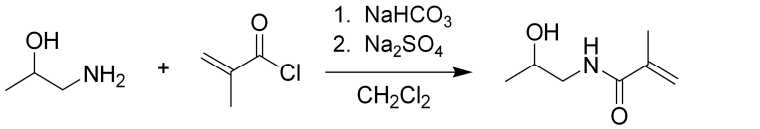
SUPPORTING INFORMATION

1. Poly(HPMA) monomer synthesis

1.1 N-2-hydroxypropyl methacrylamide



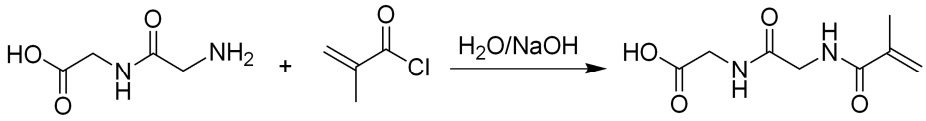
N-2-hydroxypropyl methacrylamide
"HPMA"

Scheme S1. Synthesis of N-2-hydroxypropyl methacrylamide monomer.

N-(2-hydroxypropyl)methacrylamide (HPMA) was synthesized by reaction of DL-1-amino-2-propanol with methacryloyl chloride as shown in Scheme S1. 1-amino-2-propanol (24.3 mL, 0.311 mol, 1 eq) and sodium bicarbonate (33.6 g, 0.40 mol, 1.3 eq) was added to CH_2Cl_2 (85 mL) and the suspension was purged with N_2, capped, and placed in an ice bath. A second solution containing methacryloyl chloride (29.5 mL, 0.304 mol, 0.98 eq) in CH_2Cl_2 (40 mL) was prepared and cooled to –20 ^oC. Next, the methacryloyl chloride solution was slowly added to the 1-amino-2-propanol solution under N_2 with vigorous stirring. After complete addition the reaction was stirred for 15 min at 25 ^oC, and anhydrous Na_2SO_4 (10 g) was added. The solution was manually stirred for an additional 10 minutes and the solution was filtered through paper. Solids were washed twice with 20 mL CH_2Cl_2 and the filtrates were combined. The combined filtrates were concentrated under vacuum to ~ 50% of the original volume then placed in a –20 ^oC freezer overnight. HPMA crystals were collected by filtration and washed with cold acetone. Product was purified by repeated crystallization from acetone. Typical yield: ~15.0 g, 30%, semi-transparent white crystals. ^1H NMR, δ ppm: 1.008 (d, 3H), 1.848 (s, 3H), 3.041 (t, 2H), 3.678 (m, 1H), 4.693 (s, 1H), 5.483 (d, 2H), 7.820 (s, 1H). Mp = 72 ^oC (sharp).

Reference: Ulbrich K, Subr V, Strohalm J, Plocova D, Jelinkova M, Rihova B. (2000) Polymeric drugs based on conjugates of synthetic and natural macromolecules I. Synthesis and physico-chemical characterisation. J. Controlled Release *64(1-3)*, 63-79.

1.2 Methacryloyl glycylglycine



methacryloyl glycylglycine
"MAGG"

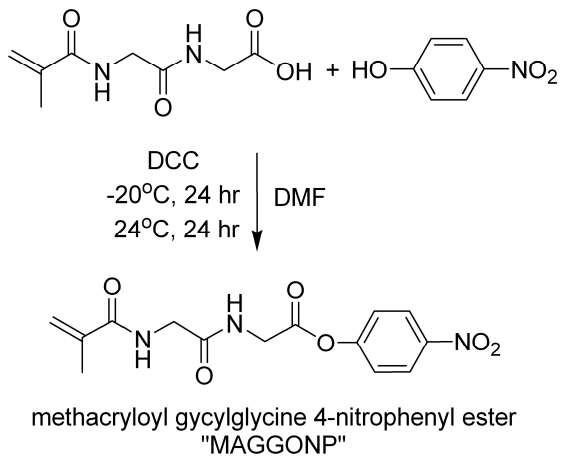
Scheme S2. Synthesis of methacryloyl glycylglycine.

N-methacryloyl glycylglycine 4-nitrophenyl ester (MAGGONP) synthesis began with preparation of methacryloyl glycylglycine (MAGG) using the Schotten-Baumann procedure in aqueous alkaline medium as shown in Scheme S2. Glycylglycine (10 g, 76 mmol, 1 eq) was dissolved in 0.5 M NaOH (15.1 mL, 76 mmol, 1 eq) over 30 minutes then cooled to 0 ^oC. Methacryloyl chloride (7.5 mL, 77 mmol, 1eq) and 5.0 M NaOH (15.1 mL, 76 mmol, 1 eq) were added dropwise and simultaneously to the glycylglycine solution while stirring at 0 ^oC. After complete addition, the reaction was stirred for an

additional hour at room temperature, then concentrated HCl was added until a dense white precipitate formed. Solids were collected by filtration and washed with cold water until the filtrate was neutral. Crude solid product was dried under vacuum and further purified by repeat crystallization from 50/50 (v/v) ethanol/water. Typical yield: ~5.0 g, 30%, small white crystals. ^{1}H NMR: (400 MHz, DMSO-d_6) δ ppm: 1.876 (s, 3H), 3.766 (d, 4H), 5.561 (d, 2H), 8.132 (t, 1H), 8.190 (t, 1H), 12.594 (s, 1H). Mp = 175-205 ^{o}C.

Reference: Kopecek J. (1977) Reactive Copolymers of N-(2-Hydroxypropyl)Methacrylamide with N-Methacryloylated Derivatives of L-Leucine and L-Phenylalanine .1. Preparation, Characterization, and Reactions with Diamines. Makromol. Chem. Macromol. Chem. Phys. *178(8)*, 2169-83.

1.3 Methacryloyl glycylglycine 4-nitrophenyl ester



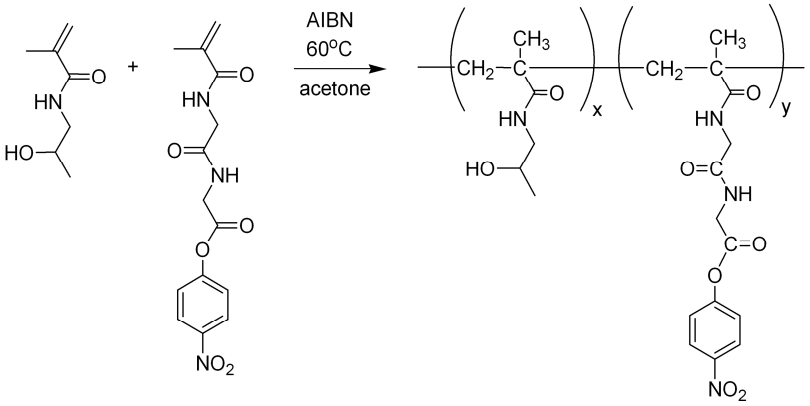
Scheme S3. Synthesis of methacryloyl glycylglycine 4-nitrophenyl ester

Methacryloyl glycylglycine (MAGG) was esterified with 4-nitrophenol using carbodiimide chemistry to yield methacryloyl glycylglycine 4-nitrophenyl ester as shown in Scheme S3. MAGG (5.23 g, 26.1 mmol, 1 eq) was dissolved in dry dimethyl formamide (DMF, 50 mL) and cooled to –15 ^{o}C. 4-nitrophenol (4.0 g, 28.71 mmol, 1.1 eq) and dicyclohexylcarbodiimide (DCC) (5.39 g, 26.1 mmol, 1.0 eq) were each dissolved in DMF (26 mL) in separate flasks and cooled to –15 ^{o}C. After all solutions were cooled, the 4-nitrophenol and DCC solutions were added dropwise to the MAGG solution sequentially while stirring. The reaction was stirred for 4 hr at –15 ^{o}C followed by 24 hr at –20 ^{o}C. Upon complete reaction, dicyclohexyl urea (DCU) byproduct was removed by filtration. The filtrate was collected and allowed to sit at room temperature for 24 hrs under N_2 and then cooled to –20 ^{o}C. The solution was again filtered to remove DCU crystals. The filtrate was retained and 100 mL cold water was added. This mixture was placed on ice for 1 hr. The pale-yellow solids were collected by filtration and dried under vacuum. Hot 50/50 water/ethanol was added to the dried solids until ~ 80% dissolved. The solution was boiled for an additional 10 minutes then filtered. Small, pale-yellow, fish-scale crystals formed slowly in the filtrate over a 3 hr period at room temperature. Solids were collected by filtration and dried under vacuum. Typical yield ~ 2.0 g, 22%, yellow crystals. ^{1}H NMR, δ ppm: 1.884 (s, 3H), 3.809 (d, 2H), 4.176 (d, 2H), 5.577 (d, 2H), 7.435 (m,2H), 8.267 (t, 1H), 8.326 (m, 2H), 8.479 (t, 1H). Mp = 143-160 ^{o}C.

Reference: Kopecek J. (1977) Reactive Copolymers of N-(2-Hydroxypropyl)Methacrylamide with N-Methacryloylated Derivatives of L-Leucine and L-Phenylalanine .1. Preparation, Characterization, and Reactions with Diamines. Makromol. Chem. Macromol. Chem. Phys. *178(8)*, 2169-83.

2. HPMA copolymer synthesis

2.1 Free radical initiated polymerization



Scheme S4. Free radical initiated copolymerization of N-2-hydroxypropyl methacrylamide and methacryloyl glycylglycine nitrophenyl ester.

HPMA copolymer was prepared by free radical initiated polymerization as previously described using 12.5% (w/v) monomer and 0.6% initiator (AIBN) in freshly distilled acetone. Reactive side chain content in the polymer product was controlled by feed ratio of MAGGONP. The following is a typical procedure to produce an HPMA copolymer with 95 mole % HPMA units and 5 mole % MAGGONP units. HPMA monomer (3.35 g, 23.4 mmol, 1 eq), MAGGONP (0.40 g, 1.2 mmol, 0.05 eq), and AIBN (0.18 g, 1.1 mmol, 0.05 eq) were added to acetone (26 mL) in a Chemglass 50 mL pressure flask containing a magnetic stirbar. The solution was degassed by sonication under N_2 flow for 5 minutes and then capped. This solution was stirred until all solids dissolved (~ 30 minutes) and placed in a hot water bath and stirred at 60 ^oC for 24 hrs. After complete reaction, solids were collected by filtration, washed with cold acetone, and dried under vacuum. Solids were further purified by repeat precipitation from 90/10 CH_2Cl_2/MeOH (v/v) into a 10-fold excess of cold acetone. Typical yield: ~ 2.2 g, 56%, cream colored powder.

Reference: Kopecek J. (1977) Reactive Copolymers of N-(2-Hydroxypropyl)Methacrylamide with N-Methacryloylated Derivatives of L-Leucine and L-Phenylalanine .1. Preparation, Characterization, and Reactions with Diamines. Makromol. Chem. Macromol. Chem. Phys. *178(8)*, 2169-83.

2.2 Detemination of Active Ester Content in HPMA Copolymers.

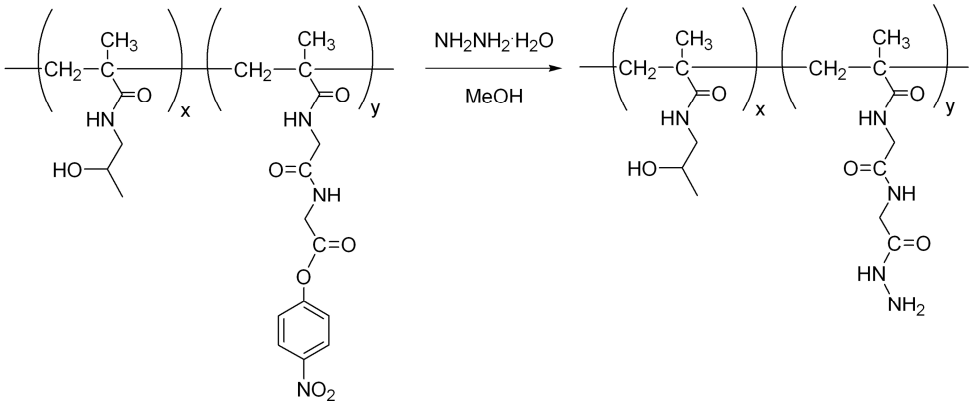
HPMA copolymer product was dissolved in 0.05 M NaOH at concentrations of 0.08-0.1 mg/mL then stirred at 37 ^oC for 3 hrs. The absorbance of the resulting bright-yellow solutions was measured at 400 nm and ester content was estimated using the molar extinction coefficient ($\varepsilon = 1.74 \times 10^4\ M^{-1}cm^{-1}$) of the released p-nitrophenolate anion and the original mass of the polymer sample. The molar extinction coefficient was determined by serial dilution of a p-nitrophenol solution in 0.05 M NaOH. No increase

in absorbance after incubation for 3 hr at 37 ^{o}C was observed, showing complete cleavage p-nitrophenol esters. The reactive ester content was determined to be 4.9% +/- 1.5%.

2.3 Molecular Weight Estimation of HPMA Copolymers

Molecular weight of synthesized poly(HPMA) copolymer was estimated following displacement of reactive ester groups with 1-amino-2-propanol by size exclusion chromatography using an HP1050 HPLC system equipped with an HP1040A refractive index detector and Biosil 125 column. The instrument was calibrated with poly(HPMA) samples of known molecular weight. For each characterization, sodium phosphate buffer (0.05 M, pH 7.4) was used as the mobile phase with a 100 μL sample injection and a 0.5 mL/min flow rate.

2.4 Hydrazide modification



Scheme S5. Conversion of reactive esters to hydrazide groups

Hydrazide groups were generated on HPMA copolymers by reaction of hydrazine with active esters present on MAGGONP side chains as previously described (Scheme S5). A representative procedure follows: HPMA/MAGGONP copolymer (5 mole % MAGGONP, 2.0 g, 0.51 mmol active esters, 1 eq) was dissolved in methanol (25 mL). Hydrazine monohydrate (267 uL, 5.1 mmol, 10 eq) was added to 25 mL methanol in a separate flask. The HPMA copolymer solution was added to the hydrazine monohydrate solution dropwise while stirring over ~ 30 minutes then purged with N_2, capped, and stirred for 3 hr at room temperature. After complete reaction, the mixture was slowly added to 75 mL cold water while stirring, then transferred to dialysis tubing (SpectraPore 7, 6-8 kDa MWCO) and dialyzed against water for 3 days with frequent changing of the dialysis water. Polymer product was isolated by lyophilization. Typical yield: ~ 1.2 g, 60%, white powder.

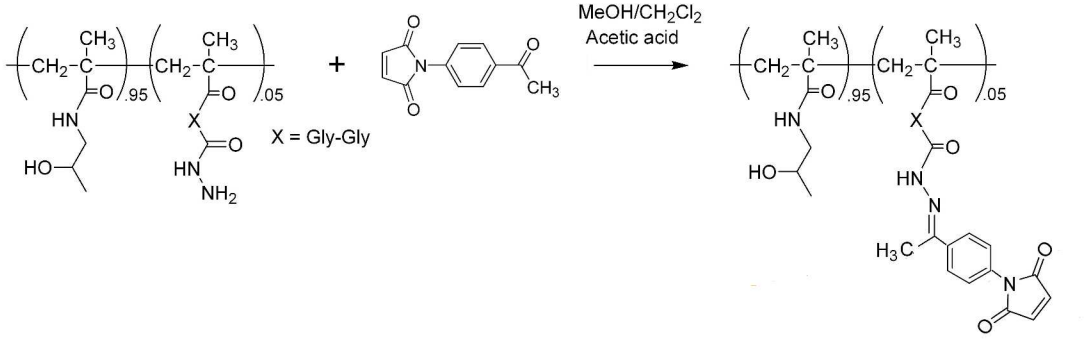
Reference: Etrych, T., Chytil, P., Jelinkova, M., Rihova, B., Ulbrich, K. (2002) Synthesis of HPMA copolymers containing doxorubicin bound via a hydrazone linkage. Effect of spacer on drug release and in vitro cytotoxicity. *Macromol. Biosci. 2*, 43-52.

2.5 Analysis of HPMA Copolymer Hydrazide Content

Hydrazide content in HPMA copolymers was estimated using a modified trinitrobenzene sulfonic acid (TNBSA) assay. TNBSA assay compared ethyl carbazate and methacryloyl glycylglycine hydrazide (MAGGH) as standards. MAGGH was synthesized by reaction of MAGGONP with hydrazine monohydrate. MAGGONP (750 mg, 2.33 mmol, 1 eq) was dissolved in methanol (90 mL) and then slowly added to a solution of hydrazine monohydrate (567 µL, 11.7 mmol, 5 eq) in 10 mL methanol. The reaction was stirred for 5 hr at room temperature under N_2. After complete reaction, solvent was removed under vacuum to yield crude solids. The resulting solids were dissolved in 90/10 CH_2Cl_2/methanol (40 mL), filtered and loaded onto a silica gel column (2.5 x 17.5 cm) equilibrated with 90/10 CH_2Cl_2/MeOH. After the sample was loaded onto the column, the elution solvent was changed to MeOH. MAGGH eluted after the visible yellow 4-nitrophenol band. Yield: 0.156 g, 31%, white powder. ^{1}H NMR, δ ppm: 1.836 (s, 1H), 3.619 (d, 2H), 3.712 (d, 2H), 4.165 (d, 2H), 5.341 (d, 1H), 5.698 (d, 1H), 8.048 (t, 1H), 8.131 (t, 1H), 8.907 (s, 1H). ^{13}C NMR, δ ppm: 18.479, 40.779, 42.402, 119.752, 139.356, 167.597, 168.011, 169.204. MS-FAB m/z = 215.1147 $[M+H]^+$(calculated m/z = 215.1144 $[M+H]^+$)

For each poly(HPMA) hydrazide content analysis, stock solutions of 0.1% w/v TNBSA, ~ 2 x 10^{-4} M MAGGH, and ~ 2 x10^{-4} M ethyl carbazate were prepared in 0.1 M $NaHCO_3$, pH 8.5. MAGGH and ethyl carbazate solutions were further diluted with 0.1 M $NaHCO_3$ to generate standards in the range of ~0.3 - 2 x 10^{-4} M. Next, hydrazide-derivatized HPMA copolymers were dissolved in 0.1 M $NaHCO_3$ at a concentration of 0.2-0.4 mg/mL. Sample or standard solutions (1.0 mL) were combined with 0.5 mL TNBSA stock solution and stirred for 1.5 – 48 hr at 37 °C. After incubation, sample absorbance was measured at 505 nm and hydrazide content was estimated from the equation of the line fit to MAGGH or ethyl carbazate standards plotted as concentration vs. absorbance.

3. Preparation of HPMA-APM conjugate



Scheme S6. Conjugation of *N*-4-(acetylphenyl) maleimide to HPMA copolymer

Hydrazide derivatized poly(HPMA), (0.20 g, 1.0 equiv, 52 µmol hydrazide) was dissolved in 50/50 methylene chloride/methanol (2 mL), and 100 mg Na_2SO_4 was added. *N*-4-(acetylphenyl) maleimide solution (34.0 mg, 3.1 equiv, 160 µmol) was prepared in 50/50 methylene chloride/methanol (2 mL) and then added to the poly(HPMA) solution dropwise. After complete addition, acetic acid (5 µL of 34% v/v solution in methylene chloride, 0.6 equiv, 30 µmol) was added and the reaction was stirred under nitrogen at room temperature for 24 hr. The reaction was terminated by precipitation of poly(HPMA) product into ether. Product was further purified by size exclusion chromatography (Sephadex LH-20, 1 x 15 cm, methanol mobile phase). Typical yield: 126 mg, 63%, white powder. Hydrazide content post-

conjugation was determined using a TNBSA assay, as described above. Crosslinker content was determined by an absorbance measurement at λ_{max} and using the molar extinction coefficients of corresponding ethyl carbazate-crosslinker hydrazone derivative at 288 nm, $\varepsilon = 1.9 \times 10^4\ M^{-1}\ cm^{-1}$. Polymer-crosslinker conjugates were analyzed by ^1H NMR and maleimide functionality was confirmed in the polymer backbone as a sharp singlet at 7.2 ppm.