Supplementary Material (ESI) for Chemical Communications

Supporting Information for:

Differences in Cytotoxicity of Poly(PEGA)s Synthesized by Reversible Addition–Fragmentation Chain Transfer Polymerization

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Experimental Section

Materials.

All materials were purchased from either Sigma-Aldrich or Fisher Scientific and were used as received unless otherwise indicated. 2,2'-Azobisisobutyronitrile (AIBN) was recrystallized twice from ethanol before use. 4-(3-hydroxy-propyl)-10-oxa-4-aza-tricyclo[5,2,1,$0^{2,6}$] dec-8-ene-3,5-dione,[1] 2-(ethyl trithiocarbonate)propionic acid,[2] ethyl-2-(phenylcarbonothioylthio)propanoate[3], and 1-phenylethyl dithiobenzoate, and furan-protected maleimide-poly(PEGA) (**2**)[4] were synthesized as previously reported. PEGA (M_n ~ 454, ≥ 99%) was purchased from Sigma-Aldrich. Merck 60 (230-400 mesh) silica gel was used for normal phase chromatography. MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was purchased from Research Products International (RPI) Corporation. NIH 3T3 mouse fibroblast cell line was purchased from ATCC. Spectra/Por Dialysis Membrane was purchase from Spectrum Laboratory, Inc. (Rancho Dominguez, CA). DI water was generated in house using a Milli-Q system. Cells were maintained in 10% Calf serum (Colorado Serum Corp., Denver, Colorado) in

DMEM (Invitrogen). Alumina (Al_2O_3) resin, neutral Act I, 50-200 μm was purchased from Sorbent Technologies.

Analytical Techniques.

^1H and ^{13}C NMR spectra were acquired on an ARX 400 MHz or 500 MHz NMR spectrometer, and spectra were processed using Topspin 1.2 NMR software. Infrared absorption spectra were recorded using a PerkinElmer FT-IR equipped with an ATR accessory. TLC plates pre-coated with silica gel 60 F254 were developed in the indicated solvent systems. Size exclusion chromatography (GPC) was conducted on a Shimadzu HPLC system equipped with a refractive index detector RID-10A, one Polymer Laboratories PLgel guard column, and two Polymer Laboratories PLgel 5 μm mixed D columns. Lithium bromide (LiBr) (0.1 M) in dimethylformamide (DMF) at 40 °C was used as the mobile phase (flow rate: 0.80 mL/min). Calibration was performed using near-monodisperse poly(methyl methacrylate) standards from Polymer Laboratories. Because of its size, the M_n of polymer **2** could not be determined accurately by ^1H NMR; thus, the value provided in Table 1 was obtained by GPC with Laser Light Scattering Detection.[4] Chromatograms were processed using the EZStart 7.2 chromatography software. Mass spectra were obtained by GC-MS on an Agilent 6890-5975 GC-MS with Autosampler.

Synthesis of benzyl 2-(ethylthiocarbonothioylthio)propanoate

2-(Ethylthiocarbonothioylthio)propanoic acid (4.2 g, 20 mmol), dicyclohexyl carbodiimide (DCC) (5.15 g, 25 mmol) and dimethylaminopyridine (DMAP) (0.24 g, 2

mmol) were dissolved in anhydrous THF (30 mL), and the solution was cooled to 0 ^{o}C. Benzyl alcohol (2.5 g, 23 mmol) was added to the reaction mixture drop wise. The reaction mixture was allowed to stir for 2 h in an ice bath. The reaction mixture was filtered and then purified via silica column chromatography (1 : 40 ethyl acetate : hexanes). δ ^{1}H NMR (400 MHz, $CDCl_3$) (Figure S1a): 7.40-7.28 (5H, m, C_6H_5), 5.18 (2H, s, OC**H**$_2$C$_6$H$_5$), 4.87 (1H, q, J = 7.3 Hz, SC**H**CH$_3$), 3.36 (2H, q, J = 7.4 Hz, CH$_3$C**H**$_2$S), 1.61 (3H, d, J = 7.3 Hz, SCHC**H**$_3$), 1.35 (3H, t, J = 7.5 Hz, C**H**$_3$CH$_2$S). δ ^{13}C NMR (500 MHz, $CDCl_3$) (Figure S1b): 221.80, 171.21, 135.56, 128.65, 128.44, 128.22, 67.54, 47.98, 31.61, 16.90, 13.06. GC-MS expected (found) ($M + K^+$): 339.11 (338.93). IR: 3032, 2928, 1735, 1497, 1452, 1376, 1303, 1237, 1153, 1080, 877, 718, 748, 697 cm^{-1}.

*Synthesis of trithiomonobenzyl-poly(PEGA) (**1**) by RAFT polymerization*.

PEGA (2.27 g, 4.95 mmol), benzyl 2-(ethylthiocarbonothioylthio)propanoate (30 mg, 0.10 mmol) and AIBN (1.6 mg, 0.0097 mmol) ([Monomer] : [CTA] : [AIBN] = 50 : 1 : 0.1) were loaded into a Schlenk tube along with 5 mL of DMF. The tube was sealed and subjected to three freeze-pump-thaw cycles. The polymerization was then initiated by immersing the Schlenk tube in a 70 °C oil bath. The polymerization was stopped at 68% conversion. The polymer was purified by dialysis against MeOH (molecular weight cut-off, MWCO 6-8,000 Da). GPC (RI) was used to determine the number-average molecular weight (M_n) and the PDI. δ ^{1}H NMR (400 MHz, $CDCl_3$) (Figure S2): 7.37-7.27 (5H, m, C_6H_5, end group), 5.13-5.01 (2H, m, C$_6$H$_5$C**H**$_2$O, end group), 4.15 (CH_2OCO, polymer), 3.84-3.42 (CH_2CH_2O, polymer), 3.36 (OCH_3, polymer), 2.29 (C**H**CH$_2$, polymer), 1.96-

1.37 (CHC**H**$_2$, polymer), 1.33 (3H, t, J = 7.42, SCH$_2$C**H**$_3$, end group), 1.14-1.12 (3H, m, CHC**H**$_3$, end group).

*Synthesis of dithio-monobenzyl-poly(PEGA) (**3**) by RAFT polymerization*

PEGA (2.37 g, 5.17 mmol), ethyl-2-(phenylcarbonothioylthio)propanoate (29.4 mg, 0.116 mmol) and AIBN (3.8 mg, 0.023 mmol) ([Monomer] : [CTA] : [AIBN] = 45 : 1 : 0.2) were loaded into a Schlenk tube along with 5 mL of DMF. The tube was sealed and subjected to three freeze-pump-thaw cycles. The polymerization was then initiated by immersion the Schlenk tube in an 80 °C oil bath. The polymerization was stopped by exposure to air after 20 h (90 % conversion). The polymer was purified by dialysis against MeOH : ethyl acetate (1 : 1) (molecular weight cut-off, MWCO 6-8,000 Da). GPC (RI) was used to determine the number-average molecular weight (M_n) and the PDI. δ ^{1}H NMR (400 MHz, CD_3CN) (Figure S3): 7.98 (2H, d, J = 9.1 Hz, *o*-C_6H_5, end group), 7.65 (1H, t, J = 7.5 Hz *p*-C_6H_5, end group), 7.48 (2H, t, J = 7.3 Hz, *m*-C_6H_5, end group), 4.15 (CH_2OCO, polymer), 3.76-3.34 (CH_2CH_2O, polymer), 3.30 (OCH_3, polymer), 2.32 (C**H**CH_2, polymer), 1.98-1.37 (CHC**H**$_2$, polymer), 1.21 (3H, t, J = 7.1 Hz, OCH$_2$C**H**$_3$, end group), 1.14-1.12 (3H, m, CHC**H**$_3$, end group).

*Synthesis of Dithiodibenzyl-poly(PEGA) (**4**) by RAFT polymerization*

PEGA (1.8 g, 3.97 mmol), 1-phenylethyl benzodithioate CTA (20.5 mg, 0.079 mmol) and AIBN (2.6 mg, 0.016 mmol) ([Monomer] : [CTA] : [AIBN] = 50 : 1 : 0.2) were loaded into a Schlenk tube along with 5 mL of DMF. The tube was sealed and subjected to three freeze-pump-thaw cycles. The polymerization was then initiated by

immersion the Schlenk tube in a 70 °C oil bath. The polymerization was stopped by exposure to air after 20 h (67 % conversion). The polymer was purified by dialysis against MeOH : ethyl acetate (1 : 1) (molecular weight cut-off, MWCO 6-8,000 Da). GPC (RI) was used to determine the number-average molecular weight (M_n) and the PDI. δ ^{1}H NMR (400 MHz, CD_3CN) (Figure S4): 7.98 (2H, d, J = 7.9 Hz, *o*-C_6H_5, end group), 7.65 (1H, t, J = 7.4 Hz *p*-C_6H_5, end group), 7.48 (2H, t, J = 7.7 Hz, *m*-C_6H_5, end group), 7.33-7.19 (5H, m, C_6H_5, end group), 4.15 (CH_2OCO, polymer), 3.76-3.35 (CH_2CH_2O, polymer), 3.29 (OCH_3, polymer), 2.31 ($CHCH_2$, polymer), 1.98-1.37 ($CHCH_2$, polymer), 1.14-1.12 (3H, m, $CHCH_3$, end group).

*Aminolysis of **3** and **4** (**3a** and **4a**)*

Polymer **3** (317 mg, 0.0184 mmol) was dissolved with 1 mL THF in a 5 ml glass vial with a stir bar. Butylamine (27 mg, 0.370 mmol) was added to the polymer solution. The reaction was allowed to proceed for 1.5 h at 24 ^{o}C; **3a** was purified by dialysis against MeOH : ethyl acetate (1 : 1) (molecular weight cut-off, MWCO 6-8,000 Da). The same aminolysis procedure was used for **4** (84 mg, 0.0044 mmol) with butylamine (6.4 mg, 0.088 mmol). The resulting polymers **3a** and **3b** were analyzed by ^{1}H NMR spectroscopy. δ ^{1}H NMR (400 MHz, CD_3CN) of polymer **3a**: 4.15 (CH_2OCO, polymer), 3.77-3.30 (CH_2CH_2O, polymer), 3.30 (OCH_3, polymer), 2.32 ($CHCH_2$, polymer), 1.98-1.37 ($CHCH_2$, polymer), 1.21 (3H, t, J = 7.1 Hz, OCH_2CH_3, end group), 1.13-1.07 (3H, m, $CHCH_3$, end group). δ ^{1}H NMR (400 MHz, CD_3CN) of **polymer 4a**: 7.33-7.19 (5H, m, C_6H_5, end group), 4.15 (CH_2OCO, polymer), 3.76-3.34 (CH_2CH_2O, polymer), 3.29 (OCH_3, polymer), 2.31 ($CHCH_2$, polymer), 1.98-1.37 ($CHCH_2$, polymer), 1.14-1.12 (3H,

m, CHC**H**$_3$, end group). Ellman's assay was employed to quantify the percent free thiol using a literature procedure:[5] **3a** 99.5%; **4a** 87.8%

*End- capping of **3a** and **4a** (**3b** and **4b**)*

Polymer **3a** (170 mg, 0.01 mmol) was dissolved in 2 mL PBS (pH 7.4) containing 10 mM TCEP at 24 ^{o}C. R-(+)-N-(-phenyl ethyl) maleimide (10 mg, 0.0494 mmol) was added to the polymer solution. The reaction was allowed to proceed for 18 h at 24 ^{o}C; **3b** was purified by dialysis against MeOH : ethyl acetate (1 : 1) (molecular weight cut-off, MWCO 6-8,000 Da). The same procedure was used for **4a** (54 mg, 0.0028 mmol) and R-(+)-N-(-phenyl ethyl) maleimide (2.8 mg, 0.014 mmol). The resulting polymers (**3b** and **4b**) were analyzed by ^{1}H NMR spectroscopy. δ ^{1}H NMR (400 MHz, CD_3CN) of polymer **3b**: 7.40-7.24 (5H, m, C_6H_5, end group), 4.15 (CH_2OCO, polymer), 3.77-3.33 (CH_2CH_2O, polymer), 3.29 (OCH_3, polymer), 2.32 (C**H**CH_2, polymer), 1.98-1.37 (CHC**H**$_2$, polymer), 1.21 (3H, t, J = 7.1 Hz, OCH_2C**H**$_3$, end group), 1.13-1.07 (3H, m, CHC**H**$_3$, end group). δ ^{1}H NMR (400 MHz, CD_3CN) of **polymer 4b**: 7.42-7.19 (10H, m, C_6H_5, end group), 4.15 (CH_2OCO, polymer), 3.77-3.34 (CH_2CH_2O, polymer), 3.29 (OCH_3, polymer), 2.31 (C**H**CH_2, polymer), 1.98-1.37 (CHC**H**$_2$, polymer), 1.14-1.12 (3H, m, CHC**H**$_3$, end group). Calculation of the end capping efficiency for **3b** was carried out as follows. First, the integration of the CH_2OCO polymer peak at δ 4.15 was compared to average integration of dithiobenzoate peaks (δ 7.98, 7.65, 7.48) of **3**. After end-capping, integration of the phenyl peaks from the maleimide cap (δ 7.40-7.24) were compared to the same reference peak (δ 4.15). Finally, the integration of dithiobenzoate peaks (δ 7.98, 7.65, 7.48) was compared to the calibrated integration

of phenyl peaks (δ 7.40-7.24) to obtain conjugation efficiency. The PEG side chain peak at δ 4.15 was compared to the peak corresponding the methyl of the α chain end (δ 1.07) to make sure that no hydrolysis of the backbone PEGs had occurred during the aminolysis reaction. A similar approach was taken for polymer **4b**, and in this case the overlapping integration of the phenyl moiety from the α chain end was taken in account in the calculations.

Synthesis of benzyl *2-bromo-2-methylpropanoate.*

Benzyl alcohol (1.296 g, 12 mmol) and triethylamine (TEA) (1.416 g, 14 mmol) were dissolved in anhydrous THF (50 mL), and the solution was cooled to 0 ^{o}C. 2-Bromoisobutyryl bromide (2.299 g, 10 mmol) was added dropwise to the reaction mixture. The reaction mixture was allowed to stir for 2 h in an ice bath. The reaction mixture was filtered and then purified via silica column chromatography (1 : 6 ethyl acetate : hexanes). δ ^{1}H NMR (400 MHz, $CDCl_3$) (Figure S5a): 7.41-7.31 (5H, m, C_6H_5), 5.22 (2H, s, O**CH_2**C_6H_5), 1.96 (6H, s, CH_3). δ ^{13}C NMR (500 MHz, $CDCl_3$) (Figure S5b): 171.51, 135.48, 128.65, 128.40, 127.95, 67.62, 55.76, 30.84. GC-MS expected (found): 256.01 (256.0). IR: 3066, 3034, 3005, 2976, 2932, 1732, 1498, 1455, 1389, 1371, 1270, 1212, 1153, 1105, 1029, 1010, 966, 904, 818, 734, 695 cm^{-1}.

Synthesis of poly(PEGA) *(**5**) by ATRP.*

PEGA (4.4 g, 9.6 mmol), benzyl 2-bromo-2-methylpropanoate (49.7 mg, 0.191 mmol), Me_6TREN (43.7 mg, 0.190 mmol) and CuCl (18.8 mg, 0.190 mmol) ([Monomer] : [Initiator] : [Me_6TREN] : [CuCl] = 50 : 1 : 1 : 1) were loaded into a Schlenk tube along

with 6 mL of anisole. The tube was sealed and subjected to three freeze-pump-thaw cycles. The polymerization was then initiated by immersion of the Schlenk tube in a 60 °C oil bath. After 5 h, the polymerization was halted by bubbling with air. Toluene (4 mL) was added to the reaction mixture and the solution flowed through an alumina plug. The solvent was removed *in vacuo*. Poly(PEGA) was precipitated into cold ether four times to produce **5a**. An additional dialysis step was performed to remove trace amount of monomer. The polymer was dialyzed against DI H_2O (molecular weight cut-off, MWCO 6-8,000 Da) to produce **5b**. GPC (RI) was used to determine the number-average molecular weight (M_n) and the PDI. δ ^{1}H NMR (400 MHz, $CDCl_3$) (Figure S6): 7.37-7.29 (5H, m, C_6H_5, end group), 5.07 (2H, s, $C_6H_5C\mathbf{H}_2O$, end group), 4.16 (CH_2OCO, polymer), 3.83-3.41 (CH_2CH_2O, polymer), 3.38 (OCH_3, polymer), 2.29 (C**H**CH_2, polymer), 1.98-1.35 (CHC$\mathbf{H}_2$ polymer), 1.25, 1.16, 1.14 (6H, m, CC$\mathbf{H}_3$, end group).

Inductively coupled plasma mass spectrometry (ICP-MS)

Copper concentrations in polymers synthesized by ATRP were measured by ICP-MS (in the Molecular Instrumentation Facility at UCLA). The samples were prepared by adding 100% v/v nitric acid for 12 h to solubilize the chemicals. The samples were then heated at 90 °C for 4 h until no precipitate was visible and samples were diluted to 2 mL. Internal standards were then added and samples were analyzed for copper content.

Cell Proliferation Study (MTT Assay)

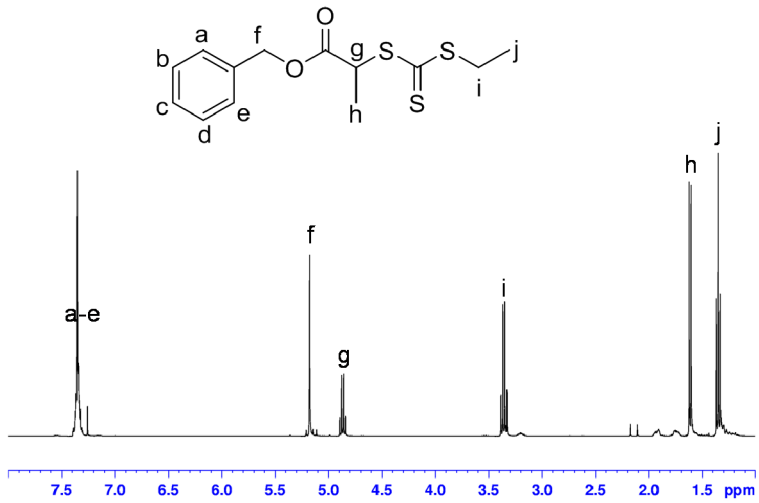
NIH 3T3 fibroblasts were seeded in 48-well plates at a density of 1~2 x 10^4 cells/well and incubated at 37 °C/5% CO_2 for 24 h. Designated polymer concentrations

(0.1, 1 and 10 mg/mL) were prepared in 10% calf serum/DMEM by serial dilution. After removing the culture media, 150 µL of each polymer solution was added to each well. For each incubation time (24 and 48 h), all polymer solutions were carefully removed and 150 µL of 10% FBS/DMEM was added to each well. Subsequently, 7.5 µl of MTT reagent (5 mg/mL in PBS) was added. After 4 h incubation at 37 °C/5% CO_2, the solution was removed from each well. The insoluble formazan salt was dissolved with 100 µL of dimethyl sulfoxide (DMSO) and 80 µL of each solution was carefully transferred to a 96 well plate. The optical density was measured using a microplate reader with detection at 570 nm. The control group consisted of cells with no polymer treatment, meaning solution with only 10% calf serum/DMEM, and five replicates were used for each polymer concentration and time point. The effect of polymer treatment on NIH 3T3 proliferation was calculated by dividing the corrected absorbance of sample (OD_{570}(sample) – OD_{570}(control)) by the absorbance of control (OD_{570}(control)):

$$\text{Cell proliferation rate}\,(\%) = \frac{OD570(\text{sample}) - OD570(\text{control})}{OD570(\text{control})}$$

The statistic significance was evaluated by one-way ANOVA. $p < 0.05$ was considered as a significant difference among the groups compared

a)



b)

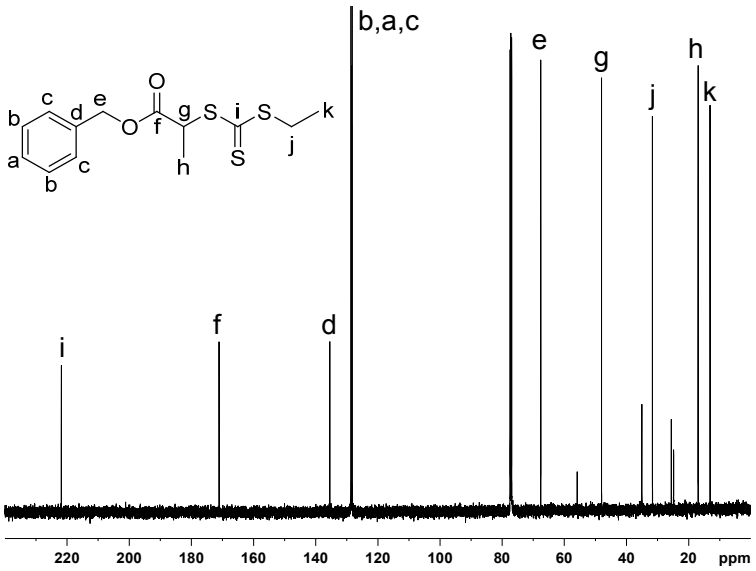


Figure S1. a) ^{1}H NMR spectrum ($CDCl_3$) and b) ^{13}C NMR of benzyl 2-(ethylthiocarbonothioylthio)propanoate.

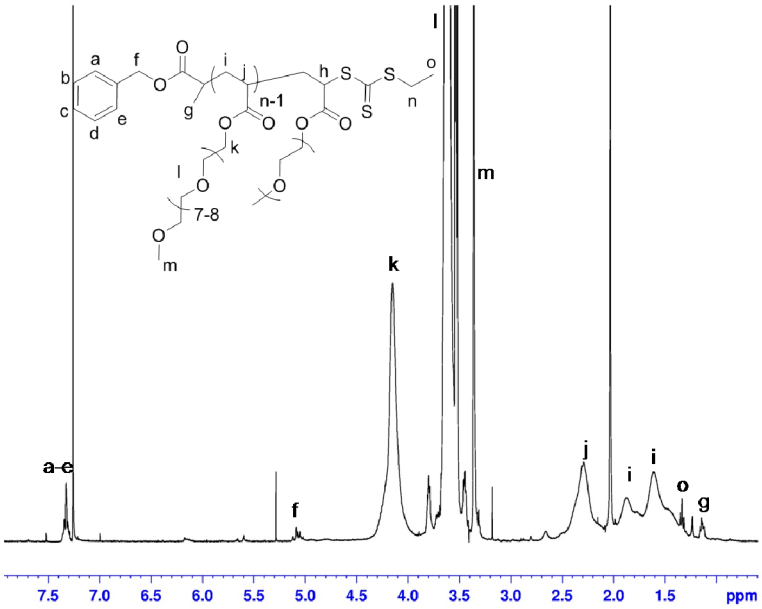


Figure S2. ^{1}H NMR spectrum ($CDCl_3$) of poly(PEGA) (**1**) synthesized with benzyl 2-(ethylthiocarbonothioylthio)propanoate by RAFT polymerization.

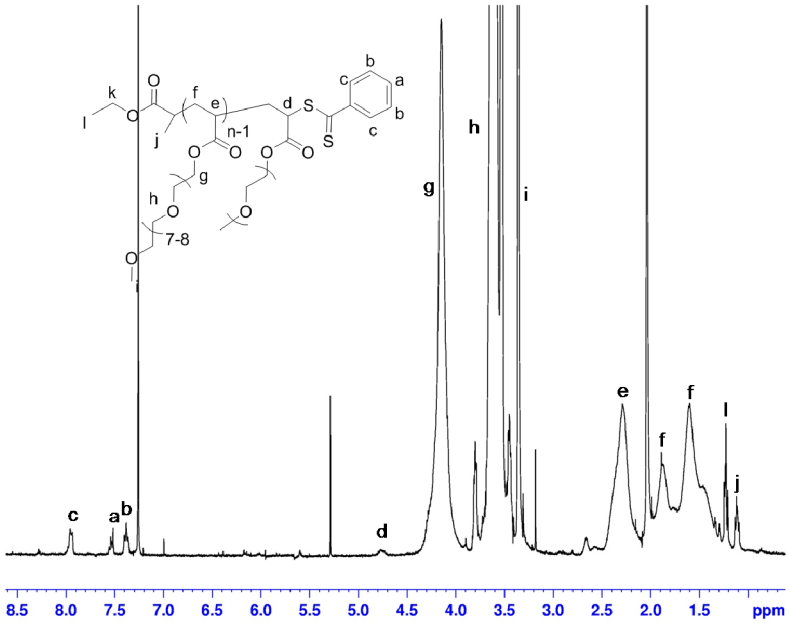


Figure S3. ^{1}H NMR spectrum ($CDCl_3$) of poly(PEGA) (**3**) synthesized with ethyl 2-(phenylcarbonothioylthio)propanoate by RAFT polymerization.

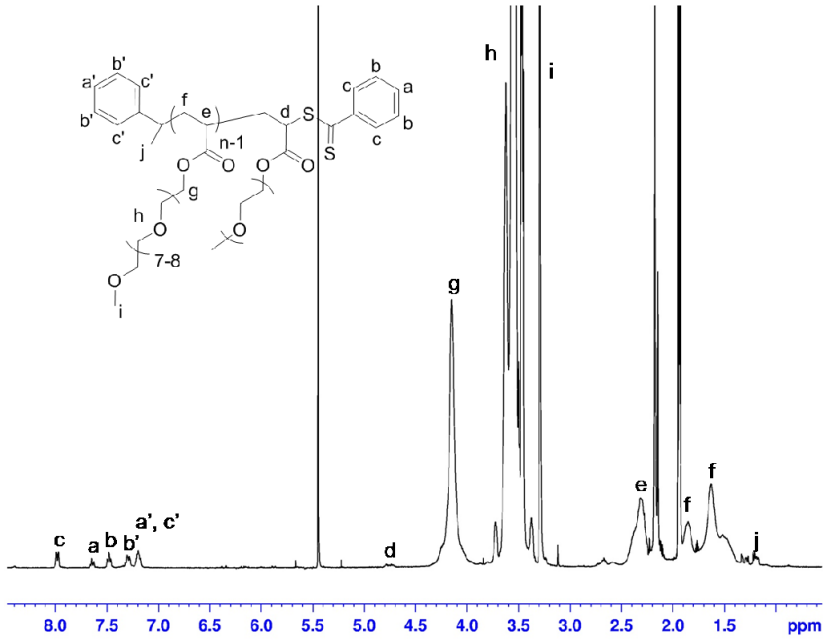
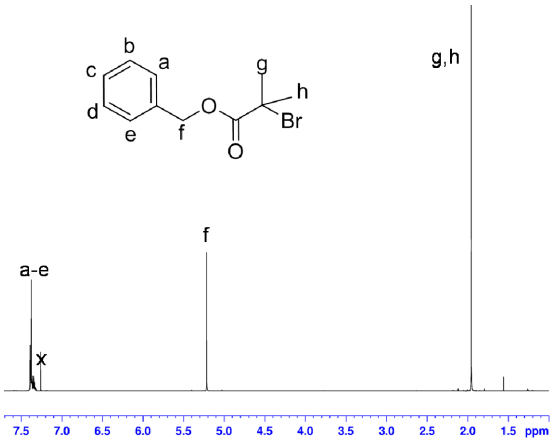


Figure S4. ^{1}H NMR spectrum (CD_3CN) of poly(PEGA) (**4**) synthesized with 1-phenylethyl benzodithioate by RAFT polymerization.

a)



b)

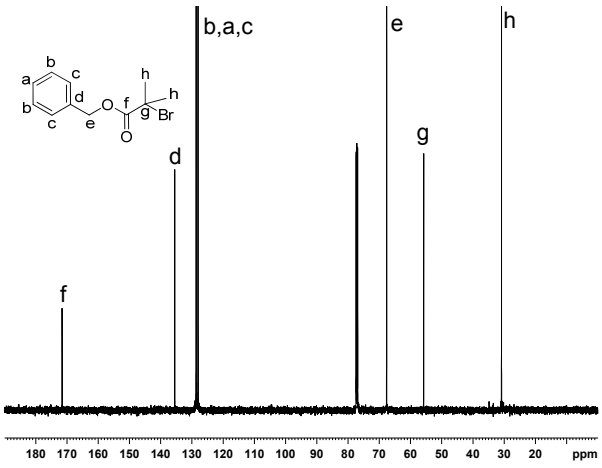


Figure S5. a) ^{1}H NMR spectrum ($CDCl_3$) and b) ^{13}C NMR of benzyl 2-bromo-2-methylpropanoate.

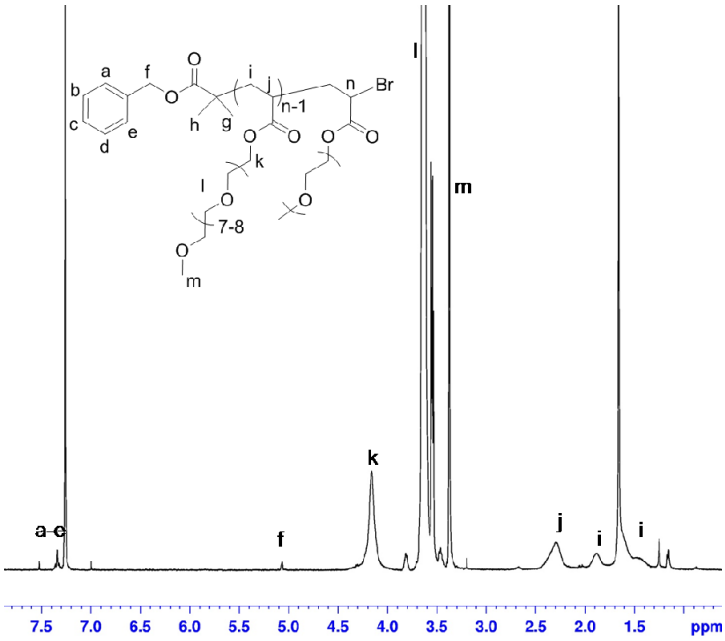


Figure S6. ^{1}H NMR spectrum ($CDCl_3$) of poly(PEGA) **5b** synthesized with benzyl 2-bromo-2-methylpropanoate by ATRP.

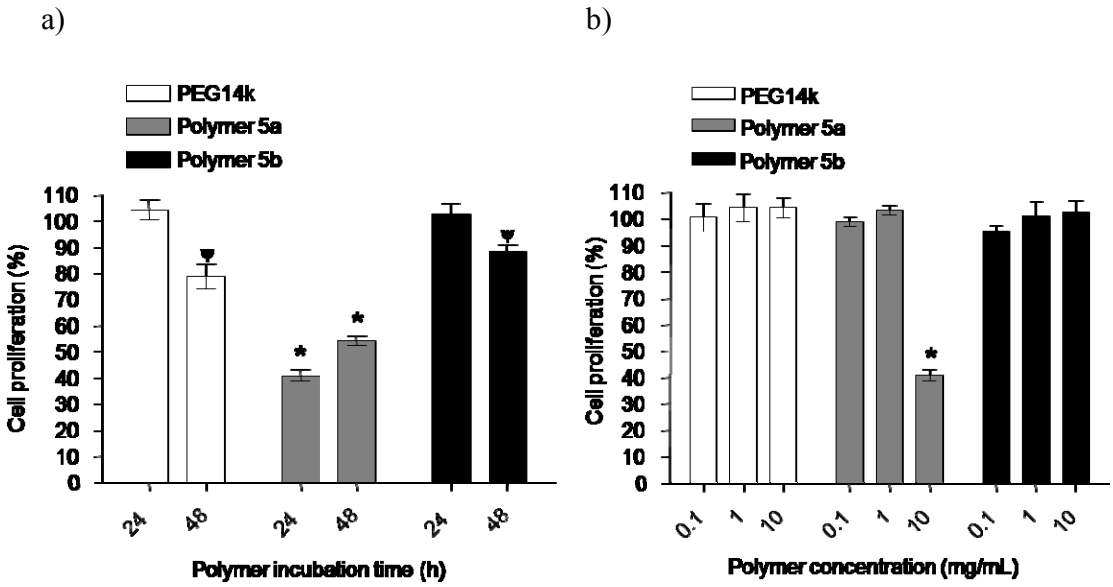


Figure S7. Cytotoxicity of poly(PEGA)s synthesized by ATRP and purified by neutral aluminum chromotography (**5a**) or by dialysis (**5b**). Data represents mean ± S.E.M. (n = 6). a) Cell proliferation rate of NIH 3T3 cells after 24 and 48 h exposure to 10 mg/ml of polymers. Significance indicated by: *$p<0.05$ versus cells exposed to PEG14k or polymer **5b**. $^{\Psi}p<0.05$ versus cells after 24 h exposure. b) Cell proliferation rate of NIH 3T3 cells after 24 h exposure to different polymer concentrations (0.1, 1 and 10 mg/mL). Significance indicated by: *$p<0.05$ versus cells exposed to polymer **5b** or PEG14k.

References

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