Supplementary information

Targeted Theranostic Prodrug Based on an Aggregation-Induced Emission (AIE) Luminogen for Real-Time Dual-Drug Tracking

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Experimental Section

General Information. Cisplatin, copper(II) sulfate ($CuSO_4$), sodium ascorbate, ascorbic acid, propargylamine, *N*, *N*-diisopropylethylamine (DIPEA), anhydrous dimethyl sulfoxide (DMSO), anhydrous dimethylformamide (DMF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin (BSA), diethyldithiocarbamate (DDTC), glutathione (GSH), lysozyme, pepsin, trypsin and other chemicals were all purchased from Sigma-Aldrich and used as received without further purification. Doxorubicin hydrochloride (DOX • HCl) was purchased from Alfa Aesar. DOX • HCl was neutralized with triethylamine to yield DOX, which was dissolved in dimethyl sulfoxide (DMSO) for further use. Deuterated solvents with tetramethylsilane (TMS) as the internal reference were purchased from Cambridge Isotope Laboratories Inc.. Alkyne-functionalized cyclic RGD (cRGD) peptide was customized from GL Biochem Ltd (Shanghai). *N*-Hydroxysuccinimide (NHS) activated *cis*, *cis*, *trans*-diamminedichlorodisuccinatoplatinum(IV) complex (NHS-Pt-NHS)[1] and 1,2-bis[4-(azidomethyl)phenyl]-1,2-diphenylethene (TPE-2N_3)[2] were synthesized following literature methods.

Dulbecco's Modified Essential Medium (DMEM) was purchased from Invitrogen. Milli-Q water was supplied by Milli-Q Plus System (Millipore Corporation, Breford, United States). 10× Phosphate-buffer saline (PBS) buffer with pH = 7.4 (ultrapure grade) is a commercial product of 1st BASE (Singapore). Milli-Q water (18.2 MΩ) was used to prepare the buffer solutions from the 10× PBS stock buffer. 1× PBS contains NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (10 mM) and KH_2PO_4 (1.8 mM).

Fetal bovine serum (FBS) and trypsin-EDTA solution were purchased from Gibco (Life Technologies, AG, Switzerland). DRAQ5™ was purchased from Biostatus (United Kingdom).

Characterization. NMR spectra were measured on a Bruker ARX 400 NMR spectrometer. Chemical shifts were reported in parts per million (ppm) referenced with respect to residual solvent. Particle size and size distribution were determined by laser light scattering (LLS) with a particle size analyzer (90 Plus, Brookhaven Instruments Co., United States) at a fixed angle of 90° at room temperature. AFM images were obtained by Dimension 3100 AFM (Veeco, CA, United States) under ambient conditions. A 0.1% trifluoroacetic acid in H_2O and acetonitrile were used as eluents for the HPLC experiments. Electrospray ionization mass spectrometry (ESI-MS) was performed on Proteome X-LTQ (Thermo Fisher Scientific, United States). Freeze drying was performed using Martin Christ Model Alpha 1-2/LD. UV-vis absorption spectra were taken on a Shimadzu Model UV-1700 spectrometer. Photoluminescence (PL) spectra were measured on a Perkin-Elmer LS 55 spectrofluorometer. All UV and PL spectra were collected at 24 ± 1 °C.

Synthesis of Amine Functionalized cRGD-TPE through Two Consecutive "Click" Reactions. TPE-$2N_3$ (15 mg, 34 μmol) and alkyne-functionalized cRGD (19.4 mg, 34 μmol) were dissolved in a mixture of DMSO/H_2O solution (v/v = 1/1, 2.0 mL). The "click" reaction was initiated by sequential addition of $CuSO_4$ (19.2 mg, 12 μmol) and sodium ascorbate (4.8 mg, 24 μmol). The reaction was continued with shaking at room temperature for 12 h. Then propargylamine (4.4 μL, 68 μmol),

$CuSO_4$ (19.2 mg, 12 μmol), sodium ascorbate (4.8 mg, 24 μmol) was added sequentially and reacted at room temperature for another 24 h. The final product was purified by preparative HPLC and lyophilized under vacuum to yield the amine functionalized cRGD-TPE as white powders in 53% yield (19.2 mg). HPLC (λ = 320 nm): purity 98.6%, retention time 10.3 minutes. ^{1}H NMR (DMSO–d_6, 400 MHz), δ (TMS, ppm): 12.24 (s, 1H), 8.22 (m, 3H), 8.01 (m, 2H), 7.78 (s, 2H), 7.10 (m, 11H), 6.94 (m, 12H), 5.43 (m, 4H), 4.62 (t, 1H), 4.41 (m, 2H), 4.10 (m, 2H), 3.13 (m, 4H), 2.90 (m, 3H), 2.65 (m, 2H), 2.38–2.27 (m, 2H), 1.75 (m, 1H), 1.46 (m, 2H), 1.35 (m, 2H). ESI-MS: *m*/*z* $[M+H]^+$ calc. 1068.495, found 1068.806.

Synthesis of Theranostic Dual-Acting Prodrug cRGD-TPE-Pt-DOX. Amine terminated cRGD-TPE (10.7 mg, 10 μmol) and doxorubicin hydrochloride (5.8 mg, 10 μmol) were dissolved in anhydrous DMSO (1.0 mL) with a catalytic amount of DIPEA (1.0 μL). The mixture was stirred at room temperature for 10 min. Then *N*-hydroxysuccinimide-activated platinum(IV) complex (7.3 mg, 10 μmol) in DMSO (0.5 mL) was added quickly to the above mixture. The reaction was continued with stirring at room temperature for another 24 h. The final product was purified by preparative HPLC and lyophilized under vacuum to yield the prodrug cRGD-TPE-Pt-DOX as red powders in 36% yield (7.6 mg). HPLC (λ = 320 nm): purity 97.3%, retention time 17.2 minutes. ^{1}H NMR (DMSO–d_6, 400 MHz): 12.24 (s, 1H), 8.38 (t, 1H), 8.24 (m, 3H), 8.08–7.88 (m, 4H), 7.72 (m, 1H), 7.57 (d, 1H), 7.20–6.98 (m, 12H), 6.96–6.79 (m, 12H), 6.46 (m, 6H), 5.43 (m, 4H), 5.24 (s, 1H), 4.93 (m, 1H), 4.57–4.68 (m, 2H), 4.30–4.51 (m, 4H), 4.15–4.07 (m, 1H), 4.07 (m, 2H), 3.97 (s, 3H),

3.79 (m, 1H), 3.62–3.53 (m, 3H),3.15 (m, 2H), 2.95 (m, 2H), 2.84 (m, 2H), 2.65 (m, 3H), 2.12 (m, 2H), 2.35–2.27 (m, 2H), 1.83 (d, 1H), 1.77–1.65 (m, 3H), 1.60–1.36 (m, 4H), 1.13 (m, 3H); ESI-MS: *m*/*z* $[M+H]^+$ calc. 2109.642, found 2109.698.

General Procedure for Drug Activation Monitoring. DMSO stock solution of cRGD-TPE-Pt-DOX diluted with a mixture of DMSO and PBS (v/v = 1/199) mixture. Next, the prodrug was incubated with ascorbic acid at room temperature and the change of fluorescence intensity was measured. The solution was excited at 320 nm, and the emission was collected from 380 to 600 nm. The release of DOX was monitored by analytical HPLC at absorbance of 254 nm and the concentration of DOX was calculated *via* a standard curve.

Cell Culture. MDA-MB-231 and MCF-7 human breast cancer cells and 293T normal cells were provided by American Type Culture Collection (ATCC). The cells were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated FBS (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Scientific) and maintained in a humidified incubator at 37 °C with 5% CO_2. Before experiment, the cells were precultured until confluence was reached.

Confocal Imaging. MDA-MB-231, MCF-7 and 293T cells were cultured in the chambers (LAB-TEK, Chambered Coverglass System) at 37 °C. After 80% confluence, the culture medium was removed and washed twice with 1× PBS buffer. Then cRGD-TPE-Pt-DOX in DMSO stock solution was added to the chamber to reach a final concentration of 20 μM. In some experiments, the cells were pre-incubated with media containing cRGD (50 μM) prior to prodrug incubation. After

incubating the prodrug at 37 °C for 2 h, the medium was replaced with fresh medium and further incubated for designated time. Then the cells were washed twice with ice-cold PBS and the cell nuclei were living stained with DRAQ5^{TM} (Biostatus) following the standard protocol of the manufacturer. The cellular apoptosis imaging measurement was carried out based on standard detection kit (Annexin V-FITC/Propidium Iodide (PI) Apoptosis Detection Kit) according to manufacturer's protocol. The cells were then imaged immediately by confocal laser scanning microscope (CLSM, Zeiss LSM 410, Jena, Germany). The images were analyzed by Image J 1.43 × program (developed by NIH, http://rsbweb.nih.gov/ij/).

Quantification of the Cellular Uptake by Fluorescence Microplate Reader. MDA-MB-231, MCF-7 and 293T cells were seeded in 96-well plates (Costar, United States) at an intensity of 4 × 10^4 cells mL^{-1}. After confluence, the medium was replaced with cRGD-TPE-Pt-DOX (10 μmol) in fresh FBS-free DMEM medium. After the designated incubation time at 37 °C, the adherent cells were washed twice with 1× PBS buffer followed by fluorescence measurement using a T-CAN microplate reader. The excitation and emission wavelengths are 488 and 591 nm, respectively.

Cytotoxicity of the Prodrug. MTT assays were used to assess the metabolic activity of MDA-MB-231 and MCF-7 cancer cells. The cells were seeded in 96-well plates (Costar, IL, United States) at an density of 4 × 10^4 cells mL^{-1}. After 24 h incubation, the medium was replaced by the prodrug suspension at different concentrations. After incubating the prodrug at 37 °C for 2 h, the medium was

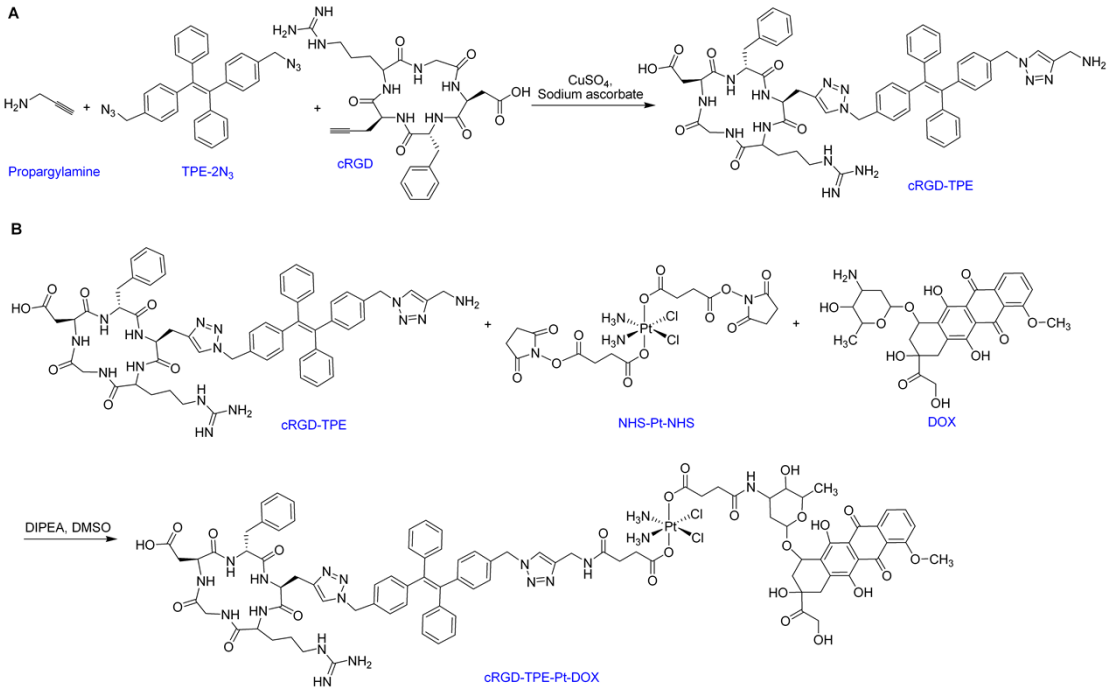
replaced with fresh medium and further incubated for designated time. Then the wells were washed twice with 1× PBS buffer, and 100 μL of freshly prepared MTT (0.5 mg mL^{-1}) solution in culture medium was added into each well. The MTT medium solution was carefully removed after 3 h incubation in the incubator at 37 °C. DMSO (100 μL) was then added into each well and the plate was gently shaken to dissolve all the precipitates formed. The absorbance of MTT at 570 nm was monitored by the microplate reader (Genios Tecan). Cell viability was expressed by the ratio of absorbance of the cells incubated with prodrug suspension to that of the cells incubated with culture medium only. The relative cell survival percentages compared to the drug-free control were plotted against the drug concentration in logarithmic scale. The data reported represent an average of three measurements from different batches. The dose-effect profiles were obtained by sigmoidal logistic fitting using Origin 8.0 (OriginLab, Northampton, MA) and the half-maximal inhibitory concentration (IC_{50}) values were determined on the basis of the fitted data.

Determination of Combination Index (C.I.). The combination therapy of cisplatin and DOX towards MDA-MB-231 cells was evaluated by the combination index (C.I.) analysis according to the previous report.[3]

1. Yuan, Y.; Kwok, R. T. K.; Tang, B. Z.; Liu, B. *J. Am. Chem. Soc.*, 2014, **136**, 2546.

2. Qin, A. J.; Lam, J. W. Y.; Tang, L.; Jim, C. K. W.; Zhao, H.; Sun, J. Z.; Tang, B. Z. *Macromolecules*, 2009, **42**, 1421.

3. Liu, S. Guo, Y. Huang, R. Li, J. Huang, S. Kuang, Y. Han, L. Jiang, C. *Biomaterials*, 2012, **33**, 4907.



Scheme S1. Synthetic route to amine-functionalized cRGD-TPE (A) and the targeted theranostic dual-acting prodrug cRGD-TPE-Pt-DOX (B).

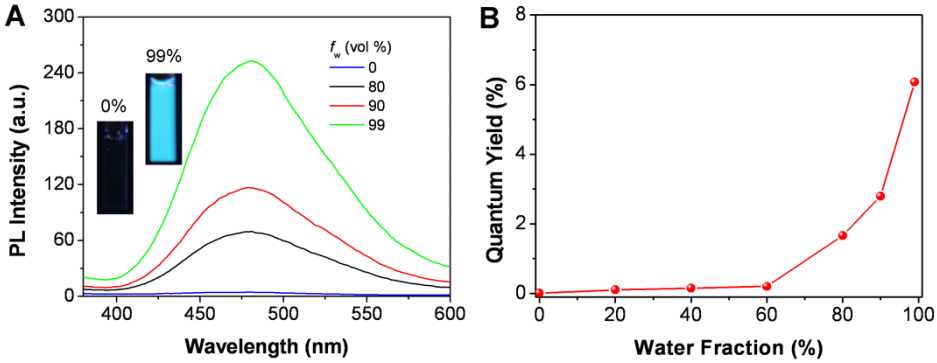


Figure S1. (A) Photoluminescence (PL) spectra of cRGD-TPE (10 μM) in DMSO/PBS (v/v = 1/199) mixtures with different fractions of water (f_w). Inset: the digital photographs of cRGD-TPE (10 μM) at water fraction of 0% and 99% under illumination of a UV lamp at 365 nm. (B) The change of the quantum yields (Φ_F) of cRGD-TPE with the increment of water fraction in DMSO/water mixture. The Φ_F values were estimated using quinine sulfate in 0.1 N H_2SO_4 (Φ_F = 54.6%) as standard.

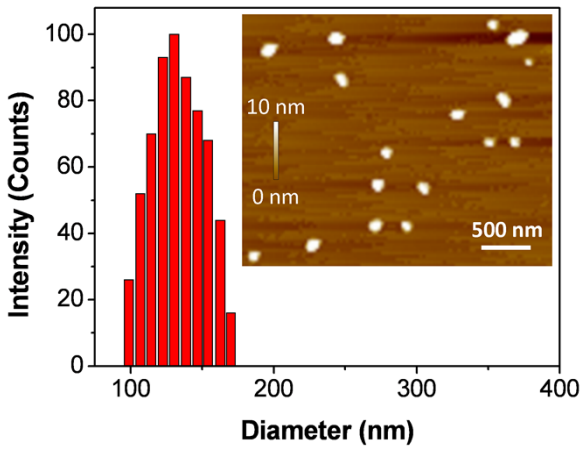


Figure S2. Laser light scattering data and AFM image (inset) of cRGD-TPE (10 μM) in DMSO/PBS (v/v = 1/199).

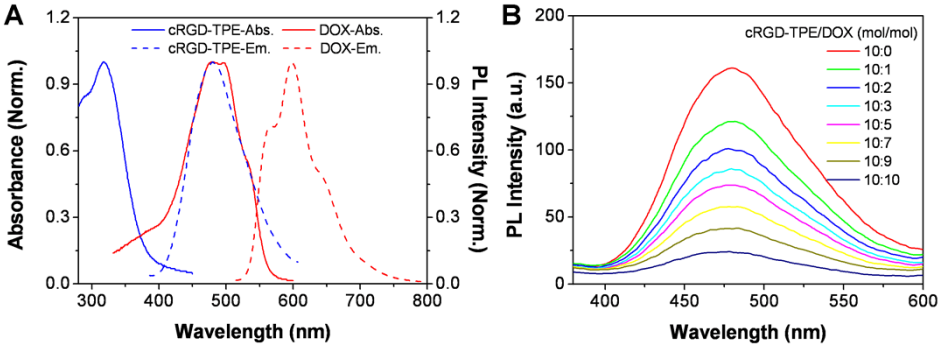


Figure S3. (A) Absorption (solid line) and emission (dashed line) spectra of cRGD-TPE (blue) and DOX (red) in DMSO/PBS (v/v = 1/199). (B) Fluorescence changes of cRGD-TPE in DMSO/PBS (v/v = 1/199) with different molar ratios of DOX. λ_{ex} = 320 nm.

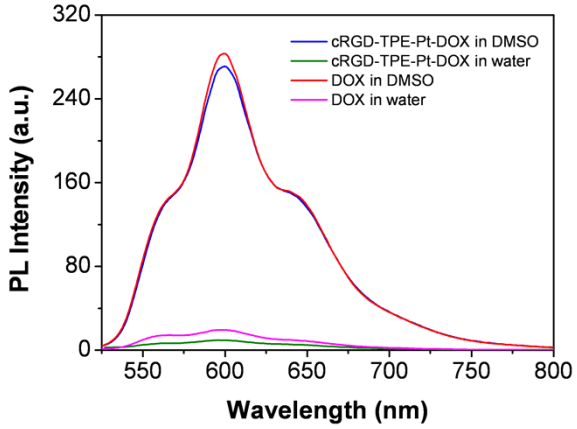


Figure S4. PL spectra of cRGD-TPE-Pt-DOX and free DOX in DMSO or DMSO/water (v/v = 1/199) upon excitation at 488 nm.

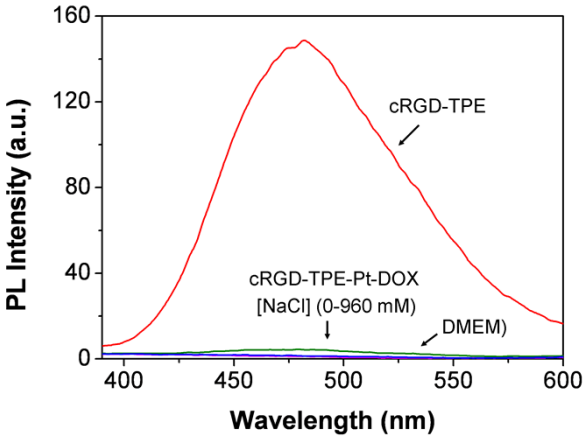


Figure S5. (A) PL spectra of cRGD-TPE-Pt-DOX in DMSO/water (v/v = 1/199) mixture with NaCl concentrations varying from 0, 240, 480 to 960 mM and in cell culture medium (DMEM). The PL spectrum of cRGD-TPE in DMSO/water (v/v = 1/199) is shown for comparison.

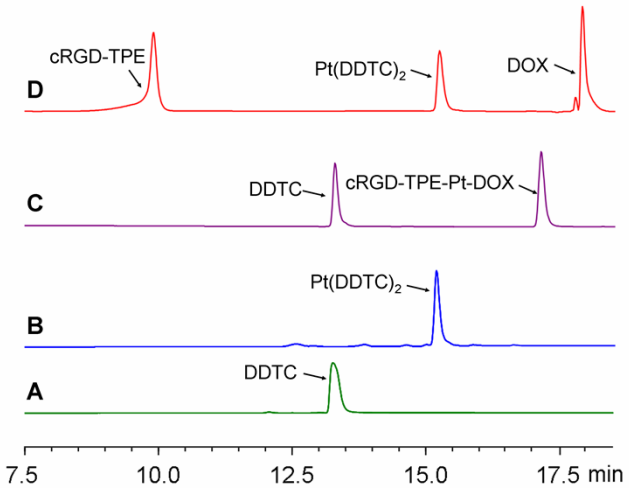


Figure S6. HPLC spectra showing the reaction of diethyldithiocarbamate (DDTC) with cisplatin and reduced Pt(IV) prodrug: (A) DDTC alone; (B) the product for reaction between DDTC and cisplatin; (C) the mixture of DDTC and cRGD-TPE-Pt-DOX; (D) the products for the reaction between DDTC and cRGD-TPE-Pt-DOX (10 μM) after treatment with 1 mM ascorbic acid for 12 h.

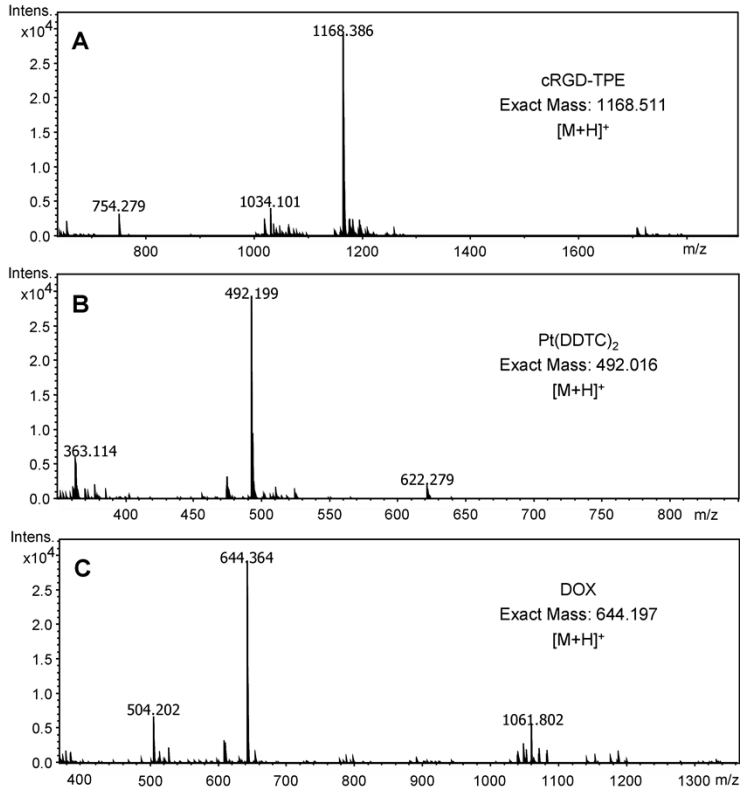
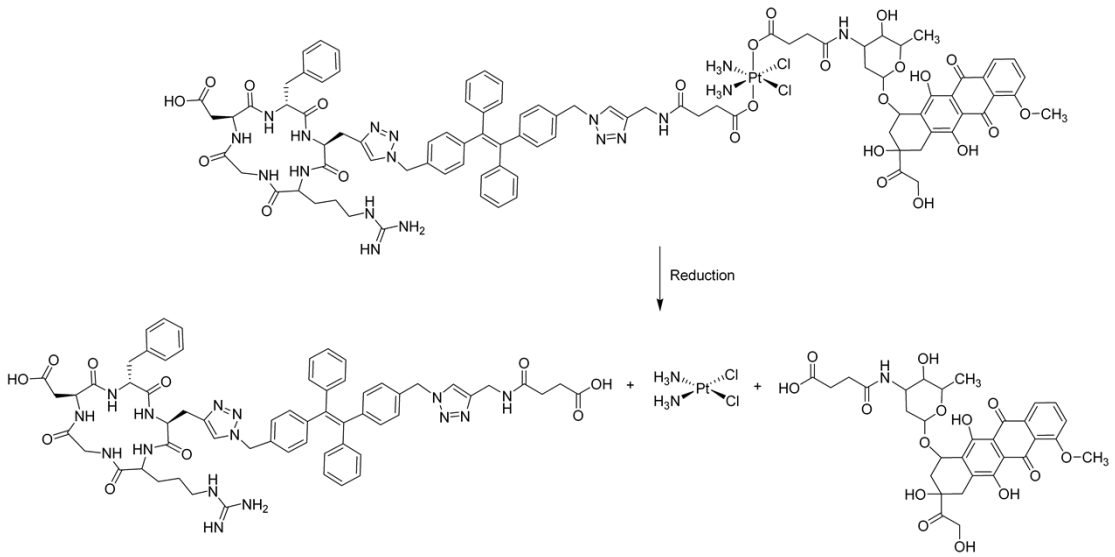


Figure S7. ESI-MS spectra of peaks in Figure S4D for cRGD-TPE (A), $Pt(DDTC)_2$ (B) and DOX (C).



Scheme S2. The reduction mechanism of cRGD-TPE-Pt-DOX.

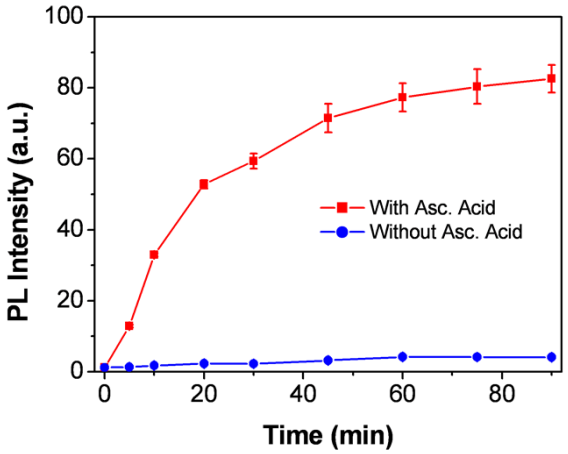


Figure S8. PL intensity at 480 nm of cRGD-TPE-Pt-DOX (10 μM) in the presence (red) and absence (blue) of ascorbic acid (Asc. Acid, 1 mM) from 0 to 90 min. Data represent mean values ± standard deviation, n = 3.

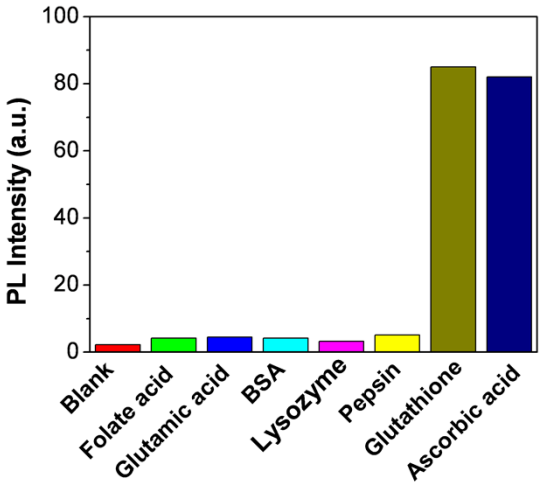


Figure S9. Fluorescence response of cRGD-TPE-Pt-DOX (10 μM) toward 1.0 mM folate acid, glutamic acid, bovine serum albumin (BSA), lysozyme, pepsin, glutathione or ascorbic acid in DMSO/PBS (v/v = 1/199).

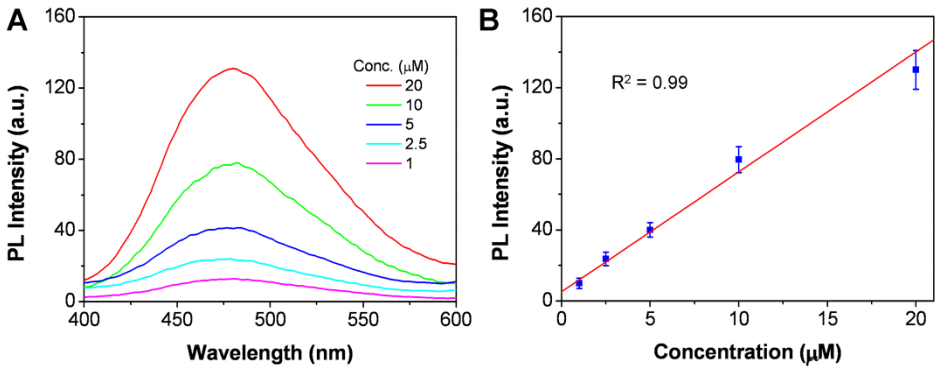


Figure S10. (A) PL spectra of cRGD-TPE-Pt-DOX at different concentrations after incubation with ascorbic acid (1 mM) in DMSO/PBS (v/v = 1/199) for 1 h. (B) Plot of PL intensities at 480 nm versus concentrations of cRGD-TPE-Pt-DOX with the incubation of ascorbic acid (1 mM) in DMSO/PBS (v/v = 1/199). Data represent mean values ± standard deviation, n = 3.

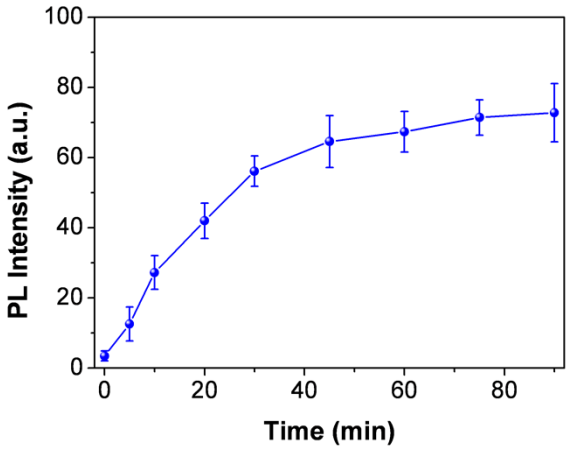


Figure S11. Time-dependent PL intensities at 480 nm of cRGD-TPE-Pt-DOX (10 μM) upon incubation with MDA-MB-231 cell lysate. Data represent mean values ± standard deviation, n = 3.

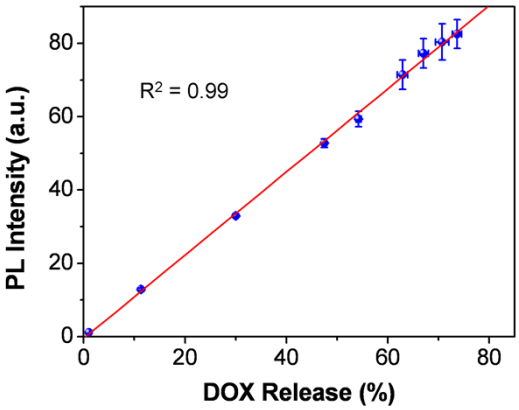


Figure S12. Correlation between released DOX to emitted fluorescence of cRGD-TPE (λ_{ex} = 320 nm, λ_{em} = 480 nm) upon reduction of cRGD-TPE-Pt-DOX with ascorbic acid.

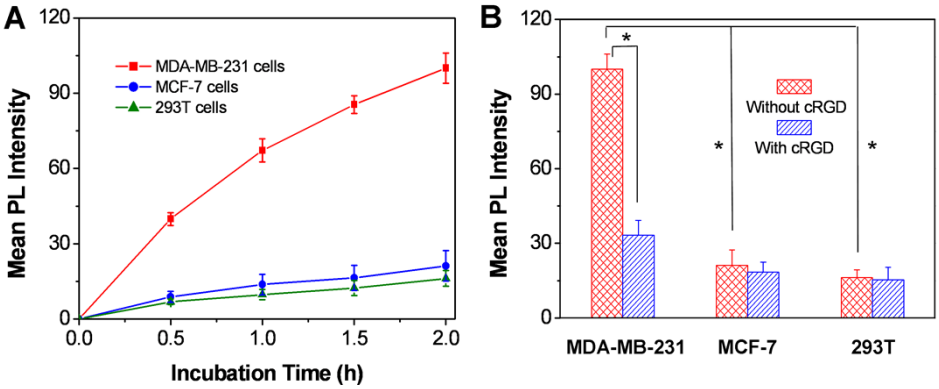


Figure S13. Evaluation of the targeting effect of cRGD-TPE-Pt-DOX to different cells. (A) Relative fluorescence intensity of cRGD-TPE-Pt-DOX (λ_{ex} = 488 nm) determined in MDA-MB-231, MCF-7 and 293T cells at different incubation time. (B) Relative fluorescence intensity of cRGD-TPE-Pt-DOX determined in MDA-MB-231, MCF-7 and 293T cells with and without cRGD (50 μM) pretreatment. The error is the standard deviation from the mean (n = 3, * is P < 0.05).

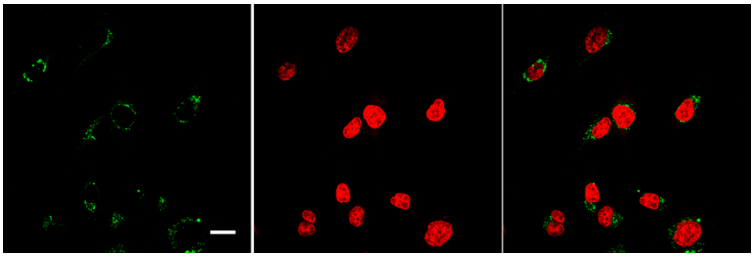


Figure S14. CLSM images of free cRGD (50 μM) pretreated MDA-MB-231 cells upon incubation with cRGD-TPE-Pt-DOX (10 μM) for 2 h. Green: DOX fluorescence; red: cell nuclei stained by DRAQ5. All images share the same scale bar (20 μm).

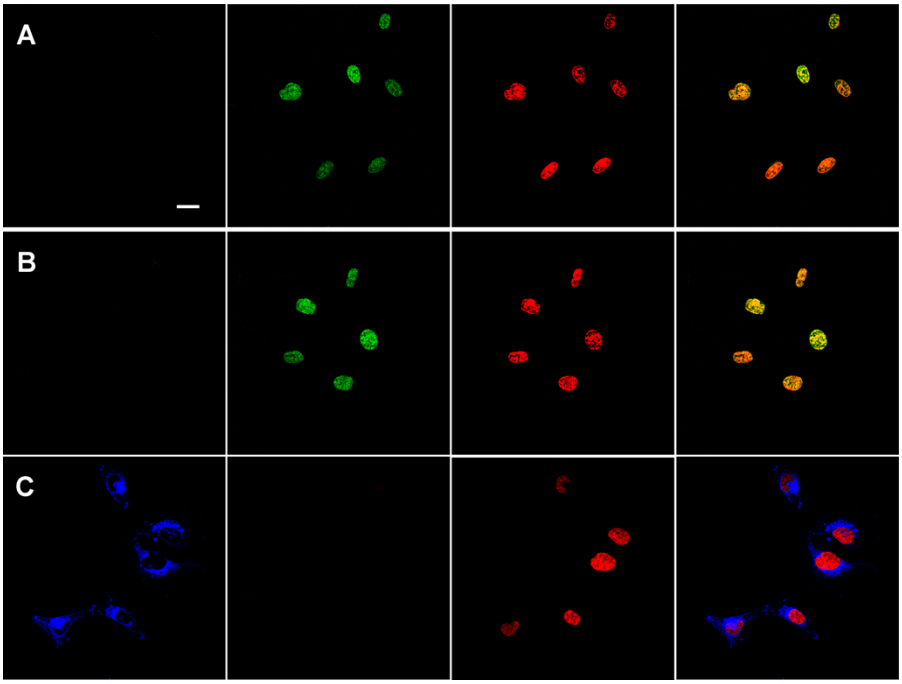


Figure S15. Confocal images of MDA-MB-231 cells after incubation with free DOX (A), succinic acid attached DOX collected by HPLC (B), and cRGD-TPE (C) for 6 h. Blue: TPE fluorescence; green: DOX fluorescence; red: cell nuclei stained by DRAQ5. All images share the same scale bar (20 μm).

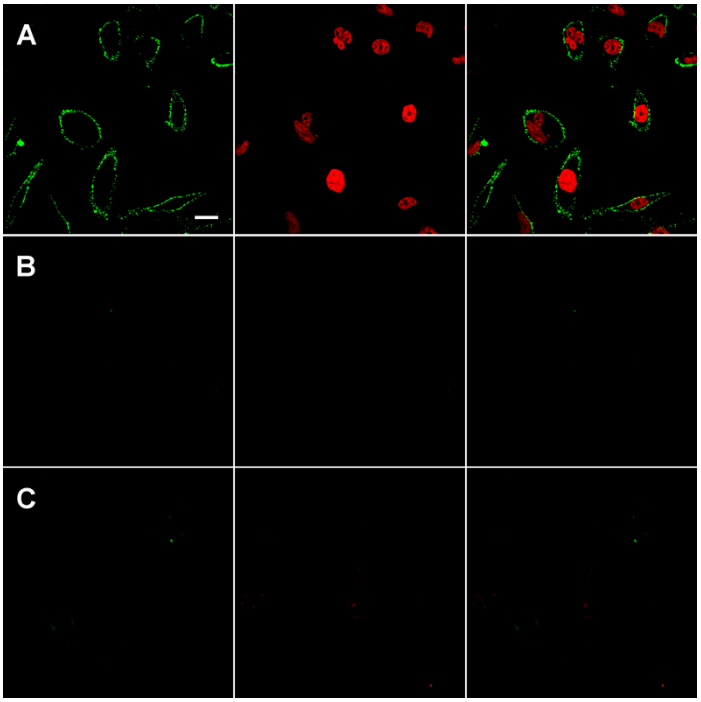


Figure S16. CLSM of cell apoptosis through Annexin V-FITC/propidium iodide stained MDA-MB-231 (A), MCF-7 (B) and 293T (C) cells after incubation with cRGD-TPE-Pt-DOX (10 μM) for 2 h followed by incubation in fresh medium for another 4 h. Annexin V-FITC/Propidium Iodide (PI) are the commonly used fluorescent probes to distinguish viable cells from apoptosis ones. Green: FITC-tagged Annexin V; red: propidium iodide. All images share the same scale bar (20 μm).

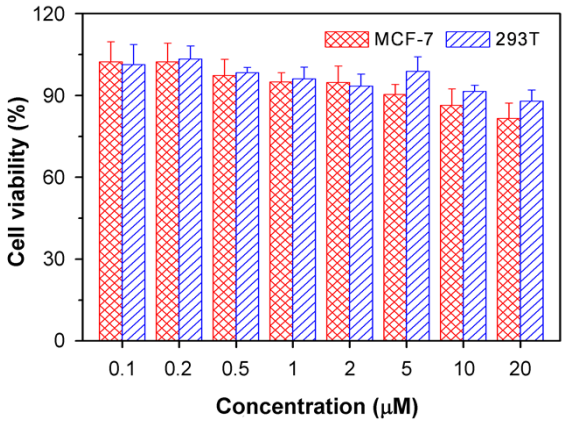


Figure S17. Relative viabilities of MCF-7 cells after being incubated with various concentrations of cRGD-TPE-Pt-DOX for 2h and further incubation in fresh medium for 72 h.

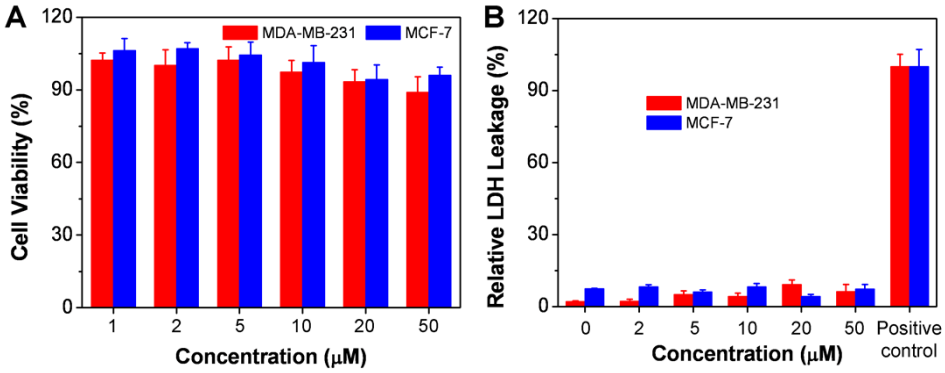


Figure S18. (A) Relative viabilities of MDA-MB-231 and MCF-7 cells after being incubated with various concentrations of cRGD-TPE for 24 h. (B) Levels of released lactate dehydrogenase (LDH) from MDA-MB-231 and MCF-7 cells after being exposed to various concentrations of cRGD-TPE for 24 h. Cells lysated with 1% Triton X-100 were used as positive controls and cells without any treatments were set as negative controls (0 μM).