Supporting Information

Indium-based and iodine-based labeling of HPMA copolymer–epirubicin conjugates: Impact of structure on in vivo fate

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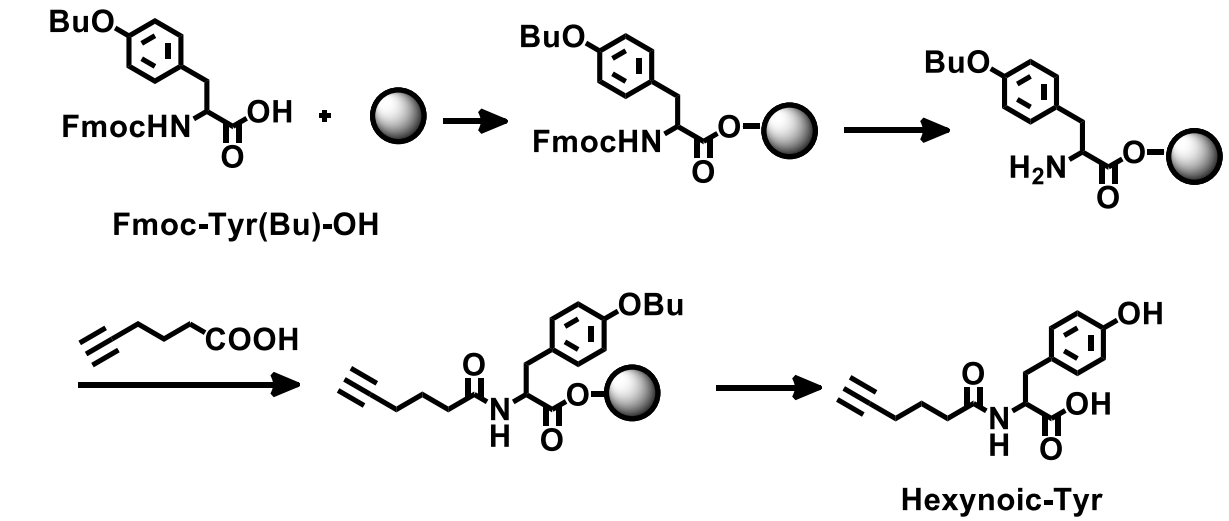


Fig. S1. Synthesis of Hexynoic-Tyr.

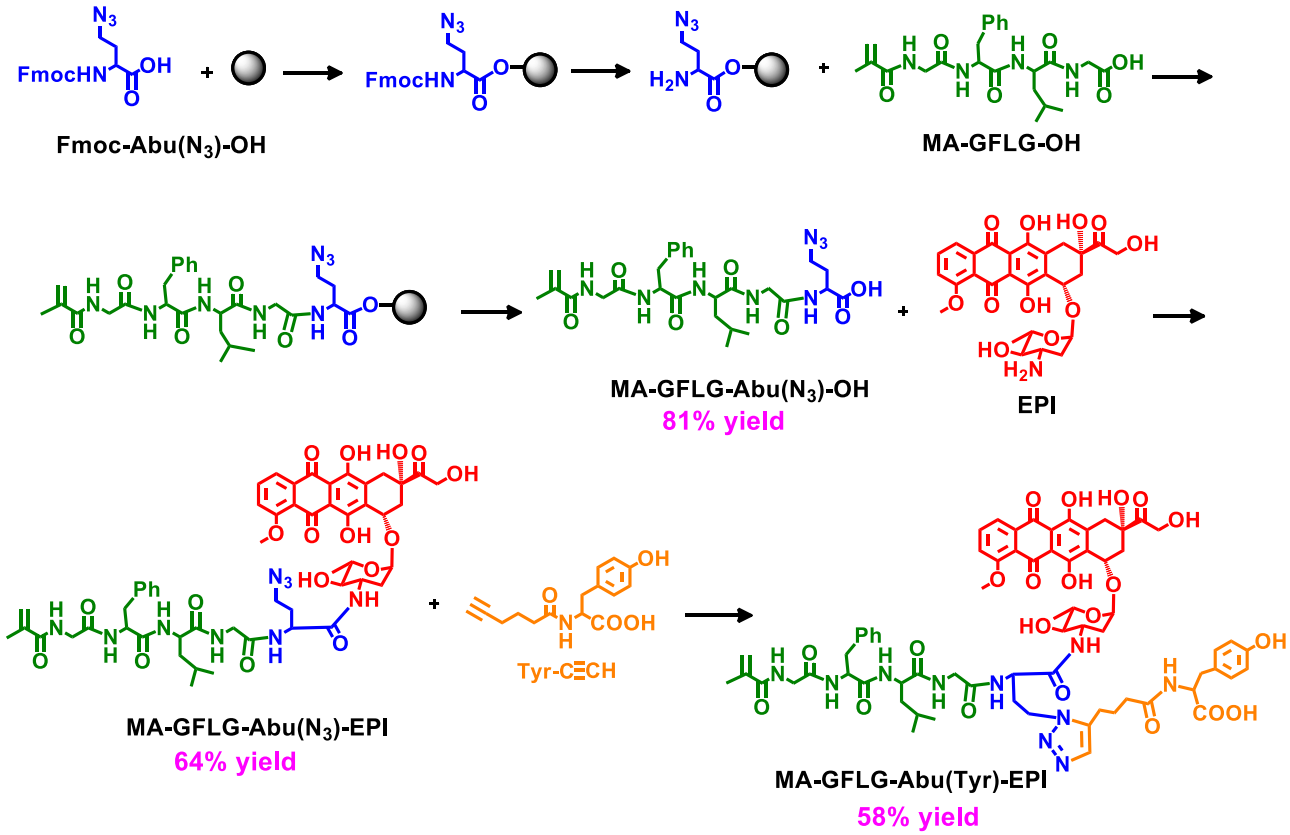


Fig. S2. Synthesis of MA-GFLG-Abu(N_3)-EPI and MA-GFLG-Abu(Tyr)-EPI.

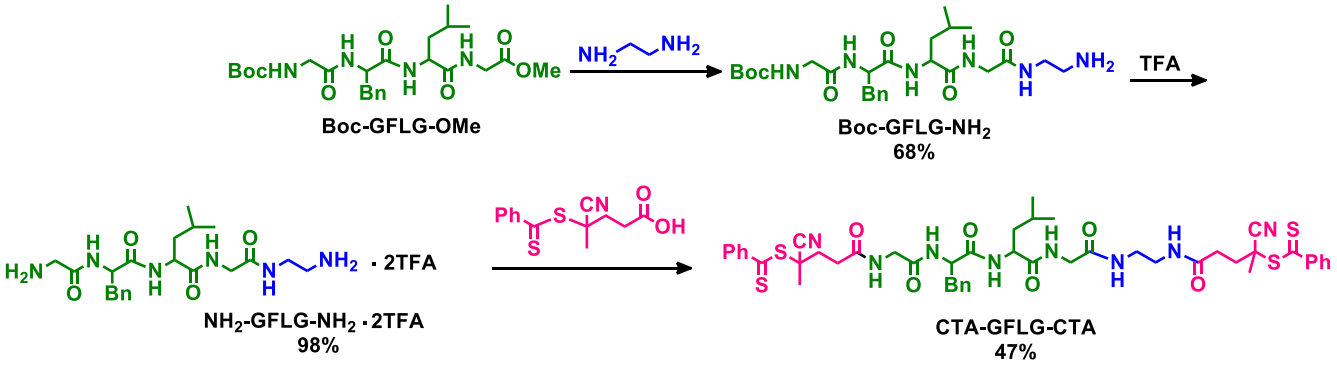


Fig. S3. Synthesis of CTA-GFLG-CTA.

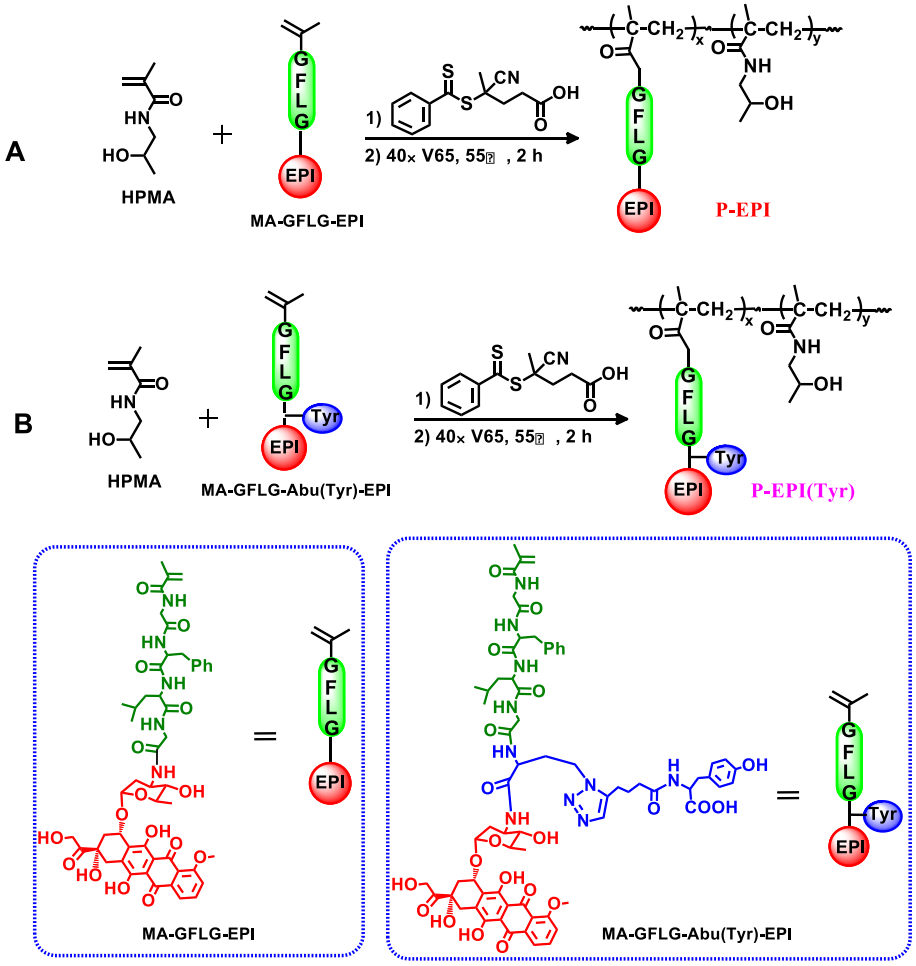


Fig. S4. Synthesis of conjugates P-EPI and P-EPI(Tyr).

Synthesis of P-DTPA

An ampoule containing 80 mg of HPMA, 4 mg of MA-GG-EPI and 10 mg of MA-GFLG-NHBoc was attached to the Schlenk-line. After three vacuum-nitrogen cycles to remove oxygen, 60 μL degassed MeOH was added and stirred at room temperature. CTA (2 mg/mL × 115 μL) in degassed MeOH and 2 mg/mL × 43 μL of VA-044 in degassed MeOH were added via syringe under magnetic stirring and bubbled with N_2 for 10 min in ice bath. The ampoule was sealed, and polymerization was performed at 44 ℃ for 22 h. The copolymer was obtained by precipitation into acetone/ethyl ether (3:1) and purified by redissolving in methanol and precipitation in acetone/ethyl ether (3:1) two more times. The copolymer was isolated as red powder and dried under vacuum. Yield: 33 mg of P-EPI-NHBoc (34%). After end-modification with 40-times excess of V-65, 33 mg of copolymer P-EPI-NHBoc was dissolved in 500 μL H_2O and added 500 μL TFA. The mixture was stirred in ice for 30 min. After removing the solvent, 31 mg red powder was obtained by precipitation into acetone/ethyl ether (3:1). The red powder (31 mg) was dissolved in 600 μL $NaHCO_3$ (0.2 M) buffer containing 10 mM EDTA (pH 8.5), then mixed with *p*-SCN-Bn-DTPA (10 mg) in 200 μL DMSO. After stirring at room temperature overnight, the sample was applied to a pre-equilibrated PD-10 Sephadex G25 column (GE Healthcare) with DI H_2O for primary purification. The fraction of 2.5–4.5 mL was collected and further purified by ultrafiltration (30,000 Da cut-off) with $NaHCO_3$ buffer three times and DI H_2O three times. The final product in DI H_2O was freeze dried to remove solvent (8.5 mg, 26%). DTPA and EPI contents of the copolymer were determined spectrophotometrically as 49 and 19 nmol/mg polymer, respectively.

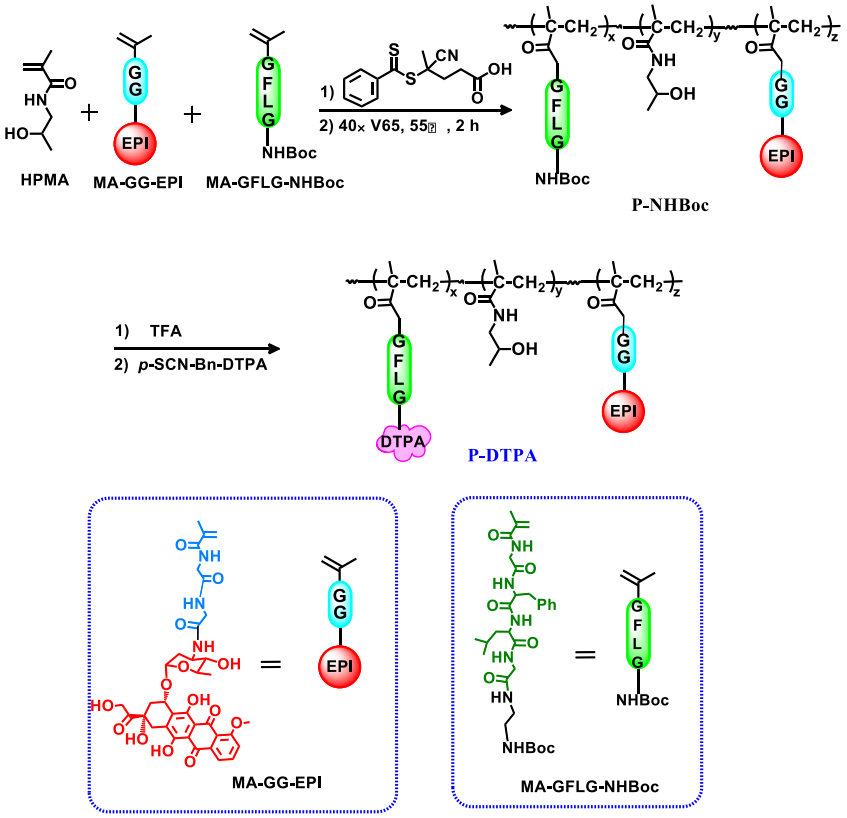


Fig. S5. Synthesis of conjugate P-DTPA.

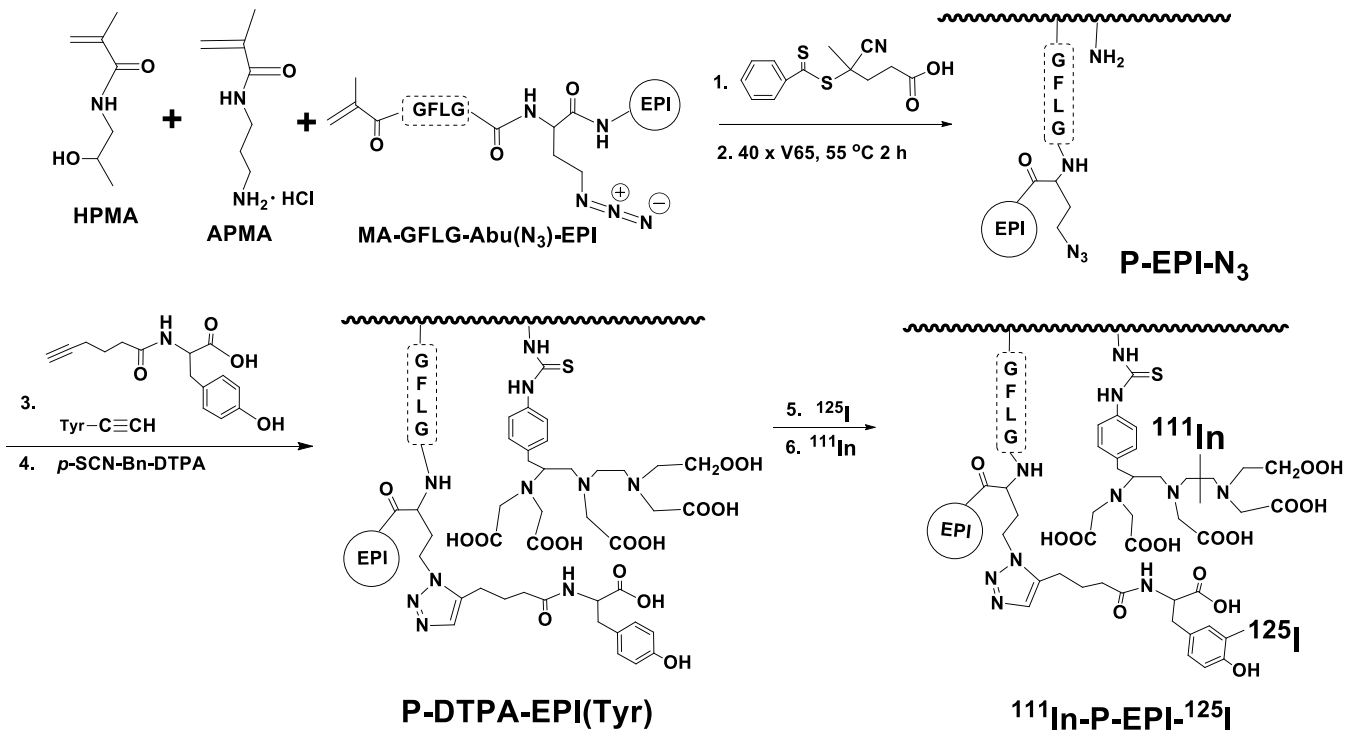


Fig. S6. Synthesis of 1st generation conjugate P-DTPA-EPI(Tyr) by copolymerization of MA-GFLG-Abu(N_3)-EPI with HPMA and APMA. Tyrosine residue was incorporated via Cu(I) assisted alkyne-azide click reaction. DTPA was attached to backbone via pendent amino group modification with *p*-SCN-Bn-DTPA. The polymer precursor was then labeled with ^{125}I and ^{111}In, consecutively.

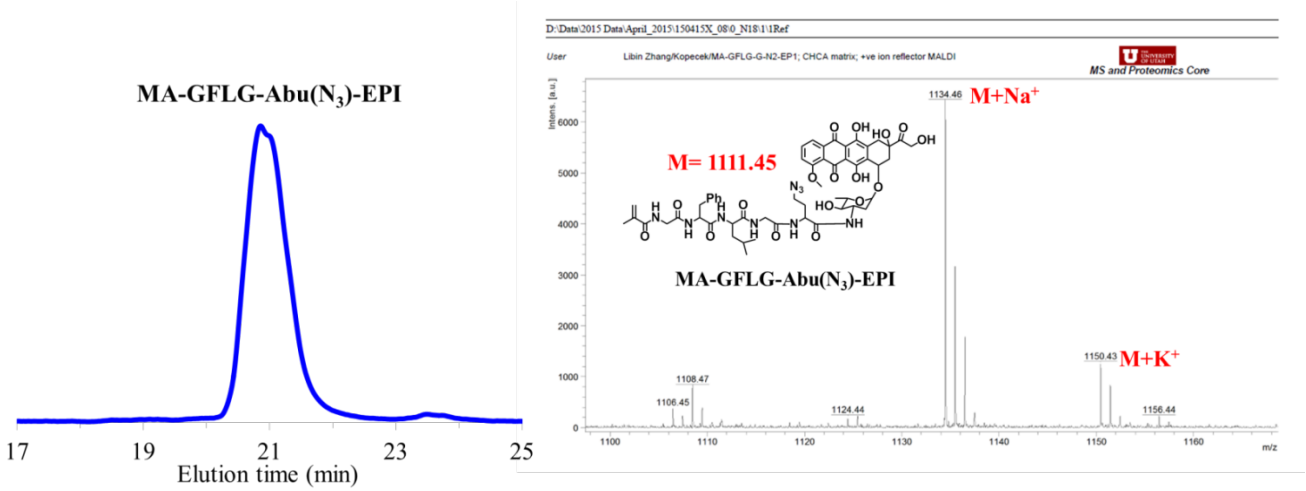


Fig. S7. HPLC (left) and MALDI-TOF-MS (right) analysis of MA-GFLG-Abu(N_3)-EPI.

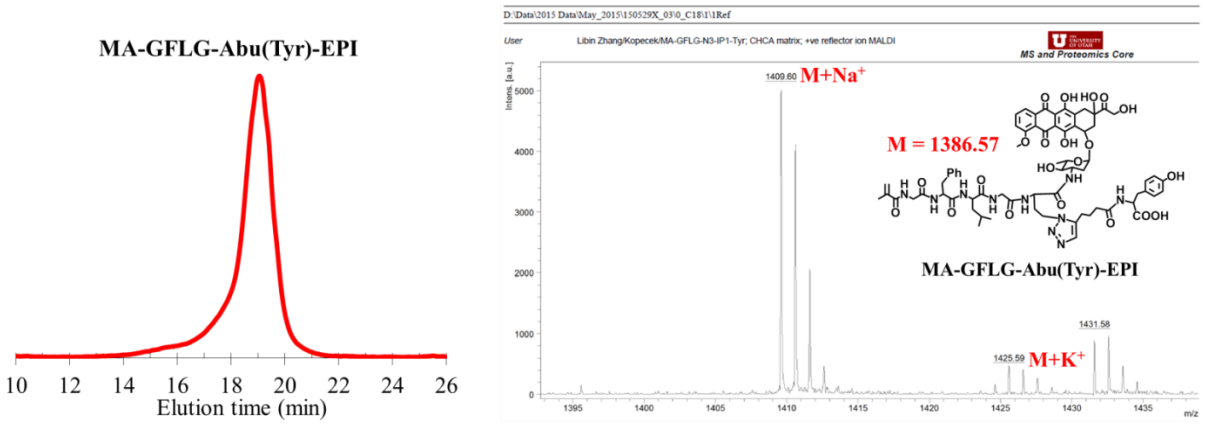


Fig. S8. HPLC (left) and MALDI-TOF-MS (right) analysis of MA-GFLG-Abu(Tyr)-EPI.

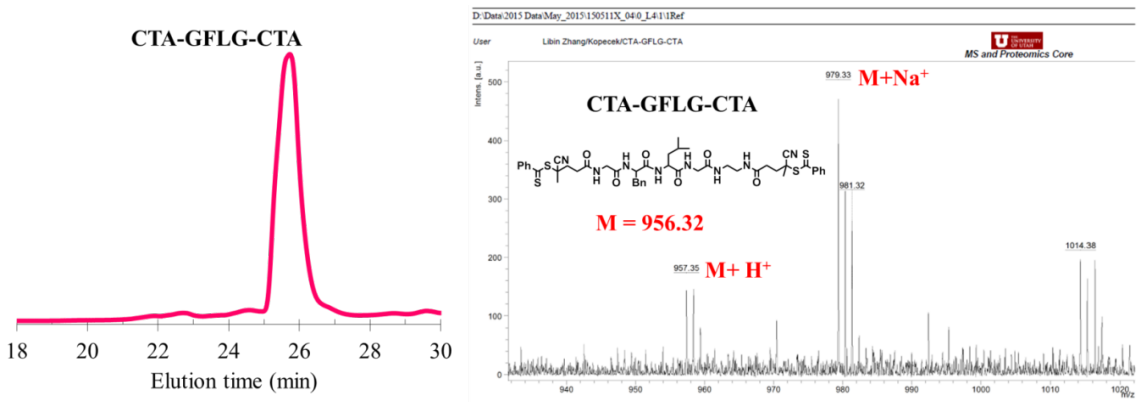
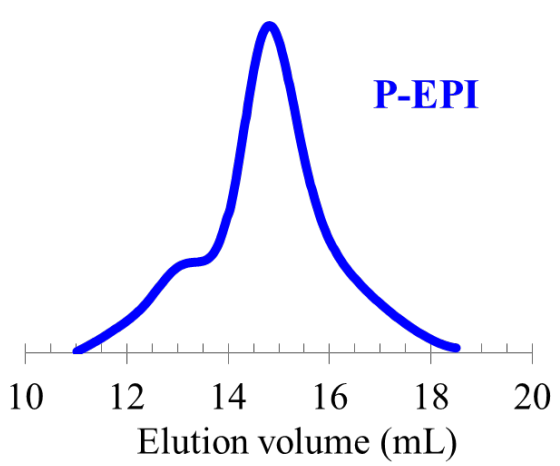
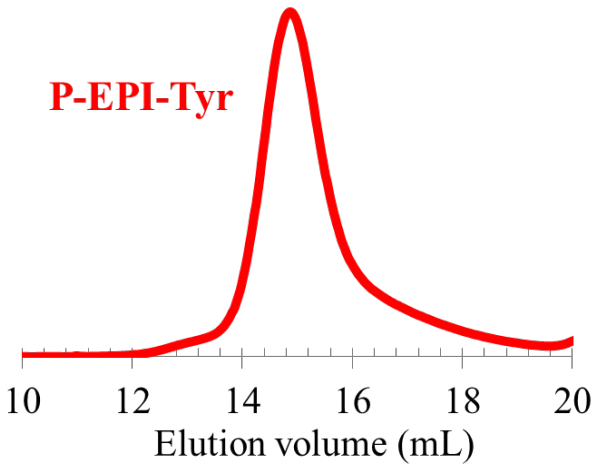
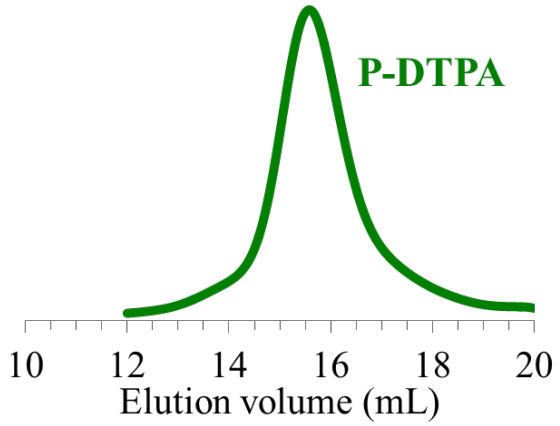


Fig. S9. HPLC (left) and MALDI-TOF-MS (right) analysis of CTA-GFLG-CTA.







	Mn (kDa)	PDI	nmol/mg
P-EPI	55.6	1.20	86 (EPI)
P-EPI-Tyr	50.7	1.09	120 (EPI)
P-DTPA	46.9	1.13	19 (EPI) 49 (DTPA)

Fig. S10. FPLC analysis and characterization of copolymer conjugates P-EPI (blue) and P-EPI(Tyr) (red).

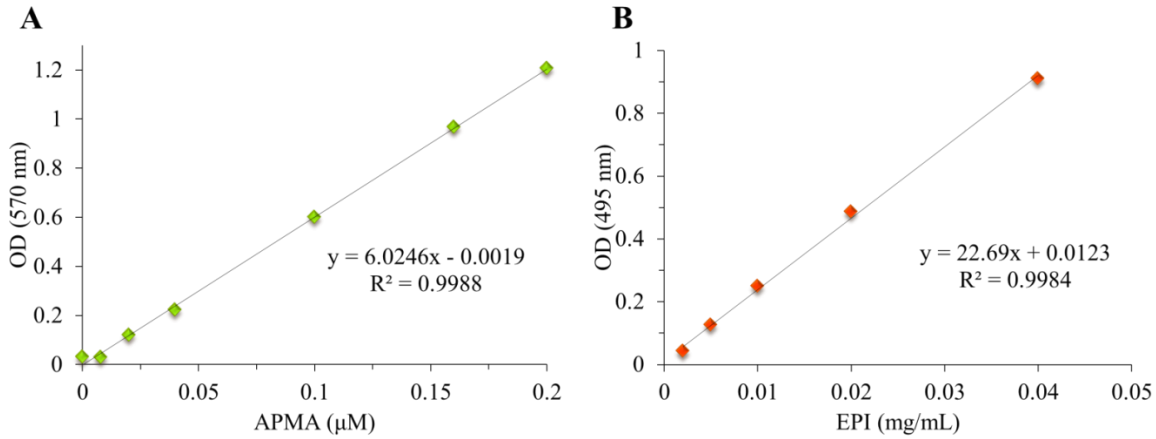


Fig. S11. APMA standard curve for ninhydrin assay (A), and EPI standard working curve (B).

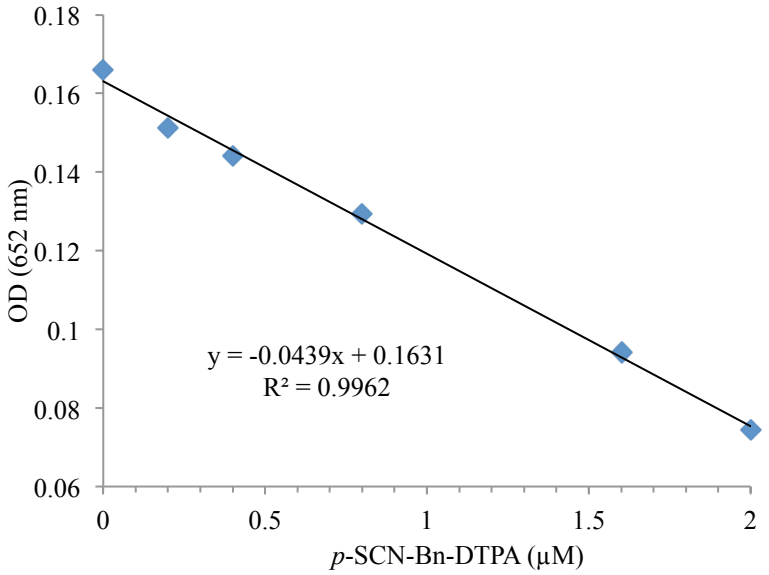


Fig. S12. Relationship between the absorbance of the yttrium complex of arsenazo **III** at 652 nm and the molarity of *p*-SCN-Bn-DTPA.

Cleavage of P-DTPA-EPI(Tyr) and 2P-DTPA-EPI(Tyr) by papain

Glutathione (35.5 mg/mL × 16.8 µL) in McIlvaine's buffer (50 mM citrate/0.1 M phosphate, pH 6.0) was added to a papain solution (1.68 mg in 168 µL McIlvaine's buffer) and activated at 37 °C for 5 min. P-DTPA-EPI(Tyr) (0.95 mg) or 2P-DTPA-EPI(Tyr) (0.7 mg) was added into a 1.5 mL tube followed by adding 60 µL of papain solution and incubated at 37 °C. After 4 h, 140 µL EtOH was added and the mixture was centrifuged for 5 min (13 000 rpm). The supernatant was analyzed by HPLC at 495 nm detection.

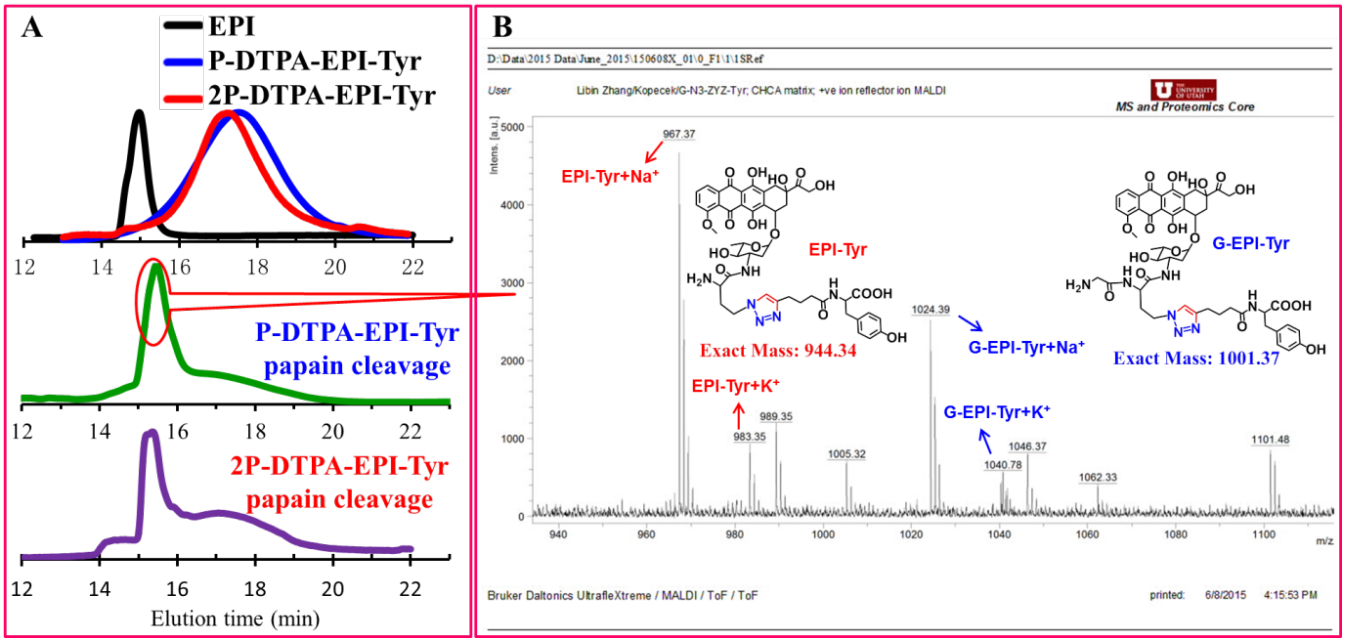


Fig. S13. (A) 1st and 2nd generation conjugates P-DTPA-EPI(Tyr) (0.95 mg) and 2P-DTPA-EPI(Tyr) (0.7 mg) were exposed to model enzyme papain solution (10 mg/mL × 168 µL). After incubation at 37 °C for 4 h, the reaction mixture was analyzed by HPLC which showed that the conjugate P-DTPA-EPI(Tyr) wide peak at 17.4 min almost disappeared accompanied by the emergence of a new peak at 15.5 min corresponding to cleavage product. (B) MALDI-TOF-MS analysis of the new peak showed that papain cleavage of the conjugate yielded two products of glycine-EPI-Tyr ($[G\text{-EPI-Tyr+Na}]^+$, 1024.39) and EPI-Tyr ($[EPI\text{-Tyr+Na}]^+$, 967.37). The results indicated that tyrosine moiety was still coupled with EPI after cleavage from P-DTPA-EPI(Tyr) by papain. Papain cleavage products of 2nd generation conjugate 2P-DTPA-EPI(Tyr) were the same as 1st generation conjugate P-DTPA-EPI-Tyr with a little slower cleavage rate.

Cleavage of P-DTPA by Tritosomes

For Tritosomes cleavage of P-DTPA, the low molecular weight products in reaction mixture were removed by ultrafiltration (10,000 Da cut-off) with DI H_2O four times. The supernatant of copolymer was analyzed using working curves (Figs. S11B and S12) to calculate the ratio of DTPA/EPI of the samples. In P-DTPA, EPI bound via nondegradable GG spacer was used as internal standard; release of DTPA resulted in the change of the DTPA/EPI ratio. The initial DTPA/EPI ratio of P-DTPA was considered as 100% before Tritosomes cleavage. From the change in the DTPA/EPI ratio the cleavage percent rate was calculated. Time intervals: 1 min, 1, 2, 4, 6, 8, 23, and 48 h.

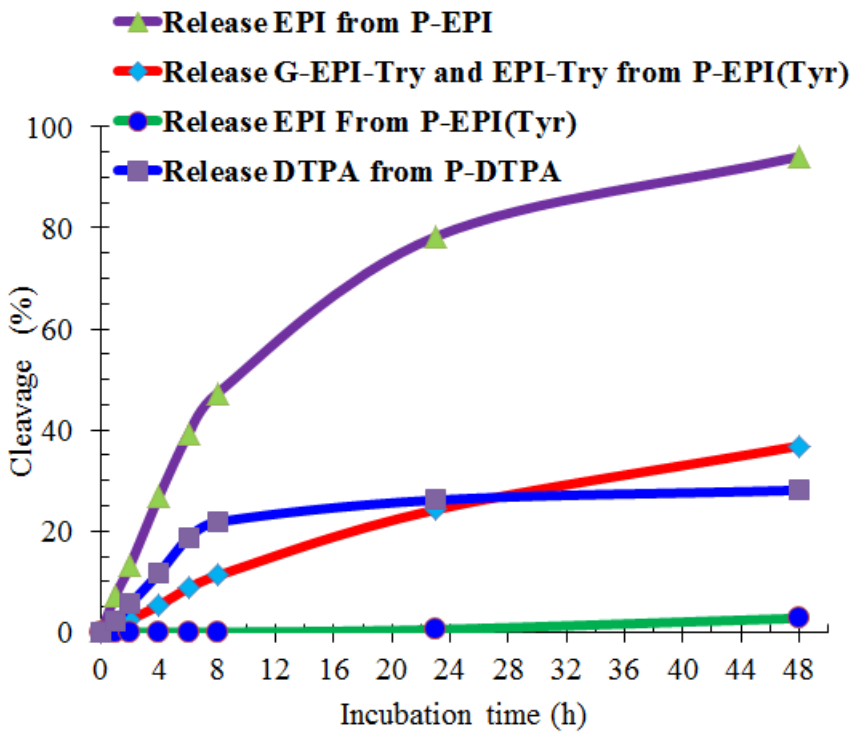


Fig. S14. Time profile of P-EPI(Tyr), P-EPI and P-DTPA cleavage by Tritosomes: Purple line – release of EPI from P-EPI; red line – release of Gly-EPI-Tyr + EPI-Tyr from P-EPI(Tyr); green line – release of unmodified EPI from P-EPI(Tyr); blue line – release of DTPA from P-DTPA. Tritosomes (480 µL) were incubated with conjugates (3.5 mg P-EPI, 2.5 mg P-EPI(Tyr) or 6.1 mg P-DTPA, 300 nmol substrate in each conjugate) in 520 µL buffer B-GSH (0.04% w/w Triton-X-100) at 37 ^{o}C. Daunomycin was used as internal standard.

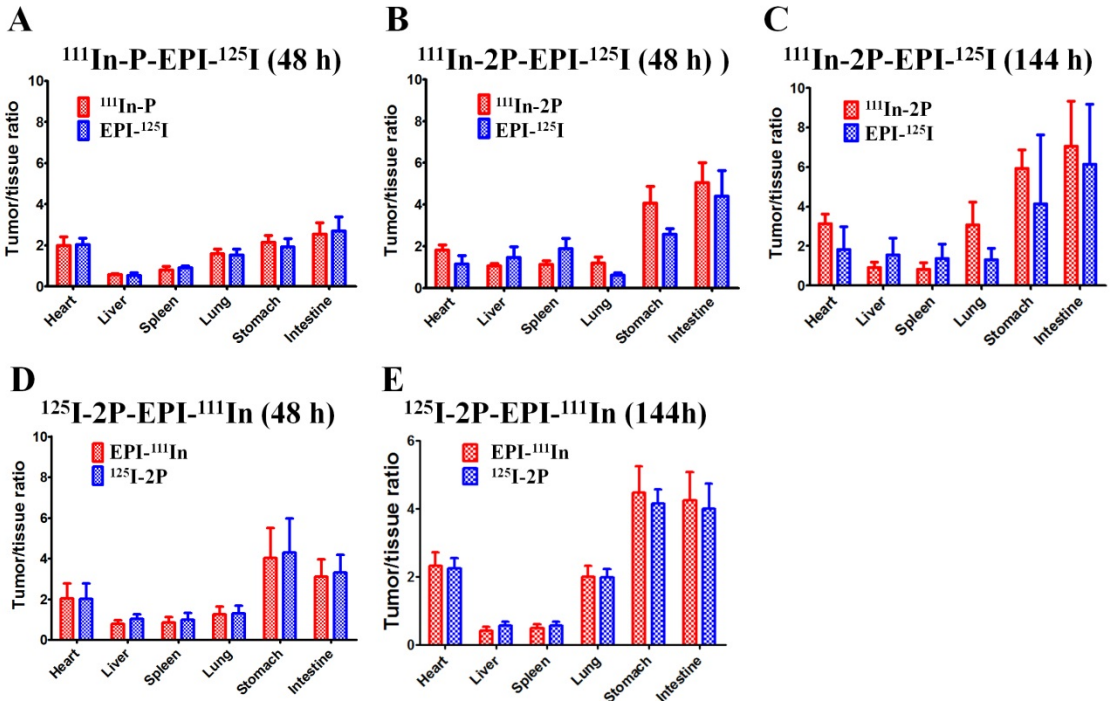


Fig. S15. Tumor-to-tissue uptake ratios of polymeric carriers and payload of HPMA copolymer-EPI conjugates in tumor-bearing mice at 48 h or 144 h after i.v. injection. Data obtained using the radioactivity count method plotted as percentage of injected dose per gram of tissue (%ID/g). All the data are expressed as mean ± standard deviation (n=5).