Electronic Supplementary Information for

Facile Synthesis of Drug-Conjugated PHPMA Core-Crosslinked Star Polymers

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Experimental

Materials. 1-Amino-2-propanol (94%), methacryloyl chloride (97%), 4,4′-azobis(cyanovaleric acid) (ACVA) (98%), anhydrous methotrexate (98%), and Spectrum/Por® Float-A-Lyzer® G2 dialysis devices with 3500 Da MWCO membranes were purchased from VWR and used as received. EMD Millipore Amicon® Ultra-0.5 centrifugal filter units with 50 kDa MWCO membranes were purchased from Fisher Scientific. *N,N′*-dicylcohexylcarbodiimide (DCC) (99%), 4-(dimethylamino)pyridine (DMAP) (99%), 2,2′-azobisisbutyronitrile (AIBN), ethylene glycol dimethacrylate (EGDMA) (98%), esterase from porcine liver (PLE) (ammonium sulphate suspension, ≥150 U/mg protein, 28.1 mg protein/mL),

tri-*n*-butylphosphine (99%), anhydrous *N*,*N*-dimethylformamide (DMF), *N*,*N*-dimethylacetamide (DMAc), and Dulbecco's phosphate buffered saline (PBS) were purchased from Sigma Aldrich. Deuterium oxide (D_2O, 99.9%), and dimethylsulfoxide-d_6 (DMSO-d_6, 99.8%) were purchased from Cambridge Isotopes. AIBN was recrystallized from methanol, EGDMA was passed through a column of basic alumina to remove inhibitors, and all other chemicals were purchased with the highest available purity and used as received. 4-Cyano-4-[(dodecylsulfanylythiocarbonyl)sulfanyl]pentanoic acid (CDTPA)[1] and bis(2-methacryloyl)oxyethyl disulfide (DSDMA)[2] were synthesized according to a literature procedure.

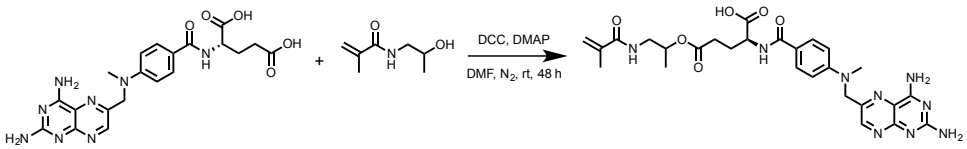
Characterization. ^{1}H NMR spectra were recorded on a Varian Inova2 500 MHz or a Varian Mercury 300 MHz NMR spectrometer using the residual solvent signal as reference. UV-Vis spectra were obtained on a Molecular Devices SpectraMax M2 multimode microplate reader. Analytical HPLC was performed using a gradient from 3:1 to 1:3 aq. trifluoroacetic acid (TFA) (0.1%):CH_3CN at 35 °C at a flow rate of 1 mL/min (Hitachi Elite LaChrom pump, column oven, and UV-Vis detector operating at 303 nm; column: 150 x 4.6 mm Phenomenex Luna 10 μm C_{18}). Preparative HPLC was performed using 3:1 to 1:1 aq. TFA (0.1%):CH_3CN at 35 °C and a flow rate of 8 mL/min (Hitachi Elite LaChrom pump, column oven, and UV-Vis detector operating at 303 nm; column: 250 x 21.2 mm Phenomenex Luna 10 μm C_{18}). High-resolution mass spectrometry to obtain accurate mass was obtained with an Agilent 6220 electrospray ionization time-of-flight mass spectrometer (ESI-TOF MS). Polymer molecular weight and molecular weight

distributions were determined by gel permeation chromatography (GPC) in *N*,*N*-DMAc with 50 mM LiCl at 50 °C and a flow rate of 1.0 mL/min (Agilent isocratic pump, degasser, and autosampler, columns: PLgel 5 μm guard + two ViscoGel I-series G3078 mixed bed columns: molecular weight range 0−20 × 10^3 and 0−100 × 10^4 g mol^{-1}). Detection consisted of a Wyatt Optilab T-rEX refractive index detector operating at 658 nm and a Wyatt miniDAWN Treos light scattering detector operating at 659 nm. Absolute molecular weights and molecular weight distributions were calculated using the Wyatt ASTRA software (PHPMA *dn/dc* = 0.0751 mL/g). Dynamic light scattering (DLS) measurements were recorded on a Zetasizer Nano ZS, (Malvern Instrument Ltd., U.K.) equipped with a He−Ne laser beam operating at 633 nm at 25 °C. Samples were prepared in pure water and filtered through a 0.45 μm syringe filter prior to analysis, and each measurement was repeated six times to obtain the average value. Transmission electron microscopy (TEM) was conducted on a Hitachi H7000 microscope operating at 100 kV. A formvar coated 200-mesh Cu grid that was freshly glow discharged (Pelco easiGlow™, Ted Pella, Inc.) was placed onto a drop of sample solution for 30 sec and wicked off with filter paper. Uranyl acetate (0.5% aqueous solution) was used as a negative stain.

Synthesis. *N-(2-Hydroxypropyl)methacrylamide (HPMA)*. To a three-neck, 2 L round bottom flask equipped with a mechanical stirring device, thermometer, and addition funnel was added 1-amino-2-propanol (75 mL, 0.96 mol). The reagent was dissolved in dichloromethane (1 L) and cooled to -5 °C in a salt-ice bath.

Methacryloyl chloride (46 mL, 0.47 mol) was added drop-wise *via* addition funnel. The reaction was stirred for 30 min at 0 °C then slowly warmed to room temperature and left to stir overnight. The reaction was filtered to remove 1-amino-2-propanol hydrochloride, and the filtrate was concentrated to 500 mL and placed in a -20 °C freezer overnight to crystallize the product. The resultant HPMA was isolated by filtration and recrystallized from acetone at -20 °C (52 g, 76% yield). ^{1}H NMR (300 MHz, D_2O, ppm) δ: 5.72 (1H, s, C*H*$_2$=C), 5.47 (1H, t, C*H*$_2$=C), 3.96 (1H, m, $CH_2CH(CH_3)OH$), 3.30 (2H, m, $CH_2CH(CH_3)OH$), 1.95 (3H, s, CH_2-$CH(CH_3)OH$), 1.19 (3H, d, $CH_2=CCH_3$). ^{13}C NMR (500 MHz, D_2O, ppm) δ: 172.05 (*C*=O), 139.01 ($CH_2=C$), 120.98 (CH_2=C), 66.20 (CH_3CHOH), 46.19 ($CH(OH)CH_2$), 19.38 ($CH_2=CCH_3$), 17.63 (CH_3CHOH).

HPMA-Methotrexate (HPMA-MTX).



Scheme S1 Synthesis of HPMA-MTX.

A 10 mL round bottom flask with magnetic stir bar was flame-dried and cooled to room temperature under N_2 flow. Methotrexate (202 mg, 0.444 mmol) was dissolved in anhydrous DMF (3 mL). DCC (110 mg, 0.54 mmol) was added with stirring, followed by DMAP (10.9 mg, 0.0892 mmol), and HPMA (317 mg, 2.22 mmol). The reaction was allowed to stir for 48 h at room temperature, at which time the white dicylohexylurea precipitate was removed by filtration. The resulting

clear solution was precipitated into ether, and the solids were isolated by filtration. The crude product was purified by preparative reverse-phase HPLC. The yellow fractions were collected, CH_3CN was removed by rotary evaporation, and the solution was lyophilized to isolate a yellow powder (73 mg, 23% yield). ^{1}H NMR (500 MHz, DMSO-d_6, ppm) δ: 9.28 (1H, s), 9.08 (1H, s), 8.72 (1H, s), 8.25 (1H, d), 8.01 (1H, t), 7.75 (2H, d), 6.82 (2H, d), 5.60 (1H, s), 5.29 (1H, t), 4.90 (3H, m), 4.36 (1H, m), 3.26 (3H, m), 3.14 (1H, m), 2.36 (2H, m), 2.08 (1H, m), 1.92 (1H, m), 3.81 (3H, s), 1.10 (3H, d). ESI-MS *m/z*; 602.2466 $[M + Na]^+$, calculated for $C_{27}H_{33}N_9O_6$, 602.2467. Analytical HPLC R_t = 4.3 min (96%).

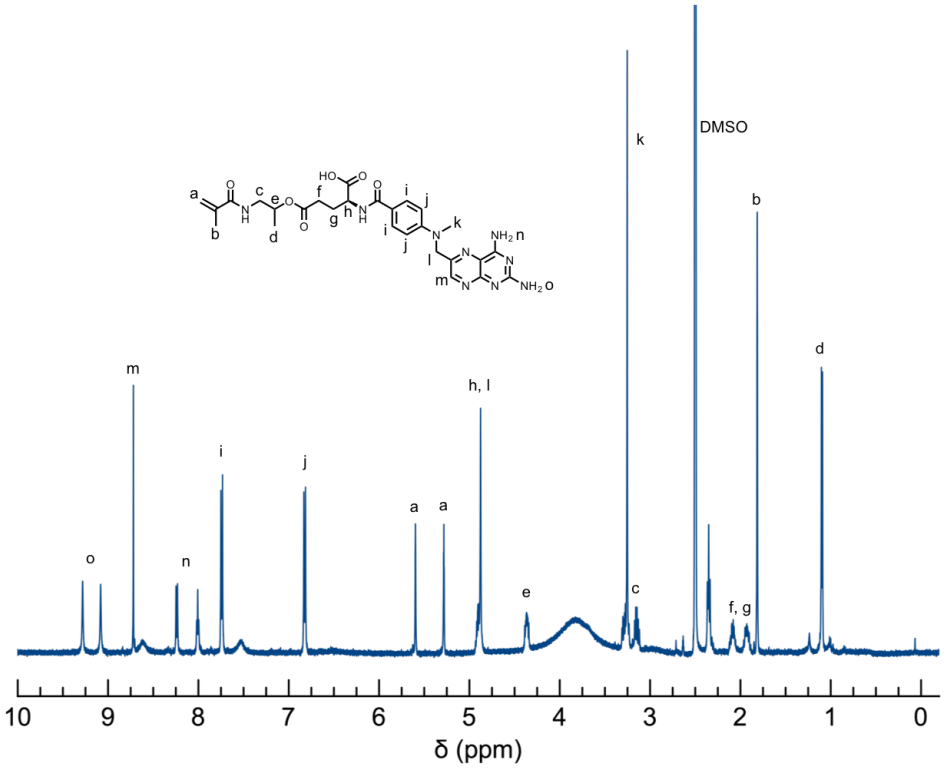


Figure S1. ^{1}H NMR spectrum and peak assignments for HPMA-MTX. (DMSO-*d*6, 500 MHz)

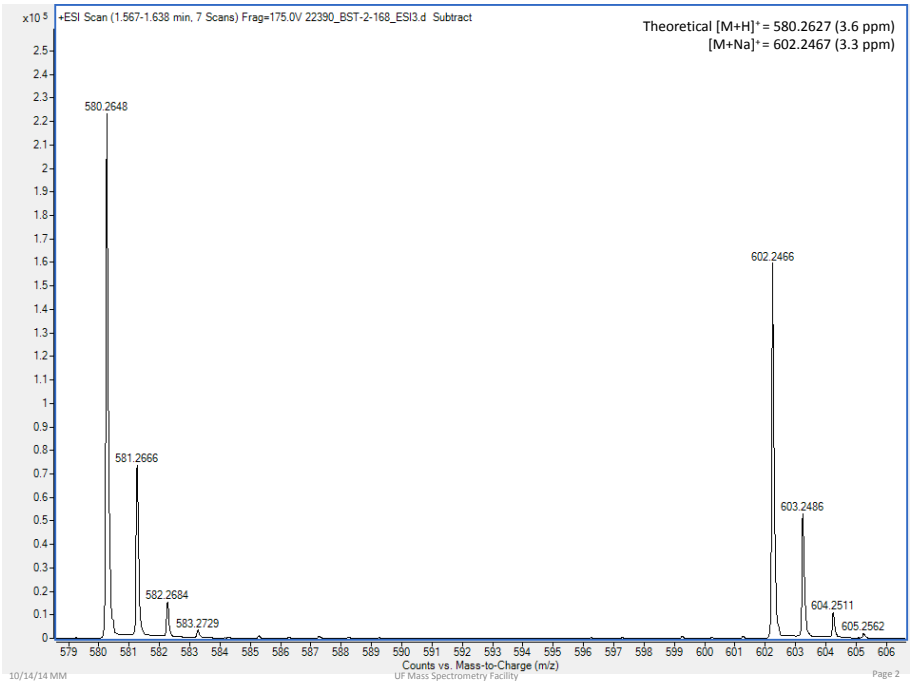


Figure S2. ESI-MS spectrum of HPMA-MTX

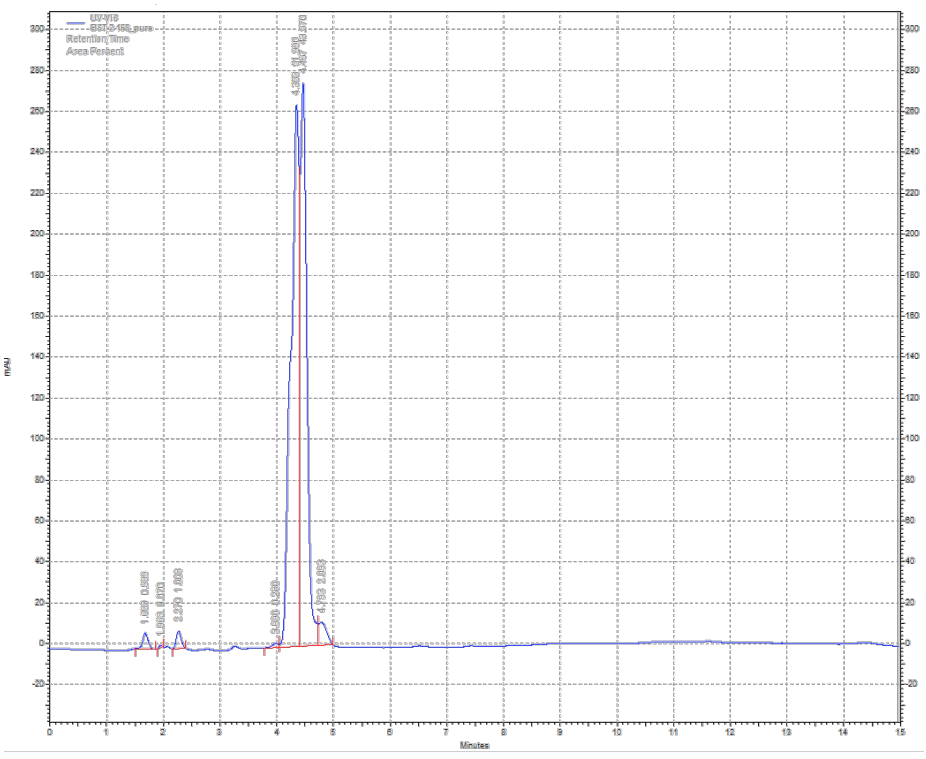


Figure S3. Analytical HPLC chromatogram of HPMA-MTX. R_t = 4.4 min, 96%.

*Poly(HPMA) (PHPMA) (**P1**)*. HPMA (3.00 g, 20.9 mmol), CDTPA (126 mg, 0.312 mmol), and AIBN (5 mg, 0.03 mmol) were added to a 20 mL scintillation vial equipped with a septum cap and magnetic stir bar ([M]:[CTA]:[I] = 67:1:0.1). The reagents were dissolved in DMAc (5 mL), purged with N_2 for 30 min, and added to a preheated heating block at 70 °C. The reaction was monitored by GPC-MALS, quenched after 3.5 h by exposing the contents to oxygen, and the polymer was purified by precipitation into cold diethyl ether (×3) and vacuum dried to yield a light yellow powder (43% conversion, $M_{n,\,GPC}$ = 6,260 g/mol, $Đ$ = 1.05).

*Synthesis of **P2.*** HPMA (15.0 g, 105 mmol), CDTPA (387 mg, 0.960 mmol), and AIBN (16.8 mg, 0.102 mmol) were added to a 50 mL Schlenk tube with a glass stopper and magnetic stir bar ([M]:[CTA]:[I] = 110:1:0.1). The reagents were dissolved in DMAc (30 mL), degassed with three freeze-pump-thaw cycles, backfilled with N_2, and added to a preheated oil bath at 70 °C. The reaction was monitored by GPC-MALS, quenched after 4 h by exposing the contents to oxygen, and the polymer was purified by precipitation into cold diethyl ether (×3) and vacuum dried to yield a light yellow powder (4.5 g, $M_{n,\,GPC}$ = 9,470 g/mol, $Đ$ = 1.05).

*Synthesis of **P3***. HPMA (2.20 g, 15.4 mmol), CDTPA (26.8 mg, 0.0664 mmol), and AIBN (1 mg, 6×10^{-3} mmol) were added to a 20 mL scintillation vial equipped with a septum cap and magnetic stir bar ([M]:[CTA]:[I] = 230:1:0.1). The reagents were dissolved in DMAc (3 mL), purged with N_2 for 30 min, and

added to a preheated heating block at 70 °C. The reaction was monitored by GPC-MALS, quenched after 4 h by exposing the contents to oxygen, and the polymer was purified by precipitation into cold diethyl ether (×3) and vacuum dried to yield a light yellow powder (1.24 g, 51% conversion, $M_{n,\,GPC}$ = 17,300 g/mol, $Đ$ = 1.24).

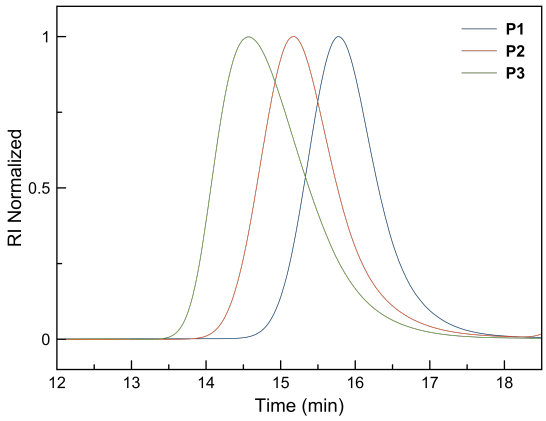


Figure S4. GPC traces of poly(*N*-(2-hydroxyproply)methacrylamide) macro-chain transfer agents of varying molecular weights using RAFT polymerization.

Synthesis of PHPMA CCS Polymers

Investigation of the effect of [crosslinker]:[unimer] ratio on star formation using EGDMA and a constant unimer MW. **P1** was used as the unimer while varying the [crosslinker]:[unimer] ratio (15:1; 10:1; and 5:1). The crude GPC chromatograms were deconvoluted using a Gaussian function in MagicPlot Pro software, and the star yield was calculated using Equation 1. The arm number, *f*, was calculated using the following equation:

$$f = \frac{M_{w,CCS} \times WF_{arms}}{M_{w,arms}} \qquad (2),$$

where WF_{arms} is the weight-fraction of the arms in the star and is given by:

$$WF_{arms} = \frac{(m_{unimer} \times p_{unimer})}{(m_{unimer} \times p_{unimer}) + (m_{CL} \times p_{CL})} \qquad (3),$$

where m_{unimer} is the mass of the unimer, p_{unimer} is the conversion of the unimer from the deconvoluted GPC chromatogram, m_{CL} is the mass of the crosslinker, and p_{CL} is the conversion of the crosslinker.

Synthesis of CCS with [EGDMA]:[unimer] = 15:1. **P1** (100. mg, 0.0160 mmol), EGDMA (47.7 mg, 0.241 mmol), and AIBN (0.3 mg, 2×10^{-3} mmol) were added to a 20 mL scintillation vial equipped with a septum cap and magnetic stir bar. The reagents were dissolved in DMAc (1 mL, [**P1**] = 100 mg/mL), purged with N_2 for 30 min, and added to a preheated heating block at 70 °C. The reaction was quenched after 24 h by exposing the contents to oxygen and analyzed by GPC-MALS (star yield = 70%, crude M_w = 1250 kg/mol, f = 100).

Synthesis of CCS with [EGDMA]:[unimer] = 10:1. **P1** (100. mg, 0.0160 mmol), EGDMA (31.7 mg, 0.160 mmol), and AIBN (0.3 mg, 2×10^{-3} mmol) were added to a 20 mL scintillation vial equipped with a septum cap and magnetic stir bar. The reagents were dissolved in DMAc (1 mL, [**P1**] = 100 mg/mL), purged with N_2 for 30 min, and added to a preheated heating block at 70 °C. The reaction was quenched after 24 h by exposing the contents to oxygen and analyzed by GPC-MALS (star yield = 60%, crude M_w = 256 kg/mol, f = 20).

Synthesis of CCS with [EGDMA]:[unimer] = 5:1. **P1** (100. mg, 0.0160 mmol), EGDMA (15.5 mg, 0.0783 mmol), and AIBN (0.3 mg, 2×10^{-3} mmol) were added to a 20 mL scintillation vial equipped with a septum cap and magnetic stir bar. The

reagents were dissolved in DMAc (1 mL, [**P1**] = 100 mg/mL), purged with N_2 for 30 min, and added to a preheated heating block at 70 °C. The reaction was quenched after 24 h by exposing the contents to oxygen and analyzed by GPC-MALS (star yield = 30%, crude M_w = 73.3 kg/mol, f = 10).

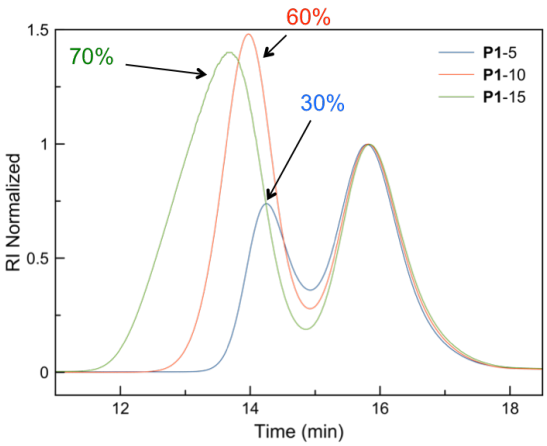


Figure S5. CCS polymers prepared using constant unimer M_n (**P1**) and varying [crosslinker]:[unimer] ratios (5:1; 10:1; 15:1, denoted as **P1**-5, **P2**-10, and **P1**-15, respectively, in the legend). The star yield of each reaction is given to show that increasing concentration of crosslinker resulted in an increase in the star yield.

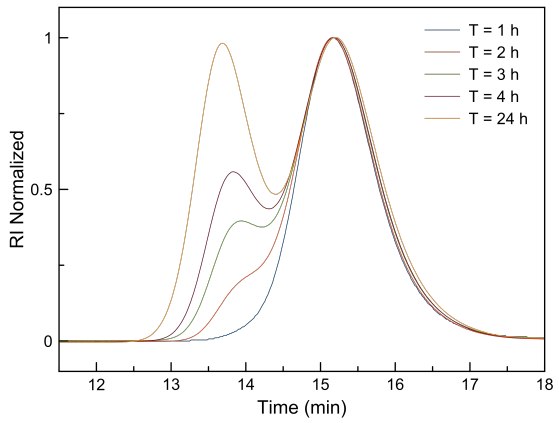


Figure S6. GPC chromatograms as a function of reaction time during CCS polymer synthesis in DMAc. ([crosslinker]:[unimer] = 10:1; unimer = **P2**).

Investigation of the effect of unimer M_n on CCS formation. The M_n of the PHPMA macroCTA was altered using **P1**, **P2**, or **P3** while holding the [crosslinker]:[unimer] ratio constant at 10:1.

*Synthesis of CCS with **P1***. The star formed using **P1** and a [crosslinker]:[unimer] ratio of 10:1 was described above, and that sample was used here in the comparison of varying unimer M_n.

*Synthesis of CCS with **P2** (**CCS1**)*. **P2** (100. mg, 0.0106 mmol), EGDMA (21.2 mg, 0.106 mmol), and AIBN (0.2 mg, 1×10^{-3} mmol) were added to a 20 mL scintillation vial equipped with a septum cap and magnetic stir bar. The reagents were dissolved in DMAc (1 mL, [**P2**] = 100 mg/mL), purged with N_2 for 30 min, and added to a preheated heating block at 70 °C. The reaction was quenched after 24 h by exposing the contents to oxygen and analyzed by GPC-MALS (star yield = 50%, crude M_w = 211 kg/mol, f = 10).

*Synthesis of CCS with **P3***. **P3** (100. mg, 5.78×10^{-3} mmol), EGDMA (11.8 mg, 5.95×10^{-2} mmol), and AIBN (0.1 mg, 6×10^{-4} mmol) were added to a 20 mL scintillation vial equipped with a septum cap and magnetic stir bar. The reagents were dissolved in DMAc (1 mL, [**P3**] = 100 mg/mL), purged with N_2 for 30 min, and added to a preheated heating block at 70 °C. The reaction was quenched after 24 h by exposing the contents to oxygen and analyzed by GPC (star yield = 10%, crude M_w = 287 kg/mol, f = 10).

Synthesis of PHPMA CCS using heterogeneous polymerizations

*Synthesis of **CCS2***. **P2** (100. mg, 0.0106 mmol), EGDMA (20.5 mg, 0.106 mmol), and ACVA (0.3 mg, 1×10^{-3} mmol) were added to a 20 mL scintillation vial equipped with a septum cap and magnetic stir bar. The reagents were dissolved in EtOH/water (1/1 v/v) (1 mL, [**P2**] = 100 mg/mL), purged with N_2 for 30 min, and added to a preheated heating block at 70 °C. The reaction was quenched after 4 h by exposing the contents to oxygen and analyzed by GPC-MALS (star yield = 70%, crude M_w = 553 kg/mol, f = 40).

*Synthesis of **CCS3***. **P2** (100. mg, 0.0106 mmol), EGDMA (20.4 mg, 0.103 mmol), and ACVA (0.3 mg, 1×10^{-3} mmol) were added to a 20 mL scintillation vial equipped with a septum cap and magnetic stir bar. The reagents were dissolved in water (1 mL, [**P2**] = 100 mg/mL), purged with N_2 for 30 min, and added to a preheated heating block at 70 °C. The reaction was quenched after 4 h by exposing the contents to oxygen and analyzed by GPC (star yield = 70%, crude M_w = 1280 kg/mol, f = 100).

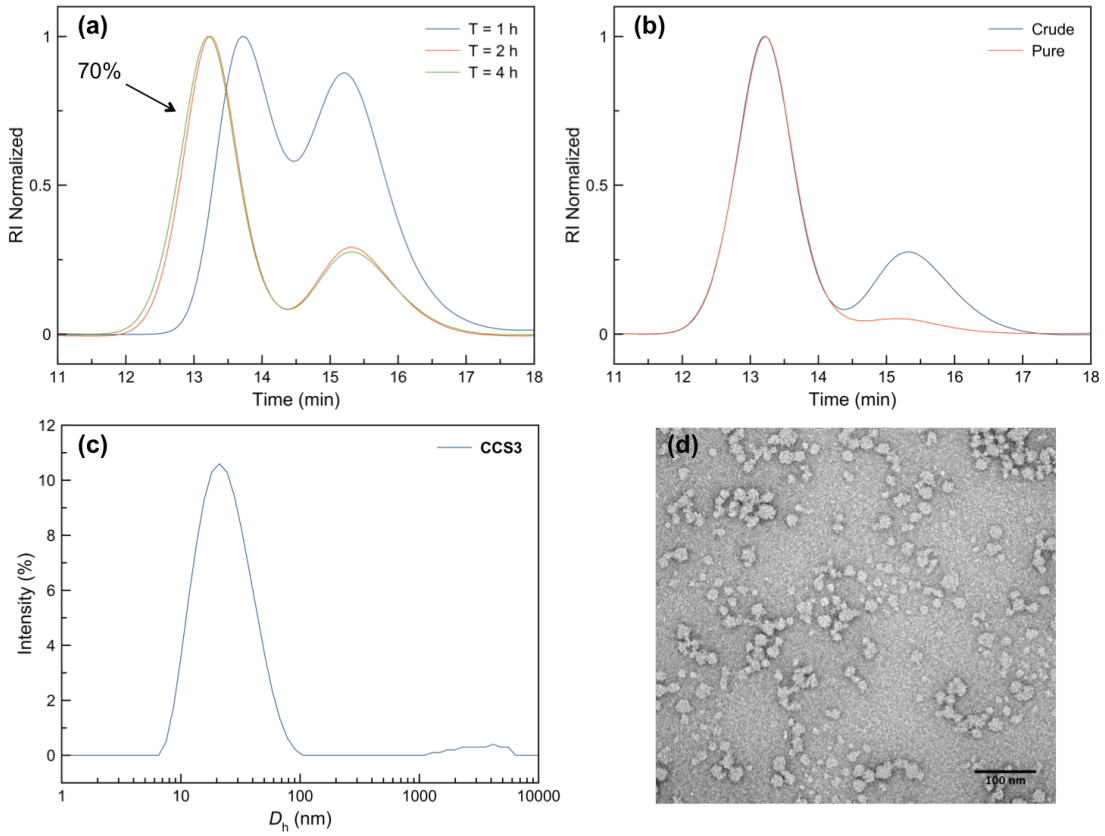


Figure S7. (a) GPC chromatograms as a function of reaction time during the synthesis of **CCS3** in pure H_2O. (b) GPC chromatograms of **CCS3** before and after purification by ultrafiltration. (c) DLS histogram of **CCS3** in pure water. (d) TEM image of **CCS3** (0.5% uranyl acetate stain; scale bar = 100 nm).

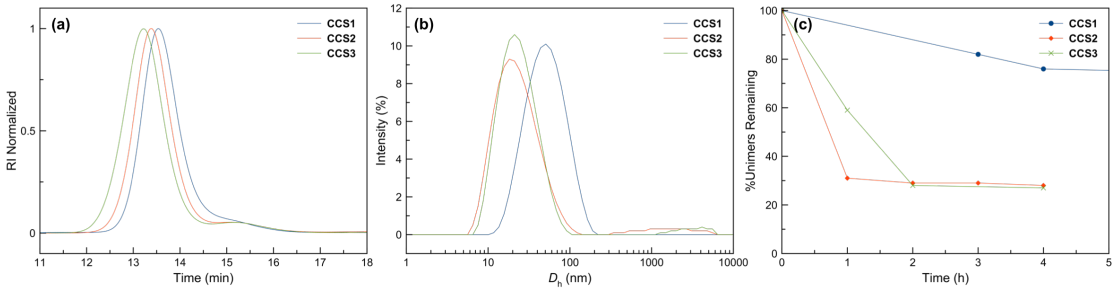
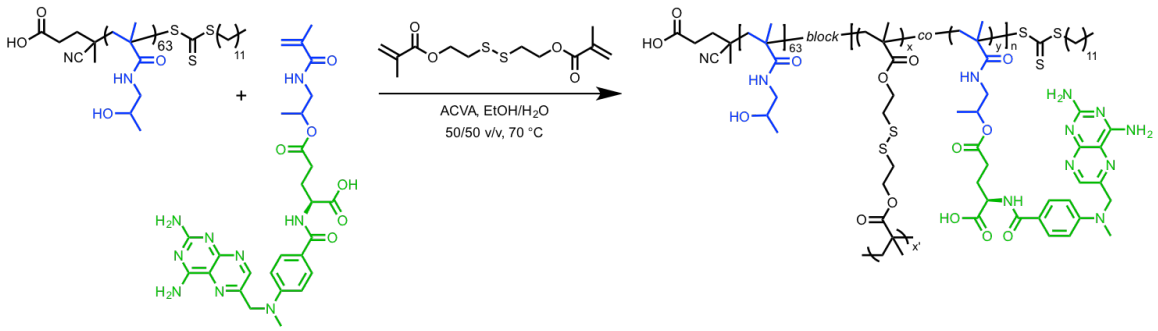


Figure S8. (a) GPC chromatograms of each purified CCS polymer prepared from reactions in DMAc, EtOH/H_2O, and pure H_2O. (b) DLS histograms in pure water of each purified CCS polymer. (c) Plot of %unimers remaining in the reaction as a function of reaction time for each CCS polymer prepared in varying solvents.

Synthesis of ***CCS4***. **P2** (100. mg, 0.0106 mmol), EGDMA (20.8 mg, 0.105 mmol), HPMA-MTX (61.4 mg, 0.106 mmol), and ACVA (0.3 mg, 1×10^{-3} mmol) were added to a 20 mL scintillation vial equipped with a septum cap and magnetic stir bar. The reagents were dissolved in EtOH/water (1/1 v/v) (1 mL, [**P2**] = 100 mg/mL), purged with N_2 for 30 min, and added to a preheated heating block at 70 °C. The reaction was quenched after 3 h by exposing the contents to oxygen and analyzed by GPC (55% conversion HPMA-MTX, star yield = 60%, M_w = 151 kg/mol, f = 10).

Synthesis of ***CCS5***. **P2** (25.4 mg, 0.00268 mmol), DSDMA (8.1 mg, 0.028 mmol), HPMA-MTX (15.3 mg, 0.0264 mmol), and ACVA (0.074 mg, 2.6×10^{-4} mmol) were added to a NMR tube equipped with a septum cap and magnetic stir bar. The reagents were dissolved in EtOH/water (1/1 v/v) (250 µL, [**P2**] = 100 mg/mL), purged with N_2 for 15 min, and added to a preheated oil bath at 70 °C. The reaction was quenched after 8 h by exposing the contents to oxygen, and the products were analyzed by GPC (star yield = 70%, M_w = 728 kg/mol, f = 30).



Scheme S2. Synthesis of **CCS5**

Table S1. Reaction conditions and molecular weight and size results during preparation of **CCS5**.

Entry	[crosslinker]:[HPMA-MTX]:[unimer]	CCS M_w (kg mol^{-1})	Star yield (%)	f	D_h (nm)	MTX wt%
CCS5	10:10:1	728	70	30	10	15

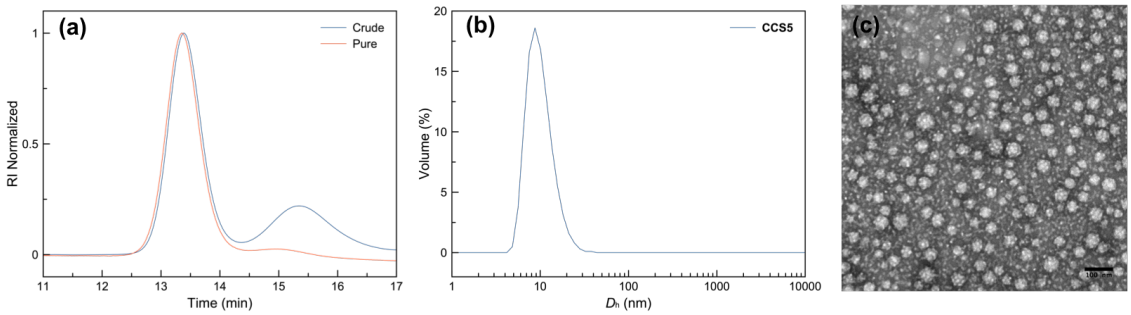


Figure S9 (a) Crude and purified GPC chromatograms, (b) DLS histogram in pure water (1 mg/mL), and (c) TEM image (0.5% uranyl acetate; scale bar = 100 nm) of **CCS5**.

Purification of CCS Polymers

Fractional precipitation. **CCS1** was purified using fractional precipitation to remove unimers and low molecular weight star polymers. The crude reaction mixture (0.5 mL) was transferred to a 1.5 mL microcentrifuge tube. Cold diethyl ether was added drop-wise with intermittent mixing until a turbid solution persisted. The tube was then centrifuged 5 min at 4,000 rpm to collect precipitated stars. The ether was removed, and the pellet was dissolved in DMAc (0.5 mL). The fractional precipitation was repeated twice more, and the purification was confirmed by GPC-MALS.

Ultrafiltration. The PHPMA CCS polymers synthesized in aqueous media were purified *via* ultrafiltration using centrifugal filter units with a 50 kDa MWCO

membrane. The crude reaction was washed with water (0.5 mL, ×5), and the purified stars were dried by lyophilization to yield a faint yellow powder for **CCS2** and **CCS3**, and a bright yellow-orange powder for **CCS4**.

In vitro drug release experiments

Drug cleavage from monomer. HPMA-MTX (1.3 mg, 2.7 × 10^{-3} mmol) was dissolved in DMSO (140 μL) to make a stock solution. To a microcentrifuge tube containing PBS (520 μL) was added the HPMA-MTX stock solution (60 μL) and PLE (40 μL, 150 U). A control experiment was used in which the HPMA-MTX stock solution (60 μL) was added to PBS (540 μL) in the absence of PLE. The reactions were left at room temperature, and aliquots (50 μL) were removed at predetermined time intervals, diluted with 3:1 aq. TFA (0.1%):CH_3CN (100 μL), and monitored by HPLC. Released MTX was determined by integrating the area of the peak at R_t = 2.1 min and using a standard curve of MTX to determine the concentration.

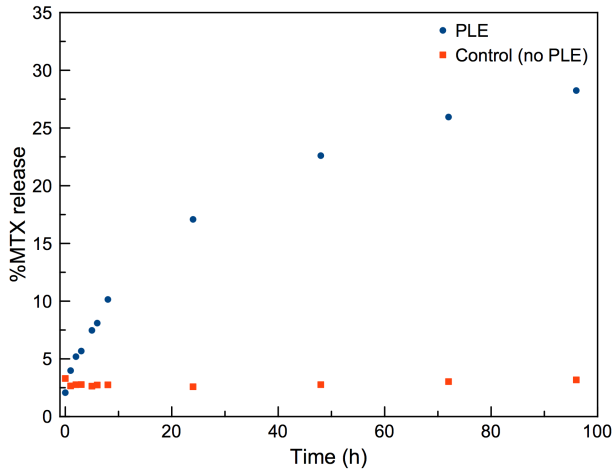


Figure S10. Percent of MTX released from HPMA-MTX with pig liver esterase (blue, 150 U) and without (orange).

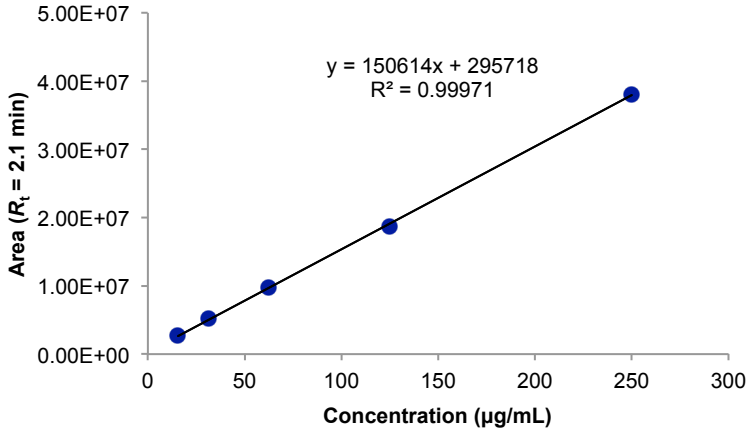


Figure S11. Calibration curve of MTX using the area under the curve at R_t = 2.1 min in the HPLC chromatogram.

*Drug cleavage from **CCS4***. **CCS4** (1.3 mg) was added to a microcentrifuge tube and dissolved in PBS (500 µL), and PLE (5 µL, 20 U) was added. A control experiment was used in which **CCS4** (1.3 mg) was added to a microcentrifuge tube and dissolved in PBS (500 µL) in the absence of PLE. The reactions were left at room temperature, and aliquots (10 µL) were removed at predetermined time intervals, diluted with 3:1 aq. TFA (0.1%):CH_3CN (90 µL), and monitored by HPLC. After 24 h with no drug release, PLE (30 µL, 120 U) was added to the reaction again, and left at room temperature for 24 h. However, no drug release was observed after 24 h.

*Drug cleavage from **CCS5***. **CCS5** (5.4 mg) was added to a 4 mL vial and dissolved in DMF (0.5 mL), and the solution was purged with N_2 for 10 min. Tri-*n*-butylphosphine (0.1 mL, 0.4 mmol) was then added, and the reaction was left to

stir at room temperature overnight. The reaction was dialyzed against water using a 3,500 Da MWCO membrane, and the polymer was isolated by lyophilisation. The recovered material (1.2 mg) was dissolved in PBS (360 μL), and PLE (40 μL, 150 U/mg) was added. The reaction was left at room temperature and monitored by HPLC by removing 40 μL of the reaction and diluting with 40 μL HPLC eluent (3:1 aq. TFA (0.1%):CH_3CN). No apparent drug release was observed after 24 h.

References

1. G. Moad, Y.K. Chong, Y. K., A. Postma, E. Rizzardo, and S. H. Thang, *Polymer,* 2005, **46**, 8458–8468.

2. K. Wang, H. Peng, K. J. Thurecht, S. Puttick, A. K. Whittaker, *Polym. Chem.*, 2014, **5**, 1760-1771.