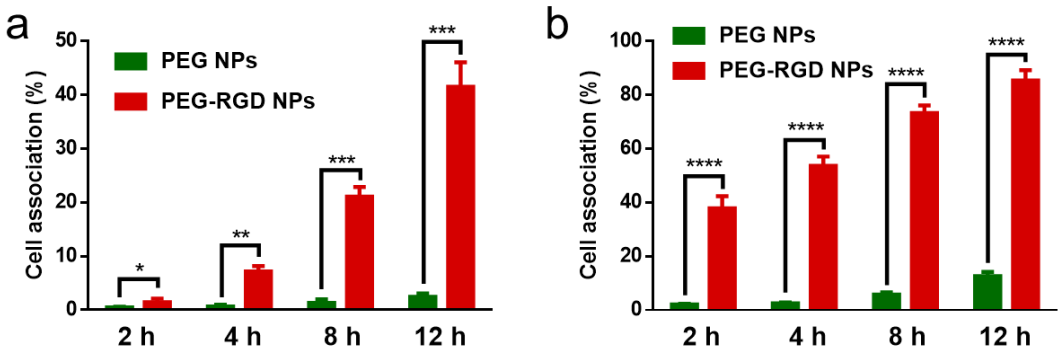


Figure S10. U87 MG cell association of AF647-labeled PEG and PEG-RGD NPs. (a) 50 μg mL^{-1}, (b) 200 μg mL^{-1}.

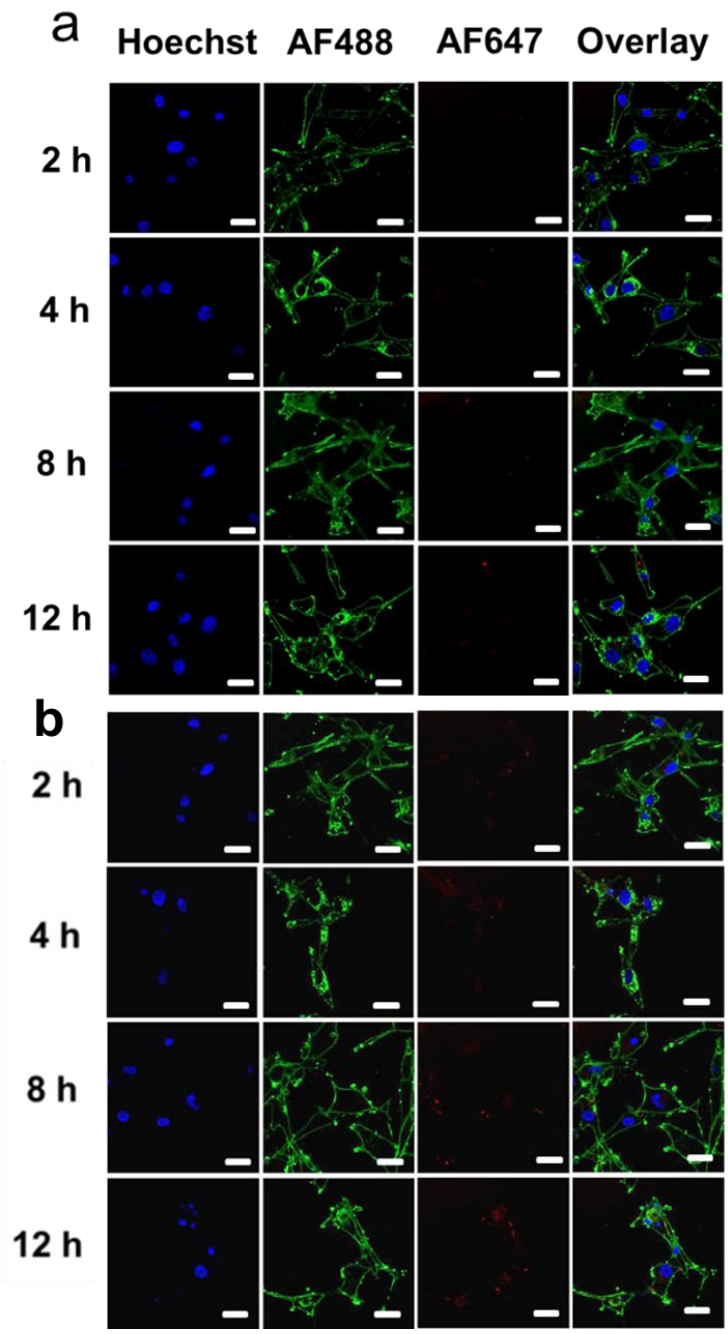


Figure S11. CLSM images of U87 MG cells after incubation with AF647-labeld PEG NPs (a) and PEG-RGD NPs (b) (100 µg mL^{-1}). Hoechst (blue) and wheat germ agglutinin Alexa Fluor 488 conjugate (green) were used to stain cell nuclei and cell membrane, respectively. Scale bars are 10 µm.

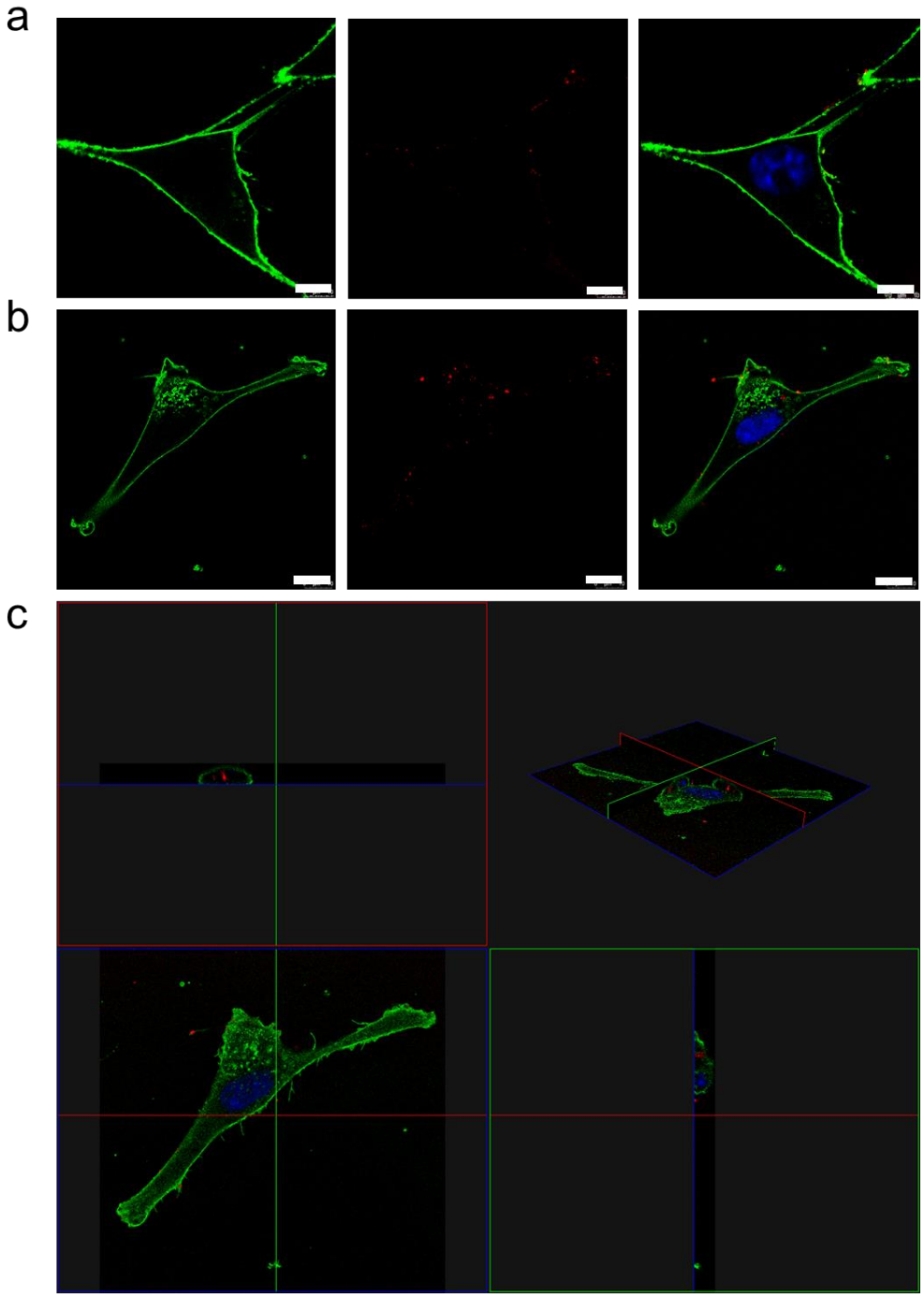


Figure S12. CLSM images of U87 MG cells after incubation with AF647-labeld PEG-RGD NPs at (a) 4 ℃ (a) and (b) 37 ℃. (c) Cross-section analysis of (b). The concentration of PEG-RGD NPs is 200 μg mL^{-1}. Scale bars are 10 μm.

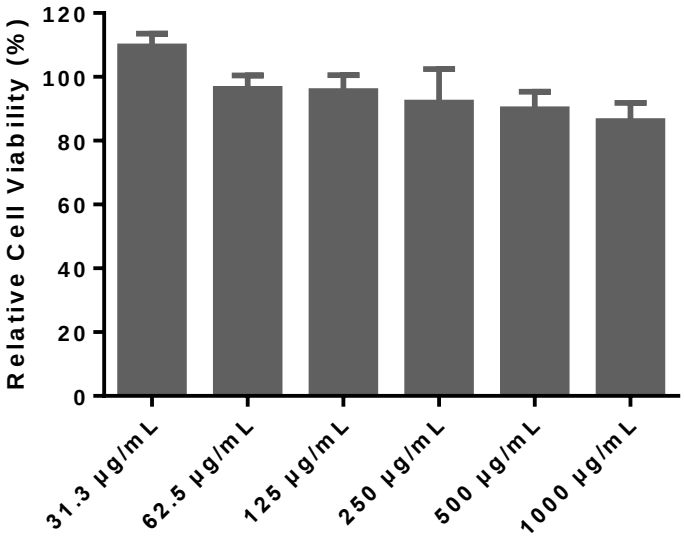


Figure S13. Cytotoxicity of PEG NPs. U87 MG cells were incubated with PEG NPs for 48 h before the MTT assay.

Table S2. Half-maximal inhibitory concentration (IC50) of free cisplatin, prodrug, and NPs against U87 MG cells.

Sample	IC50 (μg mL^{-1})
Cisplatin	3.9
Prodrug	6.3
Pt@PEG-RGD NPs	1.7
Pt@PEG NPs	3.8

References:

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