Programmed photosensitizer-conjugated supramolecular nanocarriers with dual targeting ability for enhanced photodynamic therapy

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Experimental section

Materials and characterizations

β-Cyclodextrin (β-CD), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) was supplied by Aladdin Reagent Co., Ltd. (Shanghai, China). Tosyl chloride (OTs) and triethylenetetramine was bought from Energy Chemical Co., Ltd. (Shanghai, China). Chlorin e6 (Ce6) was provided by J&K Scientific Ltd. Holo-transferrin, cathepsin B (CTSB) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Aldrich. The designed guest molecule Ad-HAIYPRH(T7) -EE-GFLG-CGKRK and Ad-HAIYPRH(T7)-EE-GGLG-CGKRK was synthesized by Top-peptide Co., Ltd (Shanghai, China). MitoTracker® Green FM was bought from Invitrogen and used following the instructions. Annexin V-FITC Apoptosis Detection Kit was purchased from BD Biosciences (Hangzhou, China). All other reagents and solvents were of analytical grade and used as received without further purification.

The ^{1}H NMR spectra were recorded on a Bruker DMX500 spectrometer operating at 500 MHz. The hydrodynamic diameter (D_h) of the nanocarriers was measured by Zetasizer Nano-ZS from Malvern Instruments equipped with a He-Ne laser at wavelength of 633 nm with an angle of 173° (25 °C). The sizes and morphologies of the resultant samples were characterized by JEM-1230 transmission electron microscopy (TEM) at an accelerating voltage of 80 kV, whereby a small drop of sample solution was deposited onto a carbon-coated copper TEM grid (230 mesh) and dried at room temperature at atmospheric pressure. The loading content of Ce6 in CD-Ce6/CGKRK-GFLG-T7was test by a Shimadzu UV-2505 spectrophotometer. Fluorescence images were acquired by using a Perkin-Elmer LS 55 fluorescence spectrometer and confocal laser scanning microscope (CLSM, Leica TS SP5, Germany). Flow cytometry was analyzed by FACSCalibur flow cytometer.

Synthesize of β-CD-Ce6

β-CD-Ce6 was obtained via a multistep synthesis routine (Scheme S1). Firstly, activating β-CD with tosyl chloride (OTs), β-CD (60g) was dissolved in deionized water

(250 mL) followed by adding sodium hydroxide solution (6.57g in 15 mL deionized water) dropwisely. After β-CD was completely dissolved, the solution was placed into an ice bath. Then, OTs (16 g in 30 ml acetonitrile) was added slowly under constantly stirring. Clear white precipitation appeared upon adding OTs. After further reacting for 4 h at room temperature, the mixture was placed into refrigerator overnight, recrystallized in water for three times and filtered to get white product (β-CD-OTs). Secondly, β-CD-OTs was further reacted with triethylenetetramine to obtain β-CD-NH_2. β-CD-OTs (1 g) was dissolved in dimethyl formamide (10 mL), then triethylamine (1mL) and triethylenetetramine (0.8 mL) was added slowly. The reaction was carried out for 72 h at 80 °C. The resultant solution was precipitated in acetonitrile for three times, followed by being dried in vacuum at 35 ºC overnight to obtain β-CD-NH_2. Finally, β-CD-NH_2 (70 mg) was reacted with Ce6 (25 mg) under the catalysis of EDC (30 mg) and NHS (20 mg) in dimethylsulfoxide (DMSO) for 24 h. Exhaustive dialysis (Mw=1000) of the resultant was conducted against DMSO for one day and distilled water for two days, the β-CD-Ce6 conjugate was obtained via lyophilization. UV-vis was applied to calculate the Ce6 content in the conjugation (DLC). Briefly, we weighted certain amount of β-CD-Ce6 and dissolved it into DMSO, then using UV-vis to test the concentration of Ce6 in the solution by comparing with a calibration curve of Ce6, finally, the Ce6 content in the conjugation (DLC) was calculated to be 30.2% according to following equation, DLC= relative molecular mass of Ce6*the concentration of Ce6 / the mass of β-CD-Ce6.

Preparation and characterization of supramolecular micelles

Typically, 3 mg β-CD-Ce6 and 5 mg Ad-T7-GFLG-CGKRK or 5 mg Ad-T7-GGLG-CGKRK were dissolved in 3 mL DMSO. After vigorously stirring for 24 h, 3 mL deionized water was added dropwisely. The reaction mixture was stirred for 24 h at room temperature in the dark and CD-Ce6/CGKRK-GFLG-T7 or CD-Ce6/CGKRK-GGLG-T7 was formed spontaneously. The prepared nanocarriers were purified by dialysis (Mw=3500) against deionized water for 2 days.

The formation of CD-Ce6/CGKRK-GFLG-T7 and CD-Ce6/CGKRK-GGLG-T7 were characterized by measuring the diameter and observing the morphology through DLS and TEM. To confirm the CTSB cleavable ability of CD-Ce6/CGKRK-GFLG-T7, 1mL micellar solution was treated with CTSB for 24 h at 37 ºC. Then, DLS, TEM and HPLC were applied to investigate the change after CTSB treatment. At last, to determine Ce6 loading content of the micelles, the micellar solution was lyophilized to remove water and weighted. Then, Ce6 content was measured using UV spectrophotometer.

Cell Culture

Michigan cancer foundation-7 (MCF-7) cells were cultured at 37 °C and 5% CO_2 environment. The cell culture medium used was Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 U mL^{-1} penicillin, and 100 mg /mL streptomycin.

Cellular uptake, targeting and ROS generation behaviors of CD-Ce6/CGKRK-GFLG-T7

To trace the cellular uptake and transferrin receptor (TfR) targeting behavior of

CD-Ce6/CGKRK-GFLG-T7, MCF-7 cells were inoculated into confocal microscopy dishes at 1.5×10^5 cells density in DMEM medium for 24 h. Cells pretreated with transferrin (25 μM) for 1 h were used as control. The culture medium was replaced with fresh medium containing CD-Ce6/CGKRK-GFLG-T7 (Ce6 5 μg mL^{-1}) followed by 4 h further incubation. Then, cells were washed with PBS, fixed with 4% paraformaldehyde and stained with 4,6-diamidino-2-phenylindole (DAPI). Images were obtained using confocal laser scanning microscope (CLSM). To quantitatively confirm above behavior, MCF-7 cells were inoculated into 24 well plates at 1.5×10^5 cells per well in DMEM medium for 24 h. Cells pretreated with transferrin (25 μM) for 1 h were used as control. The culture medium was replaced with fresh medium containing CD-Ce6/CGKRK-GFLG-T7 (Ce6 5 μg mL^{-1}) followed by 1, 2 and 4 h further incubation, respectively. Then, the cells were washed with PBS, trypsinized and resuspended in 1mL PBS for flow cytometry measurements

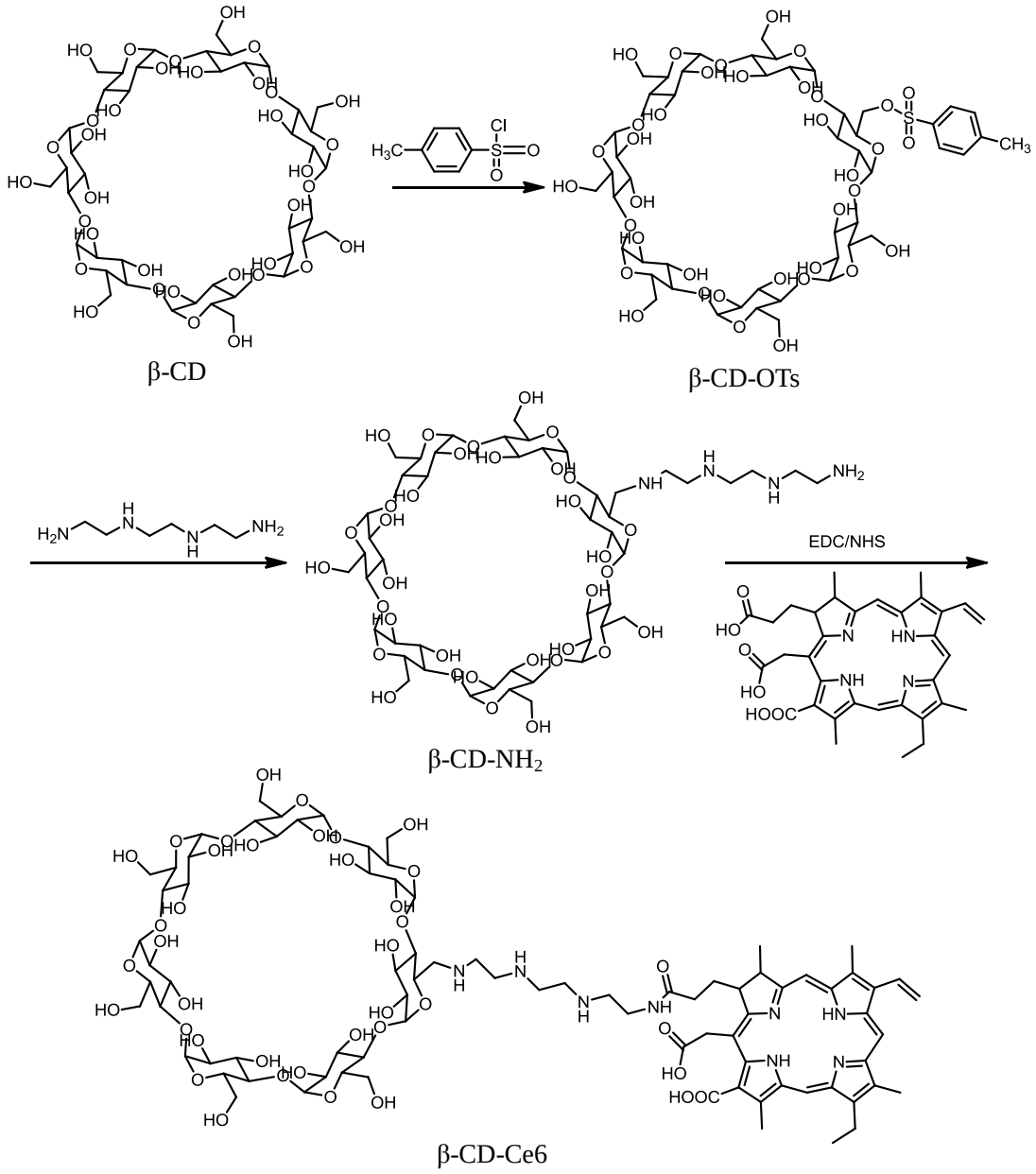
To measure the subcellular localization of CD-Ce6/CGKRK-GFLG-T7, MCF-7 cells were inoculated into confocal microscopy dishes for 24 h. Then, the culture medium was replaced with fresh medium containing CD-Ce6/CGKRK-GFLG-T7 or CD-Ce6/CGKRK-GGLG-T7 (Ce6 5 μg mL^{-1}), followed by 12h further incubation. Then, cells was stained with MitoTracker® Green FM and imaged directly via CLSM.

The generation of intracellular ROS was measured via fluorescence spectrometer using DCF-DA as the sensor. MCF-7 cells were incubated with CD-Ce6/CGKRK-GFLG-T7 or CD-Ce6/CGKRK-GGLG-T7 (2 μg mL^{-1}) and then the medium was replaced with fresh serum free medium containing DCF-DA for 20 min. light irradiation was performed subsequently (0.500 W, 30s). The cells were washed with pre-warmed fresh serum free medium and observed as soon as possible via fluorescence spectrometer. For quantitative the ROS generation, cells after washing were trypsinized and resuspended in 1mL PBS for flow cytometry measurements.

Cell apoptosis and cytotoxicity of CD-Ce6/CGKRK-GFLG-T7

MCF-7 cells were inoculated into 24 well plates at 1.5×10^5 cells per well in DMEM medium for 24 h. Then, the medium was replaced with fresh medium containing CD-Ce6/CGKRK-GFLG-T7 or CD-Ce6/CGKRK-GGLG-T7 (2 μg mL^{-1}). After 12 h further incubation, light irradiation was performed (0.500 W, 2 min). Cells were incubated for another 4 h before being collected. The collected cells were resuspended in 200 μL of binding buffer, and Annexin V-FITC and PI were added, followed by further incubation in darkness for 20 min at room temperature and then analyzed using flow cytometry.

The cytotoxicity of CD-Ce6/CGKRK-GFLG-T7 in MCF-7 cells was determined via MTT assay. Briefly, MCF-7 cells were seeded on a 96-well plate at a density of 1.5×10^4 per well for 24 h. CD-Ce6/CGKRK-GFLG-T7 with various concentrations were added to each well. 12 h later, the medium was replaced with fresh medium followed by light irradiation (0.500W, 2 min). Subsequently, the cells were incubated for another 24 h. Then, 20 μL MTT (5 mg mL^{-1}) was added to each well and the cells were further cultured at 37 °C for 4 h. Finally, the medium was replaced with 150 μL DMSO and the OD values were measured using a microplate reader.



Scheme S1 Detailed synthetic route of β-CD-Ce6

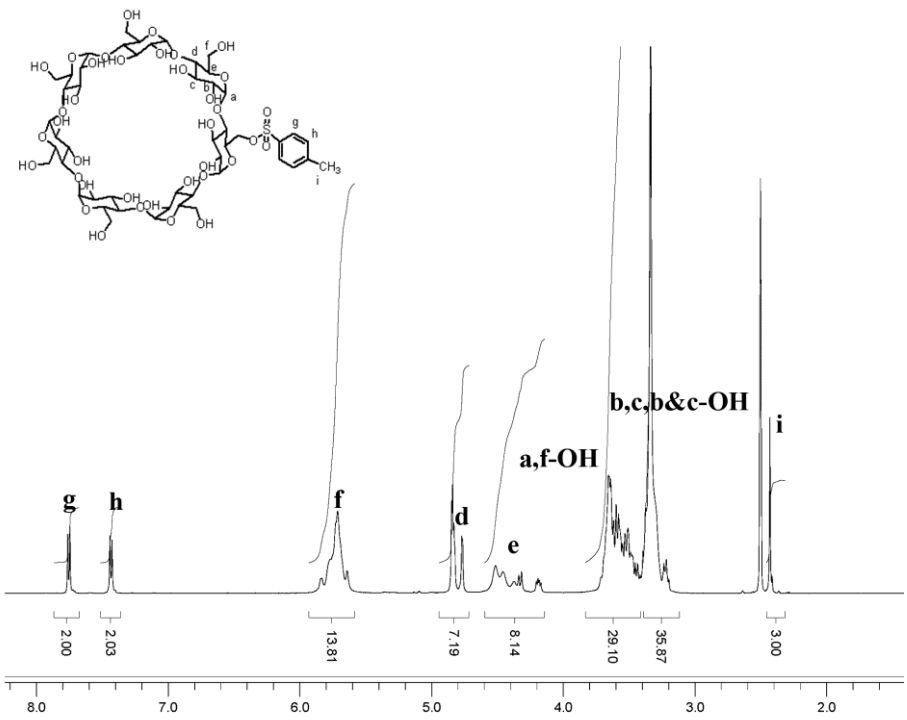


Fig. S1 ^{1}H NMR spectra of β-CD-OTs.

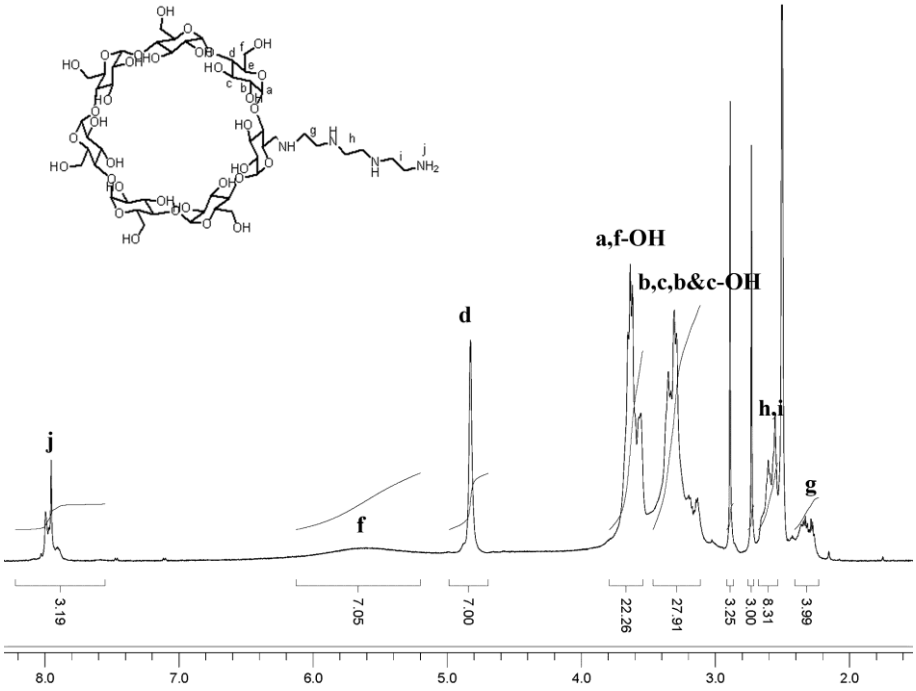


Fig. S2 ^{1}H NMR spectra of β-CD-NH_2.

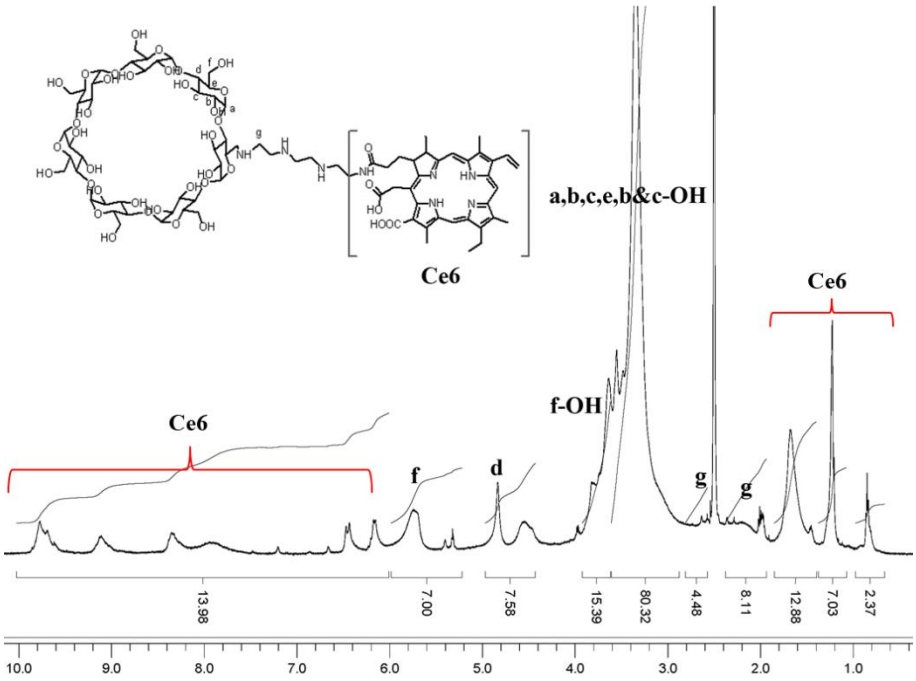


Fig. S3 ^{1}H NMR spectra of β-CD-Ce6.

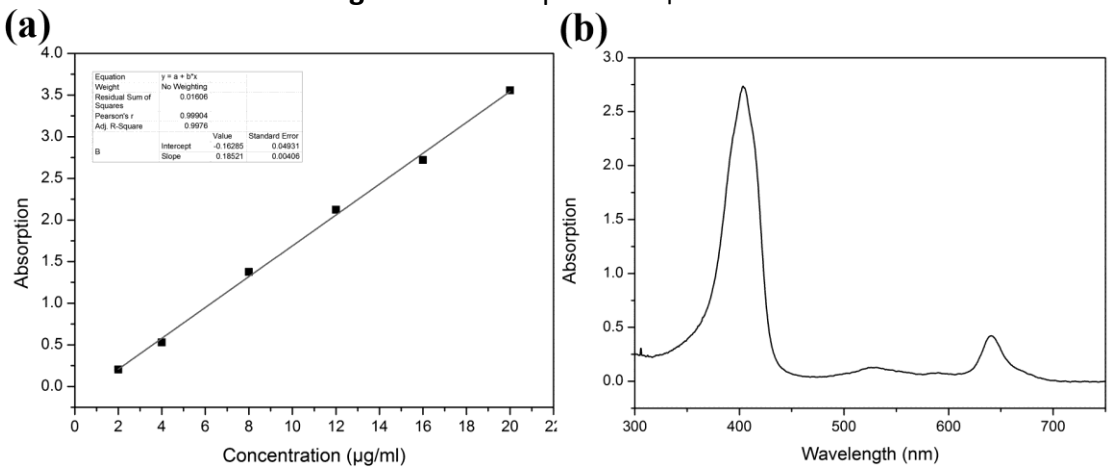


Fig. S4 (a) The calibration curve of Ce6 by UV-vis and (b) the corresponding UV spectrum of the β-CD-Ce6 solution

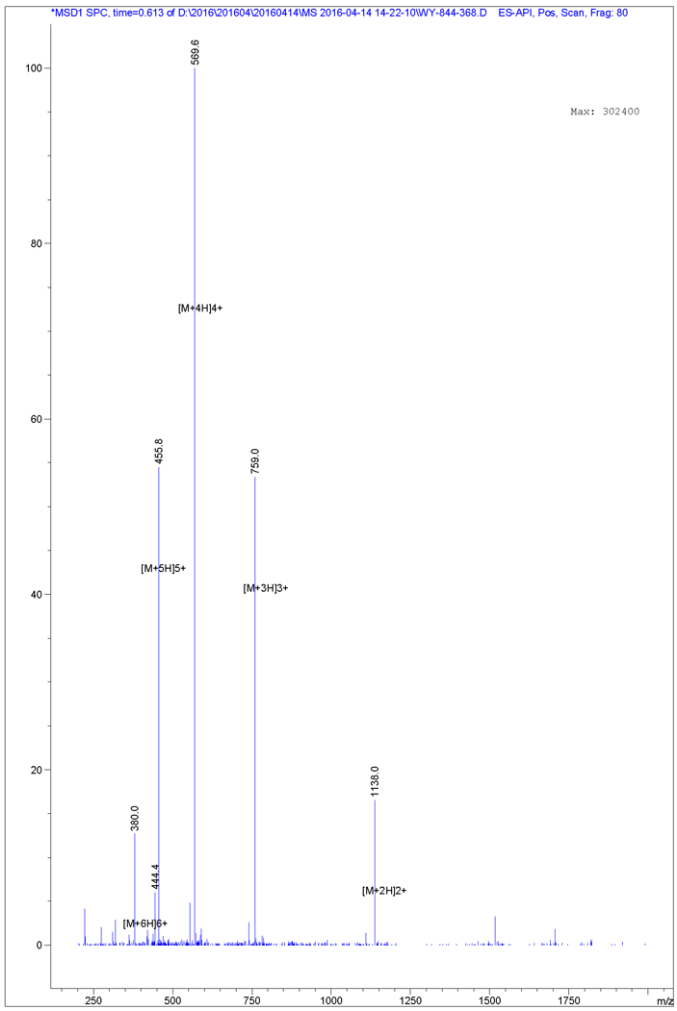


Fig. S5 MS analysis of Ad-CGKRK-GFLG-EE-T7

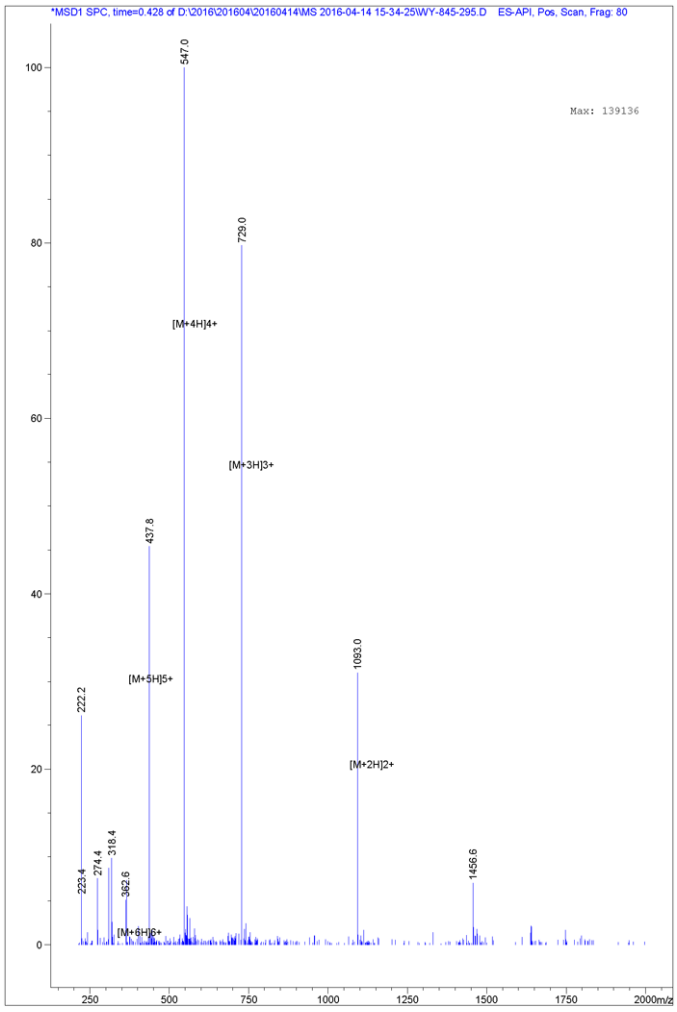


Fig. S6 MS analysis of Ad-CGKRK-GGLG-EE-T7

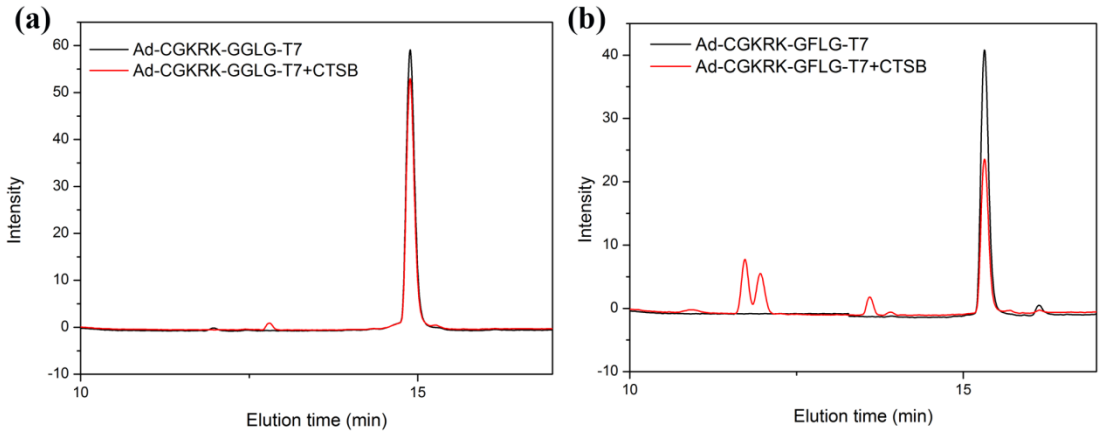


Fig. S7 HPLC analysis of (a) Ad-CGKRK-GGLG-EE-T7 and (b) Ad-CGKRK-GFLG-EE-T7 after 24 h CTSB treatment at pH 5.5 under 37 °C.

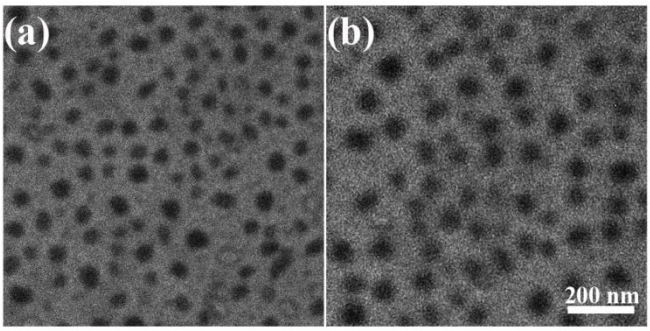


Fig. S8 TEM images of (a) CD-Ce6/CGKRK-GGLG-T7 and (b) CD-Ce6/CGKRK-GFLG-T7.

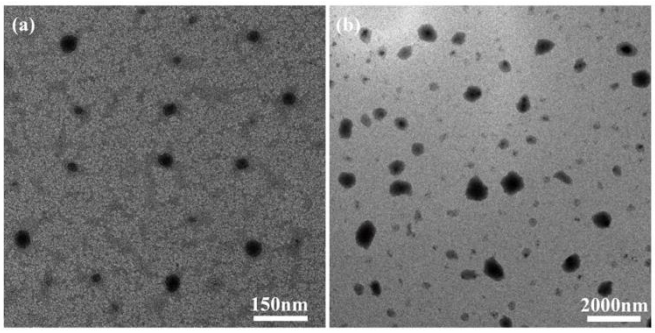


Fig. S9 TEM images of (a) CD-Ce6/CGKRK-GGLG-T7 and (b) CD-Ce6/CGKRK-GFLG-T7 after CTSB treatment.

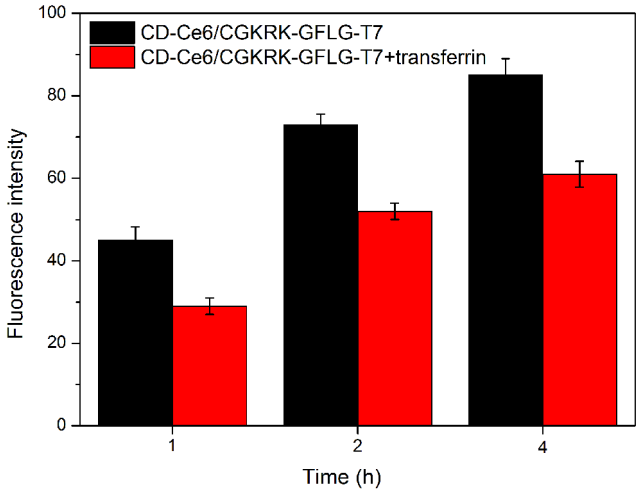


Fig. S10 Competitive inhibition effects of T7 peptide demonstrated internalization of CD-Ce6/CGKRK-GFLG-T7 in MCF-7 cells through transferrin receptor-mediated endocytosis by flow cytometry (n=3).

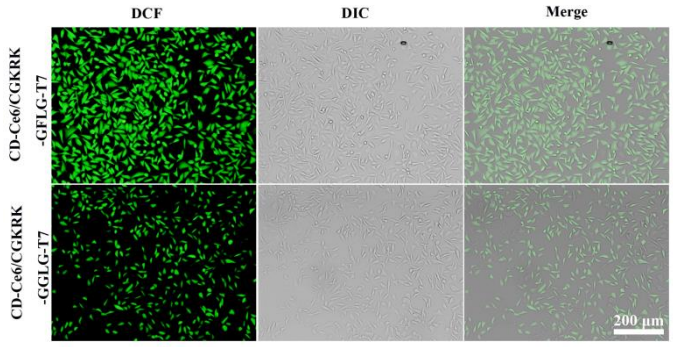


Fig. S11 The generation of intracellular ROS by MCF-7 cells incubated with CD-Ce6/CGKRK-GGLG-T7 or CD-Ce6/CGKRK-GFLG-T7 for 12h followed by light irradiation (0.500W, 0.5 min). DCF-DA was used as an ROS indicator.

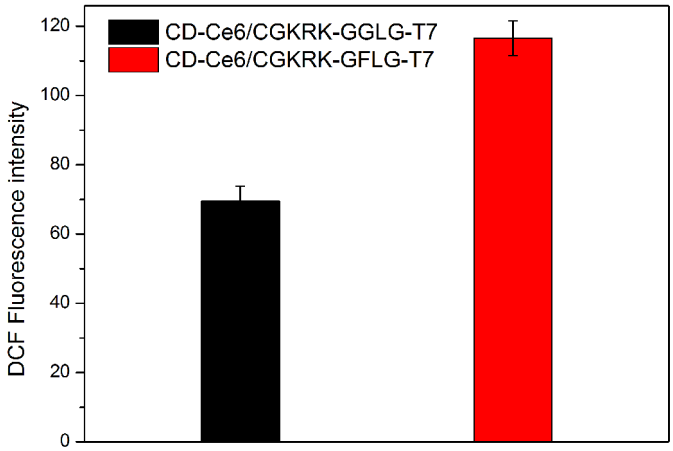


Fig. S12 Quantitative ROS generation in MCF-7 cells after treating with various nanaocarrier through flow cytometry (n=3).

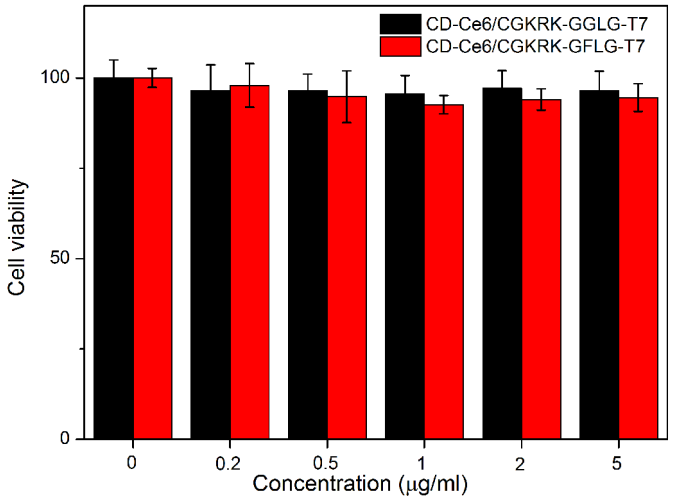


Fig. S13 Cell viability of MCF-7 cells upon treatment with various nanaocarrier of different concentrations in the dark for 24 h.