Supporting Information:

Enhancing Accumulation and Penetration of HPMA Copolymer Doxorubicin Conjugates in 2D and 3D Prostate Cancer Cells via iRGD Conjugation with an MMP-2 Cleavable Spacer

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Materials and Methods

1. Materials

1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU), protected amino acids and 2-chlorotrityl chloride resin were purchased from AAPPTEC (Louisville, KY). 2,2'-Azobis(2,4-dimethyl valeronitrile) (V-65) was purchased from Wako (Japan). Thallium (III) trifluoroacetate ($Tl(OOCF_3)_3$), *N*-methylmorpholine (NMM), triisopropylsilane (TIPS), *N*,*N*-diisopropylethylamine (DIPEA) and piperidine were purchased from Sigma Aldrich (St. Louis, MO). Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was purchased from Thermo Scientific (Waltham, MA). General solvents were purchased from Fisher Scientific (Fair Lawn, NJ). Trifluoroacetic acid (TFA) was purchased from Acros (Fair Lawn, NJ). Monomers HPMA[1], MA-GFLG-DOX[2], and MA-GG-OH[3] were prepared as described in literature.

2. Preparation of monomer MA-GG-PLGLAG-iRGD and free iRGD

The synthesis of MA-GG-PLGLAG-iRGD started from manual attachment of the Fmoc-Cys(Acm)-OH to resin by mixing 2-chlorotrityl chloride resin (400 mg, 1.1 mmol/g, 100-200 mesh) with Fmoc-Cys(Acm)-OH (200 μmol, 117mg) at room temperature for 2 h. The remaining active groups in resin were capped with a mixture solution of dichloromethane (DCM) : methanol (MeOH) : DIPEA = 17 : 2 : 1.

Next, protected amino acids (Fmoc-Asp(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Pro-OH) or MA-GG-OH were loaded to resin sequentially using a PS3™

peptide synthesizer (Protein Technologies, Tuscon, AZ). The general procedure consists of de-protection and coupling steps: (i) the Fmoc protecting groups were removed by mixing resin bound peptide with 20% piperidine in DMF for 5 min; (ii) the coupling reaction was conducted by mixing three equivalents (600 µmol) of protected amino acid (Fmoc-AA(XXX)-OH) or MA-GG-OH with resin bound peptide in the presence of HATU (228 mg, 600 µmol).

Then, a solution of thallium (III) trifluoroacetate ($Tl(OOCF_3)_3$) (254 mg, 468 µmol) in DMF was added to the resin bound linear peptide and the mixture kept at room temperature under shaking. The $Tl(OOCF_3)_3$ solution was removed by filtration, and the remaining resin was washed with DMF and MeOH, respectively.

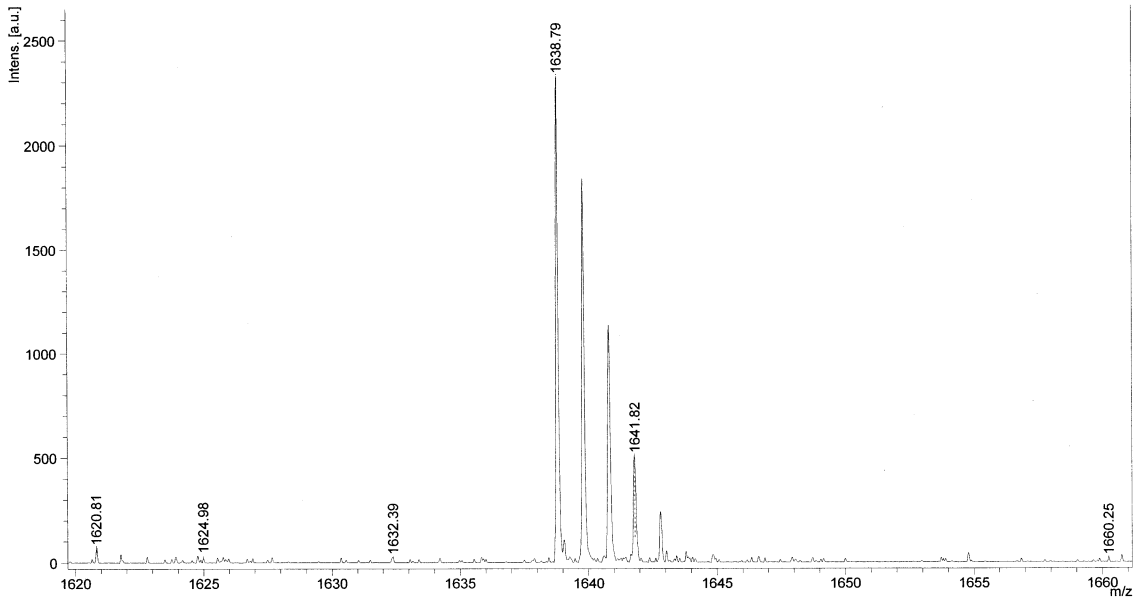

Figure S1. Mass spectrum of monomer MA-GG-PLGLAG-iRGD.

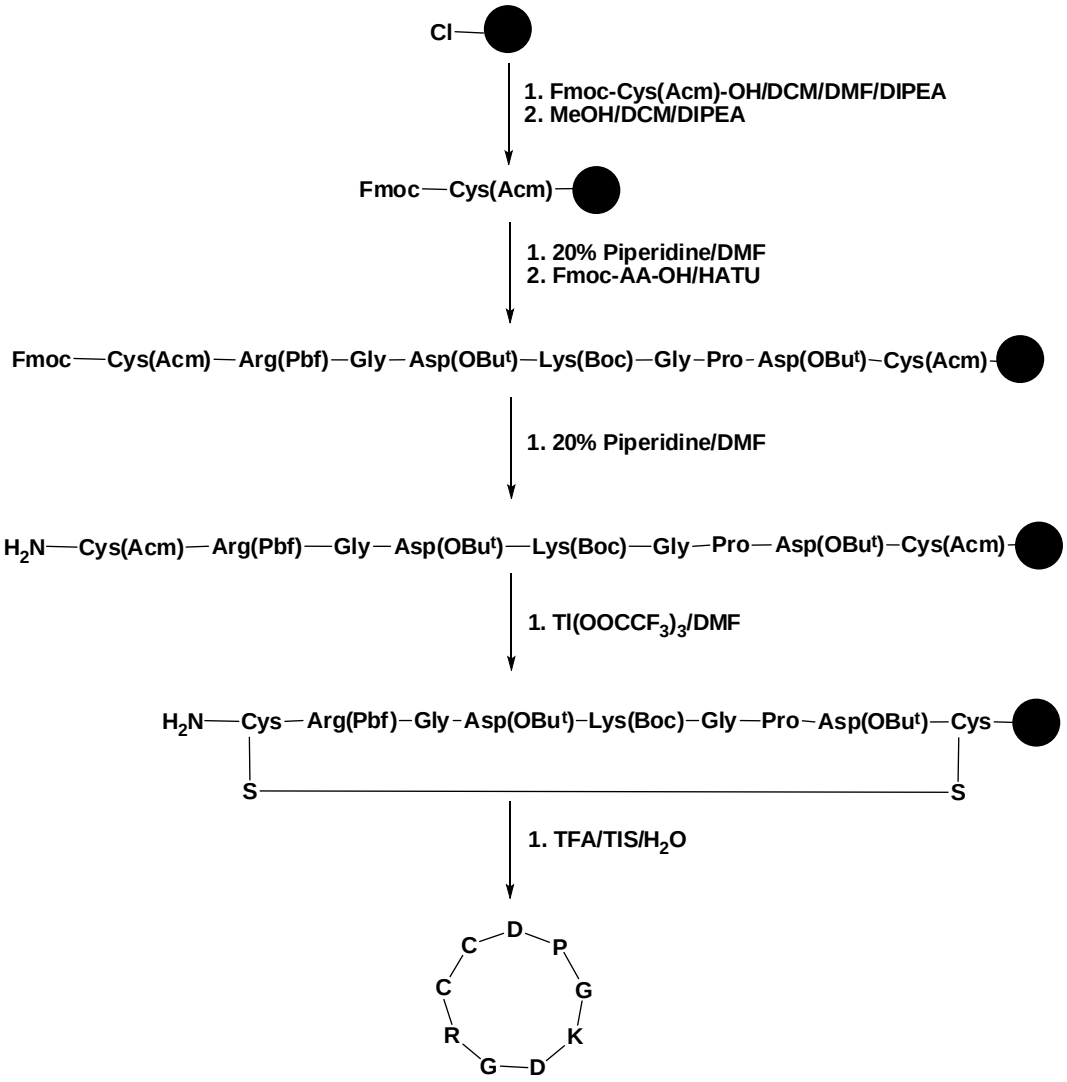

Figure S2. Synthetic scheme for free iRGD.

Finally, the crude product MA-GG-PLGLAG-iRGD was obtained by mixing the dried resin bound peptide with 10 mL of cleavage solution (TFA/TIPS/H_2O = 95/2.5/2.5) for 3 h. After removing most of the solvent under vacuum, the remaining residue was added into cold diethyl ether to precipitate the product. After centrifugation, the crude product was dried in air and purified on a HPLC system with detection wavelength (220 nm).

As shown in Figure S2, the synthesis procedure of iRGD was similar as that of MA-GG-PLGLAG-iRGD with minor modification.

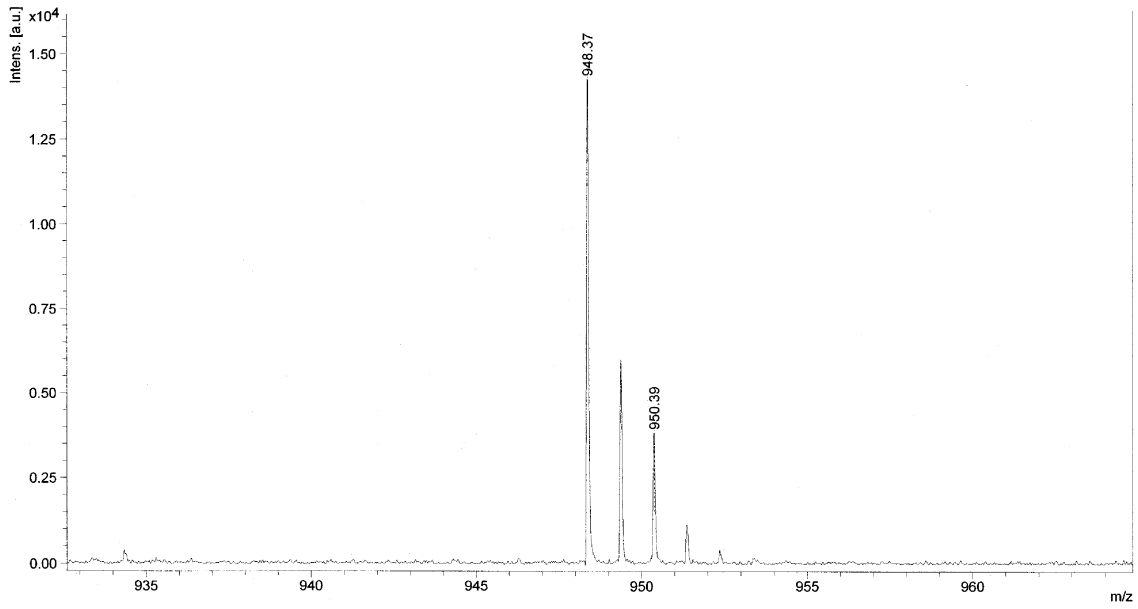

Figure S3. Mass spectrum of free iRGD.

3. Preparation and molecular weight measurement of polymer conjugate P-DOX

The synthetic scheme of P-DOX is shown in Figure S4. Monomer HPMA (416 mg, 0.97 mmol), monomer MA-GFLG-DOX (89 mg, 0.03 mmol), initiator V-65 (30 mg, 0.036 mmol) and a mixture of dimethyl sulfoxide (DMSO) and water were added to an ampoule. The mixture was bubbled with nitrogen for 30 min, and the ampoule was sealed. The polymerization was conducted at 51 ^{o}C for 10 h. The polymer was precipitated in a mixture of diethyl ether and acetone. The crude product was purified on Superose 6 HR10/30 preparative column in an AKTA FPLC system (Pharmacia) equipped with refractive index (Optilab rEX) and light scattering MiniDawn TREOS detectors (Wyatt Technology, Santa Barbara, CA). Sodium acetate (0.1 M) in a mixture of 30%

acetonitrile/70% DI water (v/v) (pH = 6.5) was used as the eluent, and the flow rate was 1 mL/min.

The molecular weight and molecular weight distribution of P-DOX was measured on a Superose 6 HR10/30 analytical column, and the flow rate was 0.4 mL/min. HPMA homopolymer fractions were used as molecular weight standards. The weight average (Mw), number average (Mn) molecular weights and molecular weight distribution (Mw/Mn) of P-DOX were 120.0 kDa; 69.8 kDa; and 1.72, respectively.

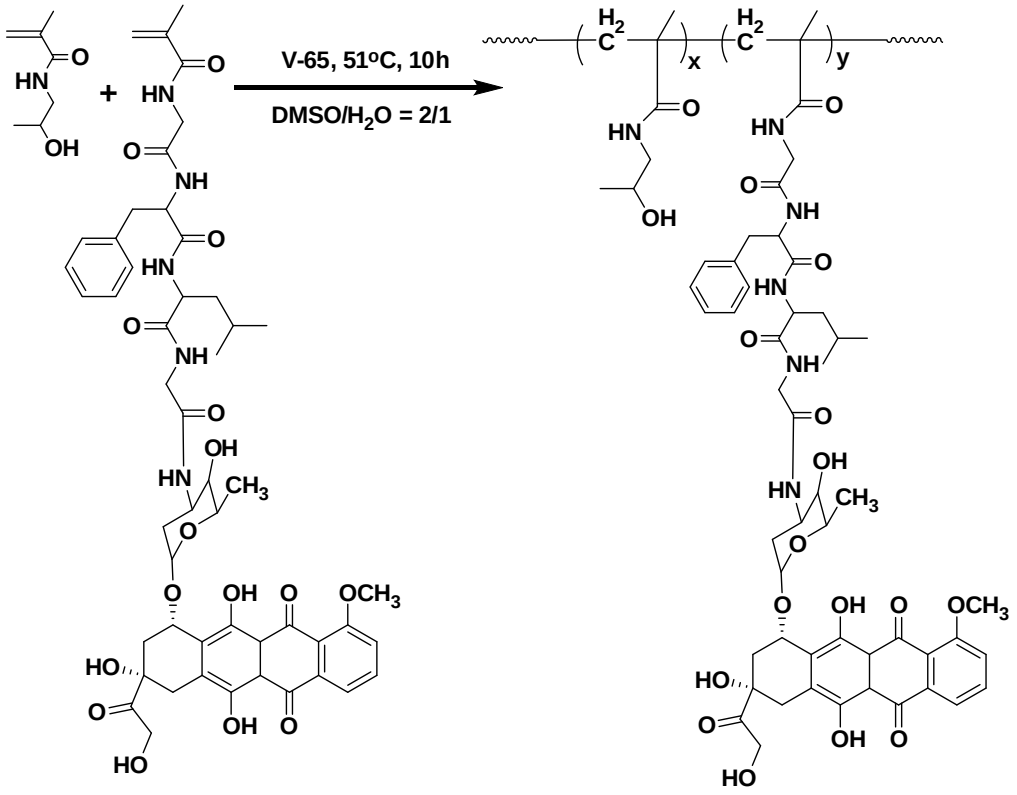

Figure S4. Synthetic scheme of polymer conjugate P-DOX.

4. Synthesis and molecular weight measurement of polymer conjugate P-DOX-PLGLAG-iRGD

The polymer conjugate P-DOX-PLGLAG-iRGD was prepared by traditional radical polymerization. Briefly, monomer HPMA (70.6 mg, 0.493 mmol), monomer MA-GFLG-DOX (15.1mg, 0.015mmol), monomer MA-GG-PLGLAG-iRGD (25 mg, 0.015 mmol), initiator V-65 (5.13mg, 0.018 mmol), and a mixture of DMSO and H_2O solvent were added to an ampoule. The ampoule was sealed after the mixture was bubbled with nitrogen for 30 min. Then the polymerization was conducted at 51 ^oC for 10 h. The purification and MW measurement methods of P-DOX-PLGLAG-iRGD are the same as for polymer conjugate P-DOX. The weight, number average molecular weight and molecular weight distribution of P-DOX-PLGLAG-iRGD were 134.3 kDa; 71.9 kDa; and 1.87, respectively.

5. Determination of the DOX content in conjugates P-DOX and P-DOX-PLGLAG-iRGD

The DOX content in conjugates P-DOX and P-DOX-PLGLAG-iRGD was calculated by measuring the DOX UV absorbance in methanol. General procedure for testing DOX UV absorbance in a solution of P-DOX-PLGLAG-iRGD in methanol: (i) P-DOX-PLGLAG-iRGD (2.26 mg) was dissolved in 10 mL methanol; (ii) 1 mL solution was transferred to a spectrophotometer cell and UV absorbance was scanned from 200 nm to 800 nm using a Varian UV-Vis spectrophotometer and methanol as the reference; (iii) the UV absorbance at 482 nm was used for DOX concentration calculation.

6. Measurement of the iRGD content in P-DOX-PLGLAG-iRGD

The iRGD content in conjugate P-DOX-PLGLAG-iRGD was determined by amino acid analysis. The first step was to hydrolyze the peptide by dissolving 2.5 mg of

conjugate P-DOX-PLGLAG-iRGD in 0.5 mL of 6 M HCl and heating at 110 ^{o}C for 24 h. The iRGD content was measured using following steps: (*i*) the solvent was removed under reduced pressure and the residue was re-dissolved in 100 µL of DI water; (*ii*) derivatization of hydrolyzed amino acid by sequential addition: 20 µL of potassium tetraborate in distilled water (150 µg/µL), 20 µL of o-phthaldialdehyde in methanol (50 µg/µL), 20 µL of mercaptopropionic acid in distilled water (0.05 mL/1 mL), and 20 µL of sample solution; (*iii*) the mixture was vortexed for 1 min and was added 0.5 mL of 0.1 M sodium acetate buffer, and then filtered; (*iv*) the fluorescence (E_x = 229 nm, E_m = 450 nm) of the hydrolyzed amino acid derivatives was measured by analytical HPLC (Agilent Technologies 1100 series, XDB-C8, 5 µm, column 4.6 x 150 mm) using gradient elution (0-2 min, 10% buffer B; 2-10 min, 10%-50% buffer B; 10-20 min, 50%-60% buffer B; 20-25 min, 60% buffer B; 25-30 min, 60%-70% buffer B; 30-35 min, 70%-90% buffer B; 35-40 min, 90% buffer B) (Buffer A: 0.05 M sodium acetate in 25 mL of acetonitrile and 975 mL of DI water, pH 6; Buffer B: 0.05 M sodium acetate in 300 mL of DI water and 700 mL of methanol, pH 6), and the flow rate was 1.0 mL/min. Aspartic acid (1 mM, 2.5 mM, 5 mM) and alanine (0.3 mM, 1 mM, 3 mM) were used for calibration.

7. Human recombinant MMP-2 catalyzed cleavage of PLGLAG spacer in polymer P-DOX-PLGLAG-iRGD

Polymer P-DOX-PLGLAG-iRGD was incubated with human-recombinant MMP-2 protein (20 µg/mL) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (100 mM HEPES, 10 mM $CaCl_2$, pH = 7.4) at 37 ^{o}C for 24 h. The cleavage products were separated and analyzed by a Waters (U.S.) e2695 system equipped with

reverse-phase C-18 analytical column as well as a Waters 2489 absorption detector (UV = 220 nm) and a Waters Qtof Micro electrospray ionization mass spectrometer. The gradient solvent condition was set as follows: solvent A, 0.1% formic acid in deionized water; solvent B, 0.1% formic acid in acetonitrile. The ratio of B was increased from 1% to 50% in 30 min with a flow rate of 1.0 mL/min. Figures S5A and S5B are the HPLC profiles of polymer P-DOX-PLGLAG-iRGD after incubation with cleavage buffer without or with MMP-2, respectively. A new peak appeared at a retention time (t_R) of 6.80 min in Figure S5B; this was the peak from the cleavage product LAG-iRGD. Its structure, calculated and measured mass are shown in Figure S6.

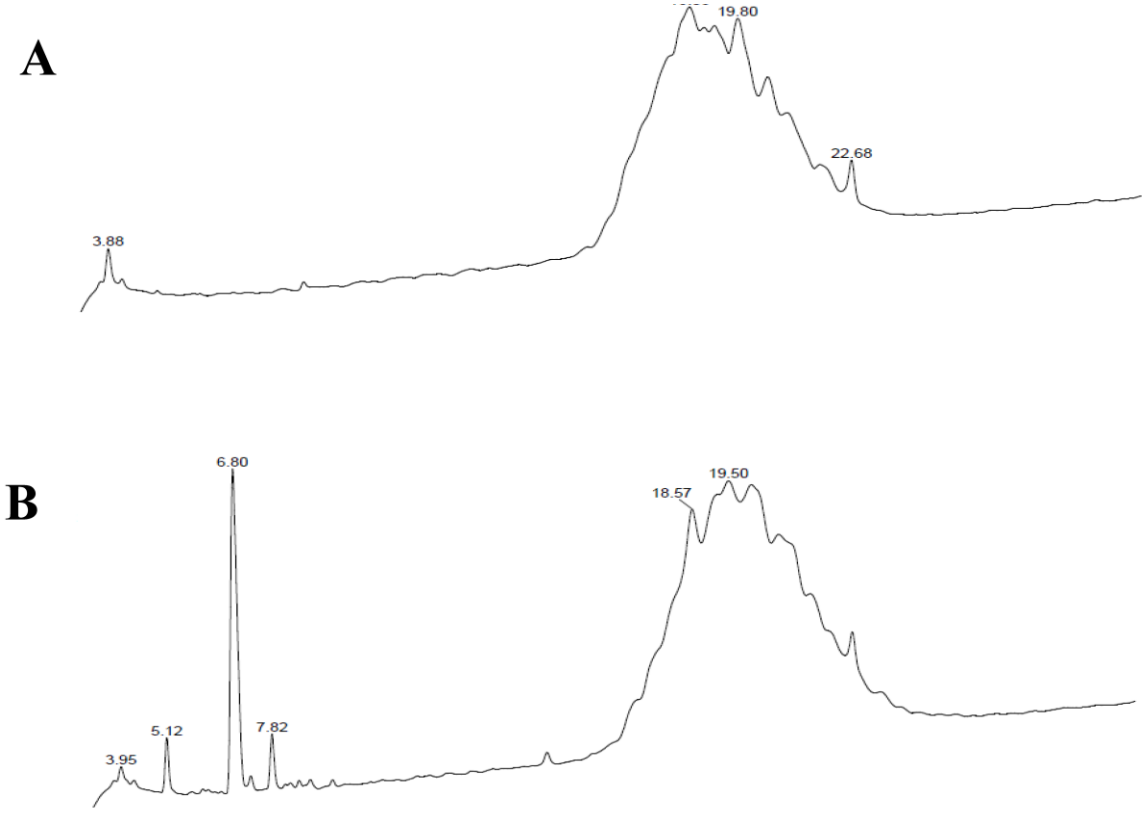

Figure S5. HPLC profiles of incubation product of polymer P-DOX-PLGLAG-iRGD without (A) or with (B) MMP-2

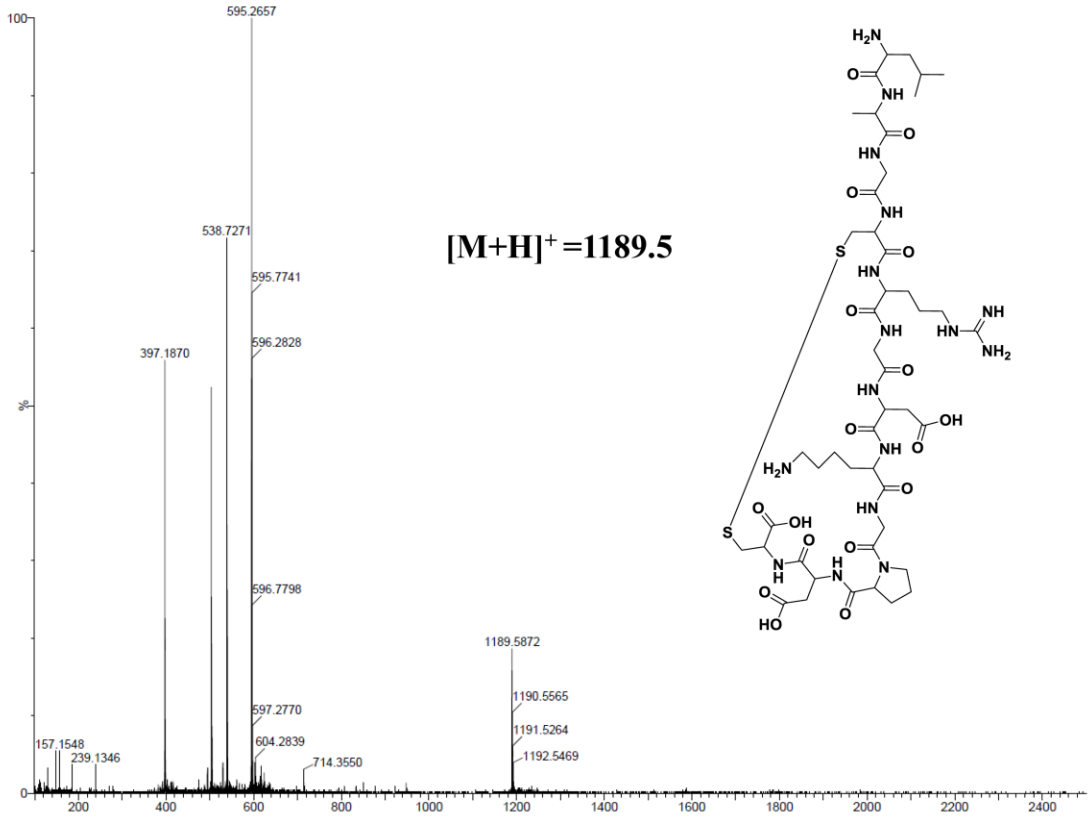

Figure S6. Mass Spectrometry of the cleavage product at elution time 6.80 min in Figure S5B

8. Cleavage of GFLG spacer with lysosomal enzyme Cathepsin B

The cleavage assay was conducted by incubation of P-DOX or MA-GG-DOX (Figure S7) with Cathepsin B in a cleavage buffer (50 mM sodium acetate, 1 mM EDTA and 5 mM dithiothreitol, pH = 5.0). The results were analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) with a flow rate of 1 mL/min and a UV wavelength of 254 nm. The gradient solvent condition was set as follows: solvent A, 0.01% TFA in deionized water; solvent B, 0.01% TFA in acetonitrile. The ratio of B was increased from 30% to 100% in 20 min. As shown in Figure S8, DOX peak appeared at a retention time of 3.162 min in the polymer P-DOX-PLGLAG-iRGD with Cathepsin B

group. However, no DOX was detected after control monomer MA-GG-DOX being incubated with Cathepsin B.

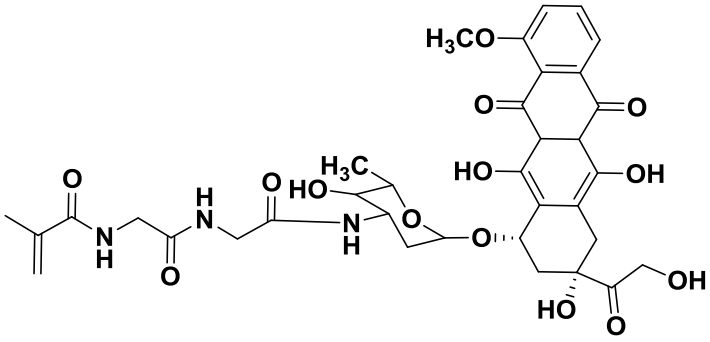

Figure S7. Structure of monomer MA-GG-DOX.

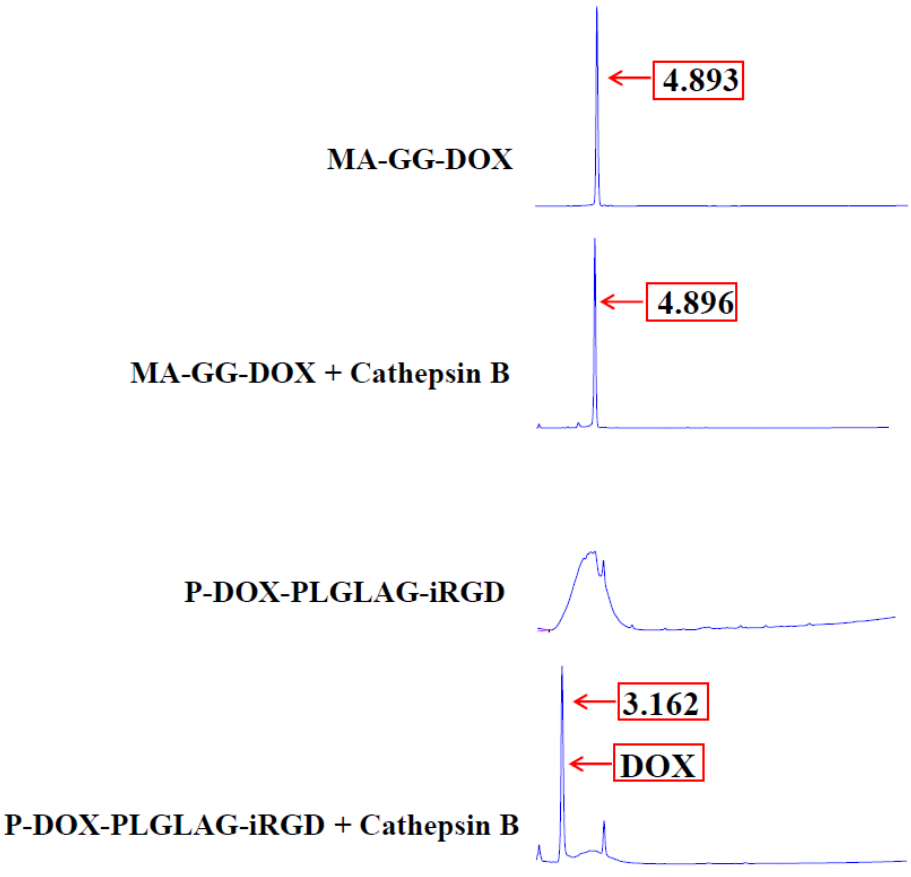

Figure S8. Detection of cleaved DOX from monomer and polymer conjugate by RP-HPLC.

9. Cell culture

Human prostate cancer DU-145 cells (ATCC, Manassas, VA) were grown in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/mL streptomycin (Gibco, Carlsbad, CA) at 37 ^{o}C in a humidified atmosphere of 5% CO_2 (v/v). Cells were passaged every 2-4 d and stopped at passage 20.

10. Fluorescence microscopy

DU-145 Cells were seeded at a density of $1*10^5$ cells/chamber in 4 chamber slide system (Fisher Scientific, Pittsburgh, PA) and grown at 37 ^{o}C for 24 h. Then, the old medium was replaced with fresh medium containing 9 μM [iRGD] equivalent of free iRGD, 10 μM [DOX] equivalent of P-DOX, 9 μM [iRGD] equivalent of free iRGD plus 10 μM [DOX] equivalent P-DOX, or 9 μM [iRGD] and 10 μM [DOX] equivalents of P-DOX-PLGLAG-iRGD for 1.5 h. After removal of the treatment media, cells were washed with PBS, and kept in PBS for confocal microscopy study. Fluorescent images were captured with a fluorescence microscope LSM710 (Carl Zeiss, Germany) using DOX (Ex 488 nm, Em 535-740 nm) filter sets.

11. In vitro accumulation of free and conjugated DOX in DU-145 cells

DU-145 cells were seeded at a density of $4*10^5$ cells/well in 6-well plates and incubated at 37 ^{o}C for 24 h to allow cell attachment. Then, the cells were treated with 9 μM [iRGD] equivalent of free iRGD, 10 μM [DOX] equivalent of P-DOX, 9 μM [iRGD] equivalent of free iRGD plus 10 μM [DOX] equivalent P-DOX, or 9 μM [iRGD] and 10

μM [DOX] equivalents of P-DOX-PLGLAG-iRGD for 24 h. The cells were washed and detached from plates with cold PBS. After being centrifuged and removed the supernatant, the cells were re-suspended in PBS and measured with flow cytometry.

12. Cell cycle arrest

The DU-145 cells were seeded at a density of $4*10^5$ cells/well in 6-well plates and incubated at 37 ^oC to allow adherence to plates. After 24 h, the medium was replaced with 9 μM [iRGD] equivalent of free iRGD, 10 μM [DOX] equivalent of P-DOX, 9 μM [iRGD] equivalent of free iRGD plus 10 μM [DOX] equivalent P-DOX, or 9 μM [iRGD] and 10 μM [DOX] equivalents of P-DOX-PLGLAG-iRGD for another 24 h. After removing the drug solution, the cells were washed and detached with cold PBS and centrifuged. The supernatant was removed and the cells were fixed with 80% ethanol for at least 2 h. After being washed with PBS, the fixed cells were re-suspended in 500 μL of propidium iodide (PI) staining solution (50 μg/mL PI, 200 μg/mL RNase) and analyzed on a flow cytometer.

13. Cytotoxicity of DOX conjugates and controls

Cytotoxicity of DOX conjugates and controls against DU-145 cells was assessed by CCK-8 assay. DU-145 cells were seeded at a density of 3,500 cells/well in a 96-well plate and cultured at 37 ^oC for 24 h. The medium was then replaced with fresh culture media containing 9 μM [iRGD] equivalent of free iRGD, 10 μM [DOX] equivalent of P-DOX, or 9 μM [iRGD] equivalent of free iRGD plus 10 μM [DOX] equivalent P-DOX, or 9 μM [iRGD] and 10 μM [DOX] equivalents of P-DOX-PLGLAG-iRGD and cultured

for 12h or 24 h. The drug solutions were replaced with 100 μL of fresh media and cultured for another 60 h or 48 h. The media was replaced with 100 μL of fresh media and 50 μL of diluted CCK-8 solution. The formazan dye formed was detected at absorbance wavelength 450 nm, and 630 nm was used as a background.

14. Multicellular tumor spheroid formation

To a 0.5% agar coated, 10-mm diameter Petri-dish was added $2*10^6$ DU-145 cells in 15 mL of RPMI media supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin and 50 μg/mL streptomycin. Cells were grown at 37 ^oC in a humidified atmosphere with 5% CO_2 (v/v). The size of spheroids was monitored with a EVOS fluorescence microscope (AMG).

15. Penetration of DOX Conjugates and cotrols in multicellular tumor spheroids

The DU-145 prostate cancer spheroids were incubated with 9 μM [iRGD] and/or 10 μM [DOX] equivalents of iRGD, P-DOX, a mixture of iRGD and P-DOX, or P-DOX-PLGLAG-iRGD for 2 h. After being washed and fixed, the DOX fluorescence in spheroids was measured with Olympus FV1000 confocal microscope using XY-stack imaging with 20 μm intervals. The images were analyzed using ImageJ software.

16. Accumulation of DOX conjugates and controls in multicellular tumor spheroids

When most of the spheroids grew to 100-300 μm diameter, the spheroids were transferred to 15 mL conical base plastic tube and allowed to settle for 2 min. The supernatant was removed, and the spheroids were transferred to 24-well plates and

incubated with 9 μM [iRGD] and/or 10 μM [DOX] equivalents of iRGD, P-DOX, a mixture of iRGD and P-DOX, or P-DOX-PLGLAG-iRGD for 24 h. After being washed with PBS three times, the spheroids were disassociated with accutase (Invitrogen) to single cells and analyzed on flow cytometer.

17. Statistical analysis

Average results are expressed as mean ± SEM or mean ± SD. The statistical difference between groups was assessed by a one-way analysis of variance (ANOVA) plus post-hoc tests using GraphPad Prism software. P values ≤ 0.05 were considered statistically significant.

References

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