SUPPORTING INFORMATION FOR:

Injectable and Tunable Poly(ethylene glycol) Analogue Hydrogels Based on Poly(oligoethylene glycol methacrylate)

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Experimental

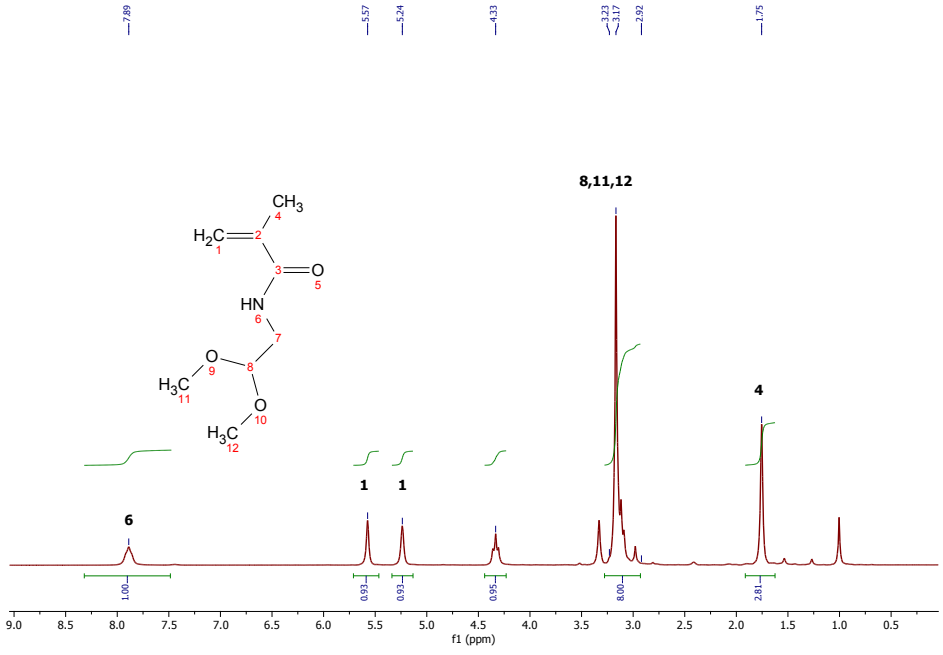
Materials: Oligo(ethylene glycol) methyl ether methacrylate with an average number-average molecular weight of 475 g/mol ($OEGMA_{475}$, Sigma Aldrich, 95%) was purified by passing it over a column of basic aluminum oxide (Sigma Aldrich, type CG-20) to remove the methyl ether hydroquinone (MEHQ) and butylated hydroxytoluene (BHT) inhibitors. Acrylic acid (AA, Sigma Aldrich, 99%), thioglycolic acid (TGA, Sigma Aldrich, 98%) and 2,2-azobisisobutryic acid dimethyl ester (AIBMe, Wako Chemicals, 98.5%), adipic acid dihydrazyde (ADH, Alfa Aesar, 98%), *N*'-ethyl-*N*-(3-dimethylaminopropyl)-carbodiimide (EDC, Carbosynth, Compton CA, commercial grade), sodium cyanoborohydride ($NaBH_3CN$, Sigma Aldrich, reagent grade), aminoacetaldehyde dimethyl acetal (Sigma Aldrich, 99%), 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO, Sigma Aldrich, 98%), methacryloyl chloride (Sigma Aldrich, purum), bovine serum albumin (BSA, Sigma Aldrich, >96%), fibrinogen from human plasma (Sigma Aldrich), Arg-Gly-Asp (RGD, Sigma Aldrich, ≥97%)and fluorescein isothiocyanate (FITC, Sigma Aldrich, 90%) were all used as received. For all experiments Milli-Q grade distilled deionized water (DIW) was used. Dimethyl sulfoxide (DMSO, reagent grade) was purchased from Caledon Laboratory Chemicals (Georgetown, ON). Hydrochloric acid (1M) was received from LabChem

Inc. (Pittsburgh, PA). 3T3mouse fibroblasts were obtained from ATCC: Cederlane Laboratories (Burlington, ON). Cell proliferation media, recovery media, and trypsin-EDTA were obtained from Invitrogen (Burlington, ON). Media contents included Dulbecco's Modified Eagle Medium-high glucose (DMEM), fetal bovine serum (FBS), penicillin streptomycin (PS), and trypsin-EDTA and were purchased from Invitrogen Canada (Burlington, ON). Thiazolyl blue tetrazolium bromide (MTT) was purchased from Sigma Aldrich (Oakville, ON). LIVE/DEAD assay for cell viability were purchased from Invitrogen Canada (Burlington).

Chemical Characterization: Aqueous size exclusion chromatography (SEC) was performed on a system consisting of a Waters 515 HPLC pump, Waters 717 plus autosampler, three Ultrahydrogel columns (30 cm × 7.8 mm i.d.; exclusion limits: 0–3 kDa, 0–50 kDa, 2–300 kDa) and a Waters 2414 refractive index detector. A mobile phase consisting of 0.3 M sodium nitrate and 0.05 M phosphate buffer (pH 7) at a flow rate of 0.8 mL/min was used for all polymers analyzed, and the system was calibrated with narrow-dispersed poly(ethylene glycol) standards ranging from 106 to 584×10^3 g/mol (Waters). ^{1}H-NMR was performed on a Bruker AVANCE 600 MHz spectrometer using deuterated chloroform as the solvent. The acrylic acid content of the polymers was determined using base-into-acid conductometric titration (ManTech Associates) using 50 mg of polymer dissolved in 50 mL of 1 mM NaCl as the analysis sample and 0.1 M NaOH as the titrant.

Synthesis of N-(2,2-dimethoxyethyl)methacrylamide (DMEMAm): The *N*-(2,2-dimethoxyethyl)methacrylamide (DMEMAm) monomer was synthesized by adding aminoacetaldehyde dimethylacetal (50 mL, 461 mmol) and 100 mg of TEMPO to a concentrated

sodium hydroxide solution (100 mL) at 10 °C. Methacryloyl chloride (47.08 mL, 486 mmol) was then added drop-wise over a period of 2 hours under nitrogen, and the resulting mixture was allowed to react for 24 hours under nitrogen at room temperature. Subsequently, the mixture was extracted with 150 mL of petroleum ether to remove impurities. The aqueous phase was then saturated with sodium chloride and extracted three times with 75 mL tert-butyl methyl ether. The organic phase was dried with magnesium sulfate, filtered, and concentrated under reduced pressure, yielding an orange oil as the final product. This product was stored in the dark at 10 °C until use. Purity (determined from ^{1}H-NMR) = >99%. ^{1}H-NMR (DMSO, 600 MHz): δ = 1.75 (s, 3H, -CH3), δ = 2.92 – 3.23 (m, 8H, O-CH3 and –N(H)-CH2), δ = 4.33 (t, 1H, -CH), δ = 5.24 (s, 1H, =CH2) , δ = 5.57 (s, 1H, =CH2) , δ = 7.89 (s, 1H, -NH).



Synthesis of the hydrazide-functionalized precursor ($PO_{100}H_y$): $PO_{100}H_y$ precursors were prepared by adding AIBMe (37 mg, 0.14 mmol), $OEGMA_{475}$ (4.0 g, 8.4 mmol), AA (0.25 g, 3.5 mmol, for $PO_{100}H_{30}$), and TGA (1 µL, 0.02 mmol) to a 50 mL Schlenk flask. Dioxane (20 mL)

was added and the solution was purged with nitrogen for at least 30 minutes. Subsequently, the flask was sealed and submerged in a pre-heated oil bath at 75°C for 4 hours under magnetic stirring. After the solvent was removed, the resulting poly(OEGMA-co-AA) polymer was purified by dialysis against DIW for a minimum of 6 (6+ hour) cycles and lyophilized to dryness. The carboxylic acid groups of POH precursor were subsequently converted to hydrazide groups via a carbodiimide-mediated conjugation of a large excess of adipic acid dihydrazide. The polymer (3.8 g) was dissolved in 100 mL DIW and added to a 250 mL round-bottom flask. ADH (2.65 g, 15.2 mmol, 5 mol eq.) was added and the pH of the solution adjusted to pH = 4.75 using 0.1 M HCl. Subsequently, EDC (1.18 g, 7.6 mmol, 2.5 mol eq.) was added and the pH maintained at pH = 4.75 by the dropwise addition of 0.1 M HCl over 4 hours. The solution was left to stir overnight, dialyzed against DIW for a minimum of 6 (6+ hour) cycles, and lyophilized. The degree of functionalization was determined from conductometric base-into-acid titration. The polymers were stored as 20 w/w% solutions in PBS at 4°C.

Synthesis of the aldehyde-functionalized precursor ($PO_{100}A_y$): $PO_{100}A_y$ precursors were prepared by adding AIBMe (60 mg, 0.26 mmol), $OEGMA_{475}$ (4.0 g, 8.4 mmol), DMEMAm (0.60 g, 3.5 mmol, for $PO_{100}A_{30}$) and TGA (1 μL, 0.02 mmol) to a 50 mL Schlenk flask. Dioxane (20 mL) was added and the solution was purged with nitrogen for at least 30 minutes. Subsequently, the flask was sealed and submerged in a pre-heated oil bath at 75°C for 4 hours under magnetic stirring. After polymerization, the solvent was removed and the poly(OEGMA-co-DMEMAm) polymer was purified by dialysis against DIW for a minimum of 6 (6+ hour) cycles and lyophilized to dryness. The acetal groups of $PO_{10}A$ were subsequently converted to aldehydes by dissolving 3.5 g of the copolymer prepared above in 75 mL DIW and 25 mL 1.0 M

HCl in a 250 mL round-bottom flask. The solution was left to stir for 24 hours, dialyzed for a minimum of 6 (6+ hour) cycles and lyophilized to dryness. The polymer was stored at 20 w/w% in PBS at 4°C.

Synthesis of RGD labelled POA: RGD labelled POA precursor ($PO_{100}A_{30}$-RGD) was prepared by incubating a solution of $PO_{100}A_{30}$ (0.6 g) and RGD (10 mg, 28.9 μmol) in 50 mL distilled deionized water for 24 hours under continuous agitation. Subsequently, sodium cyanoborohydride (18.2 mg, 0.29 mmol, 10 mol eq. to RGD) was added and the solution was stirred for another 48 hours. The solution was dialyzed for a minimum of 6 (6+ hour) cycles and lyophilized to dryness. The polymer was stored as 20 w/w% solution in PBS at 4°C.

Synthesis of fluorescein-isothiocyanate labelled proteins: Fluorescein-isothiocyanate (FITC)-labelled bovine serum albumin (BSA-FITC) and fibrinogen (Fib-FITC) were prepared by dissolving 50 mg of the protein in a 100 mL carbonate buffer at pH = 9.0. FITC (1 mg) was added and the solution was incubated at room temperature for at least 12 h under gentile mechanical agitation. The FITC-labelled protein was subsequently dialyzed against distilled deionized water 6 (6+ hour) cycles and lyophilized to dryness. The isolated conjugated protein was stored at -4°C in the dark. For both proteins, a calibration curve was prepared to relate their concentration in PBS to the fluorescence signal measured at λ = 495nm and 535nm, with linear calibration curves (R^2>0.99) observed in the concentration range of 2 to 10 μg/mL and 10 to 100 μg/mL respectively for BSA and fibrinogen.

Preparation of injectable hydrogels: POEGMA hydrogels were prepared via co-extrusion of

hydrazide-functionalized (POH) and aldehyde-functionalized (POA) precursors dissolved in 10 mM PBS. Intensive mechanical mixing of both polymer precursor solutions was achieved through the use of a double barrel syringe fitted with a static mixer at the outlet (Medmix Systems). Hydrogel disks for all *in vitro* testing were prepared by extrusion of the reactive polymer precursors through the double barrel syringe into cylindrical silicone rubber molds (diameter = 7 mm, volume = 300 μL) and incubated at room temperature for at least 12 hours to ensure complete gelation prior to testing.

Swelling kinetics: Swelling of POEGMA hydrogels was determined at 22°C in 10 mM PBS at pH 7.4. Hydrogels (n = 4) were placed into cell culture inserts that are then placed in a 12-well cell culture plate and completely submerged with PBS (4 mL/well). At predetermined time intervals, the cell culture inserts were removed from the well, the PBS drained, and the hydrogel gently dried to wick off non absorbed PBS prior to weighing of the hydrogel. Subsequently, the hydrogels were resubmerged in a fresh 4 mL of PBS solution and tested repeatedly until equilibrium swelling was reached (generally ~30 hours). Error bars represent the standard deviation of the replicate measurements. The mass-based swell ratio (Q_m) was calculated by dividing the mass of the hydrogel at any given time point (m_h) by the dry mass of polymer in the hydrogel (m_p = initial hydrogel mass × (1 – water content)). In the legend of Fig. 1, "initial and equilibrium swelling" are defined as the mass-based swell ratio upon preparation of the hydrogels (initial) and after incubation in PBS for 30 hours (equilibrium).

Degradation kinetics: Degradation of POEGMA hydrogels was determined at 37°C in 100 mM HCl at pH 1.0; these acid-catalyzed conditions were used to compare the degradation properties

of the hydrogels on a more measurable time frame. Hydrogels (n = 4) were placed into cell culture inserts that are subsequently placed in a 12-well cell culture plate and completely submerged with the HCl solution (4 mL per well). At predetermined time intervals, the cell culture inserts were removed from the well, the PBS drained and the hydrogel gently dried to wick off non absorbed solution prior to weighing of the hydrogel. Subsequently, the hydrogels were resubmerged in fresh HCl solution (4 mL/well) until the hydrogel was completely degraded (i.e. no separate phase was observed between the hydrogel and the HCl bath solution). Error bars represent the standard deviation of the replicate measurements.

Hydrogel rheology: The rheological properties of the hydrogels were measured using an ARES rheometer (TA Instruments) operating under parallel-plate geometry with a plate diameter of 7 mm and a plate spacing of 1 mm. Rheological properties were measured by first conducting a strain sweep from 0.1−100% strain at 1 Hz to identify the linear viscoelastic range of the hydrogels. A strain was then selected from within this linear range and set as a constant to perform a frequency sweep from 1 to 100 rad/s to measure shear elastic (G′) and loss (G″) moduli. All measurements were conducted at 22 °C and in triplicate, with error bars representing the standard deviation of the replicate measurements.

Cytotoxicity assay: The cytocompatibility of POH and POA precursors (n = 4) was quantified using a MTT assay. NIH 3T3 fibroblasts were maintained in tissue culture flasks in DMEM supplemented with 10% FBS and 1% penicillin. Cytotoxicity of the linear polymers (at concentrations ranging from 200 to 2000 μg/mL) was evaluated using an MTT assay over a 1-day exposure time. NIH 3T3 fibroblasts were plated at density of 1.0×10^4 cells per well in a 24-

well plate and maintained in DMEM media supplemented with 10% FBS and 1% penicillin. Cell viability was then characterized by removing the polymer solution, adding the MTT solution, and incubating over four hours. The absorbance of the MTT solution was read using a Biorad microplate reader (model 550) at 570 nm, normalized against a 630 nm baseline, and compared to that measured in cell-only wells in which no materials were added to estimate relative cell viability. Each experiment (hydrogels as well as controls) were done in quadruplicate, with reported errors representing the standard deviation of the replicates.

In vitro protein adsorption assay: Protein absorption to the POEGMA hydrogels was assayed in 96 well plates. POH and POA polymer solutions (150 mg/mL) were sterilized and 60 μL of each precursor solution was extruded into each well and left overnight to ensure complete gelation. Once gelation was complete, 60μL of 10 mM PBS was added to each well and hydrogels were allowed to swell to equilibrium prior to protein addition (over 30 hours). Unabsorbed PBS was then removed, and 60μL of either BSA-FITC or Fib-FITC solution (125, 250, 500, 1000 and 2000 μg/mL) was added. The hydrogels were incubated for 2 hours at 37°C. After 2 hours, the hydrogels were rinsed to remove unadsorbed protein and the fluorescence signal was measured using a VICTOR 3 multi-label microplate reader and compared to the stock solution controls. Each experiment (hydrogels as well as controls) were done in quadruplicate, with reported errors representing the standard deviation of the replicates.

In vitro cell adhesion assay: Cell adhesion to the POEGMA hydrogels and RGD-functionalized POEGMA hydrogels was assayed in 48-well plates using 3T3 fibroblasts as a model cell line. Hydrogels were directly extruded into each well, with 100 μL of each sterilized polymer

precursor solution (150 mg/mL in 10 mM PBS) added and then left overnight to ensure complete gelation. Gels were then washed with DMEM culture media prior to cell addition. Cells were plated on top of the hydrogels at a density of 2.0×10^4 cells per well together with 400 µL of DMEM and incubated for 24 hours at 37ºC. After incubation, a LIVE/DEAD assay was conducted to visualize cells using microscopy and quantify adhesion. After staining, each well was washed three times with sterile 10 mM PBS to remove any non-adherent cells from the gels. Once washed, the resulting fluorescence of the cells on the gels was quantified using a VICTOR 3 multi-label microplate reader and compared to the cell-only TCPS control. All experiments were conducted in quadruplicate and multiple images (minimum 10) were taken per well for analysis, with error ranges reported representing the standard deviation associated with the cell counts in the replicate measurements. Cell morphology on the hydrogels was visualized using a Zeiss Axiovert 200M fluorescence/live cell imaging microscope.

In vivo tolerability assay: The *in vivo* toxicity of the POEGMA hydrogels was assessed using a mouse subcutaneous injection model. A total of four BALB/c mice (22-24 g, Charles River Laboratories) were injected with 0.35 mL samples of a 150 mg/mL hydrogel precursor (30 mol% reactive groups) using a double-barrel syringe. Four additional mice were injected with 0.15M NaCl to serve as controls for comparing the tissue response to the hydrogels. Animals were also visually observed to identify any systemic toxic response. Both treated and control animals were sacrificed after 3 days (acute response) and 1 month (chronic response) after injection. A tissue sample that includes skin, underlying tissue, and residual material was recovered from the animals and subjected to histological analysis using hematoxylin and eosin staining. Animals were cared for in compliance with protocols approved by the Animal Research Ethics Board at

McMaster University and regulations of the Animals for Research Act of the Province of Ontario and the guidelines of the Canadian Council on Animal Care.

Table S1. Chemical characterization of the various POEGMA precursors

	Functional Group [-]	Theoretical Functional Monomer [mol%] [a]	Actual $OEGMA_{475}$ [mol%]	Actual Functional Monomer [mol%][b]	M_n [$x10^3$ g·mol^{-1}][c]	$Đ$ [-][d]	Average # of Functional Groups/Chain
$PO_{100}H_{20}$	$NHNH_2$	20.0	81.8	18.2	16.9	2.66	8
$PO_{100}H_{25}$	$NHNH_2$	25.0	77.9	22.1	18.1	2.43	10
$PO_{100}H_{30}$	$NHNH_2$	30.0	72.8	27.2	19.4	2.35	16
$PO_{100}H_{40}$	$NHNH_2$	40.0	64.4	35.6	19.1	3.15	20
$PO_{100}A_{20}$	CHO	20.0	82.7	17.3	19.5	3.15	7
$PO_{100}A_{25}$	CHO	25.0	75.2	24.8	17.9	2.87	9
$PO_{100}A_{30}$	CHO	30.0	71.9	28.1	19.3	2.43	12
$PO_{100}A_{40}$	CHO	40.0	60.2	39.8	20.3	3.21	17

Nomenclature: PO_xH_y; x represents the mol fraction of $OEGMA_{475}$ of the OEGMA monomers used and y represents the theoretical mol fraction of functional monomer (hydrazide or aldehyde). [a] Theoretical degree of functionalization in mol%, [b] Experimental degree of functionalization as determined from conductometric base-into-acid titration for the hydrazide precursors or from ^{1}H-NMR for the aldehyde precursors, [c] Determined using aqueous GPC with a mobile phase consisting of 0.3 M sodium nitrate and 0.05 M phosphate buffer at pH 7, [d] Dispersity ($Đ$) as determined from aqueous GPC

Table S2. Gelation times of the various POEGMA hydrogels measured at 37°C.

Hydrazide precursor	Aldehyde precursor	Concentration [mg/mL]	Functionality [mol%]	Gelation time [min]
$PO_{100}H_{30}$	$PO_{100}A_{30}$	100	30	160
$PO_{100}H_{30}$	$PO_{100}A_{30}$	125	30	85
$PO_{100}H_{30}$	$PO_{100}A_{30}$	150	30	45
$PO_{100}H_{30}$	$PO_{100}A_{30}$	175	30	10
$PO_{100}H_{30}$	$PO_{100}A_{30}$	200	30	2
$PO_{100}H_{20}$	$PO_{100}A_{20}$	150	20	420
$PO_{100}H_{25}$	$PO_{100}A_{25}$	150	25	140
$PO_{100}H_{40}$	$PO_{100}A_{40}$	150	40	0.75

Table S3. Average 3T3 fibroblast cell counts per mm^2. $n = 6$ for the polystyrene control and POEGMA hydrogel and $n = 4$ for POEGMA-RGD hydrogel.

Days [-]	Control [1/mm^2]	POEGMA [1/mm^2]	POEGMA-RGD [1/mm^2]
1	2400 ± 130	6 ± 1	36 ± 1
5	2560 ± 127	0 ± 1	104 ± 25
7	2664 ± 100	0 ± 1	91 ± 10

Table S4. Summary of literature sources cited for the comparison of protein adsorption between POEGMA hydrogels and PEG-modified surfaces.

Reference	Protein	Loading Solution [μg/mL]	Incubation Time [hr]	Temperature [°C]	Adsorption [ng/cm^2]
11a	BSA	100	3	RT	Min. 360
11b	BSA	1500	0.33	37	< 3000
	Fib	1500	0.33	37	3800
11c	Fib	1, 100, 300, 500 and 1000	3	RT	Max. 200
11d	Fibronectin	20	1	37	Min. 50
11e	BSA	1000	2	37	1320
Our work	BSA	125,250,500,1000 and 2000	2	37	Max. 100
	Fib	125,250,500,1000 and 2000	2	37	Max. 460

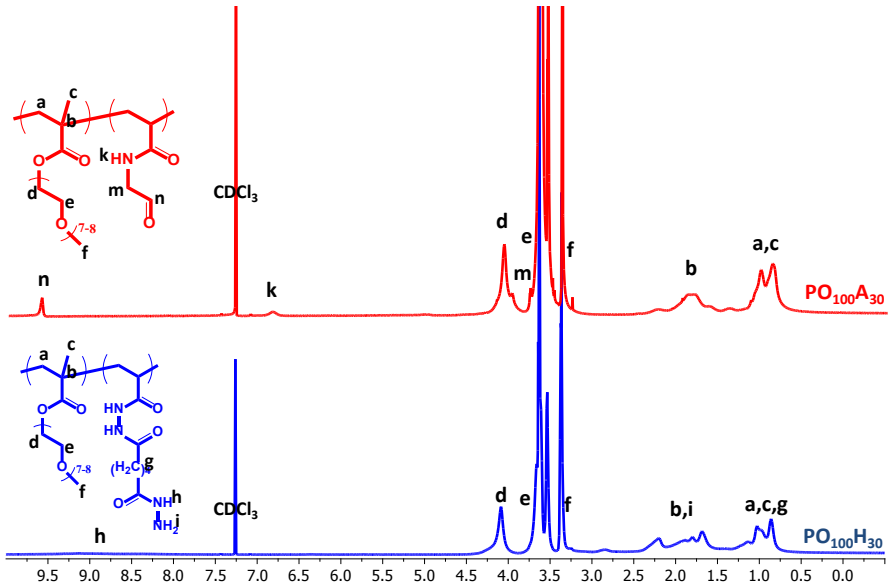


Figure S1 ^{1}H-NMR spectra of $PO_{100}H_{30}$ (blue, bottom) and $PO_{100}A_{30}$ (red, top).

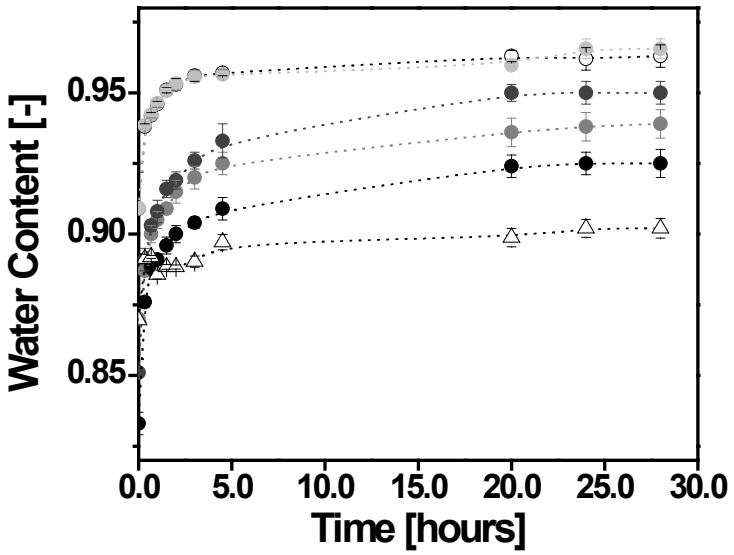


Figure S2. Swelling kinetics of the POEGMA hydrogels prepared at different precursor concentrations and precursor degrees of functionalization. (○) 100 mg/mL, 30 mol%, (●) 125 mg/mL, 30 mol%, (●) 150 mg/mL, 30 mol%, (●) 175 mg/mL, 30 mol%, (●) 200 mg/mL, 30 mol% and (△) 150 mg/mL, 40 mol%.

Note that the Q_m of hydrogels prepared from $PO_{100}H_{20}$ and $PO_{100}A_{20}$ precursors could not be determined under the experimental conditions due to the rapid dissolution of these hydrogels.

The hydrogel prepared at 175 mg/mL precursor concentration ($PO_{100}H_{30}$ and $PO_{100}A_{30}$) is not consistent with the trend of decreasing swelling with increasing precursor concentration reported in the article and undergoes a dramatic change in appearance and a tripling in weight as it swells in PBS (see Fig. 1H of the manuscript); this result was consistent across three independent preparations of this hydrogel. While the reason for this behavior remains unclear, swelling in hydrogels prepared from the same precursors at different mass distributions should primarily be governed by a combination of the osmotic pressure influences (higher concentration = higher osmotic pressure) and the cross-link density (higher concentration = higher number of cross-links forming, as chains are on average closer together); the 175 mg/mL precursor concentration may shift the balance of these effects to favour osmotic swelling relative to additional cross-linking.

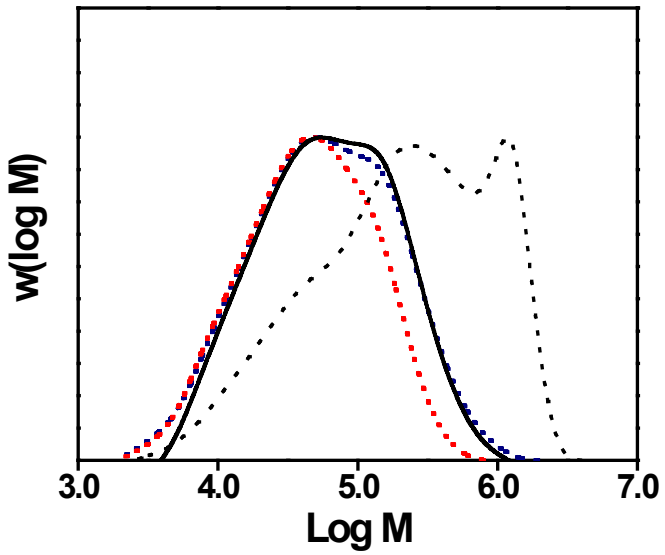


Fig S3. Molecular weight distributions of $PO_{100}H_{30}$ (blue), $PO_{100}A_{30}$ (red) and the hydrogel degradation products after intermediate degradation (black, dotted line) and complete degradation (black, solid line) as measured by aqueous size exclusion chromatography.

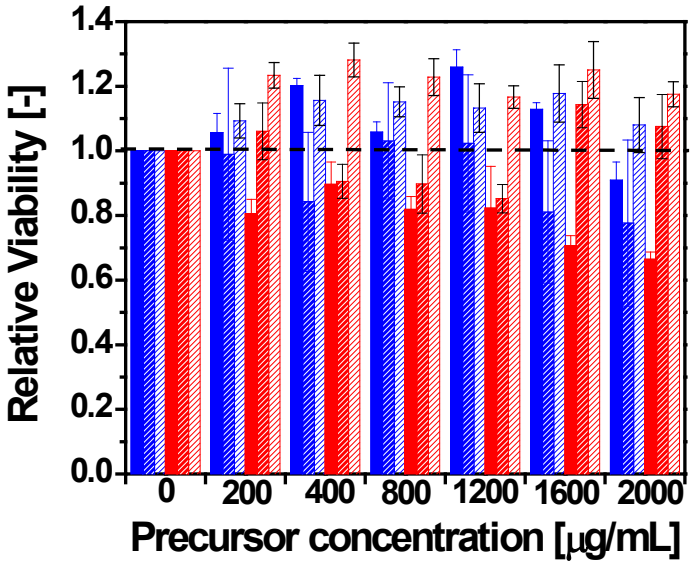


Figure S4. Relative cell viability of $PO_{100}H_{30}$ (blue) and $PO_{100}A_{30}$ (red) as determined from an MTT assay on 3T3 mouse fibroblasts

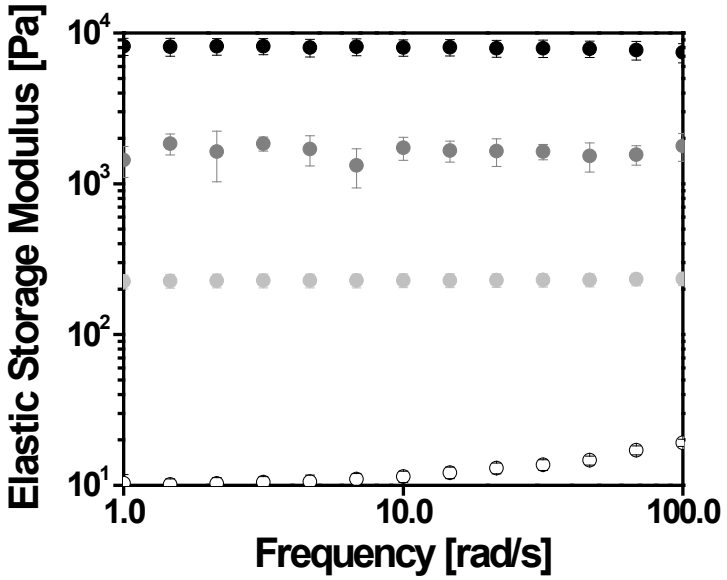


Figure S5. Elastic storage modulus of the POEGMA hydrogels prepared at 150 mg/mL with precursors with varying degree of functionality. (○) 20 mol%, (◐) 25 mol%, (◉) 30 mol% and (●) 40 mol%.

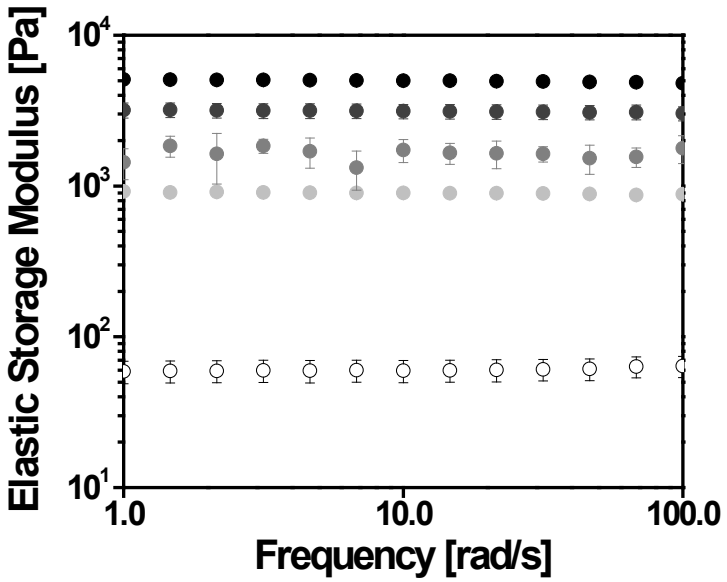


Figure S6. Elastic storage modulus of the POEGMA hydrogels prepared at varying precursor concentrations with from precursors functionalized with 30 mol% functional groups. (○) 100 mg/mL, (◐) 125 mg/mL, (◉) 150 mg/mL, (●) 175 mg/mL and (●) 200 mg/mL.

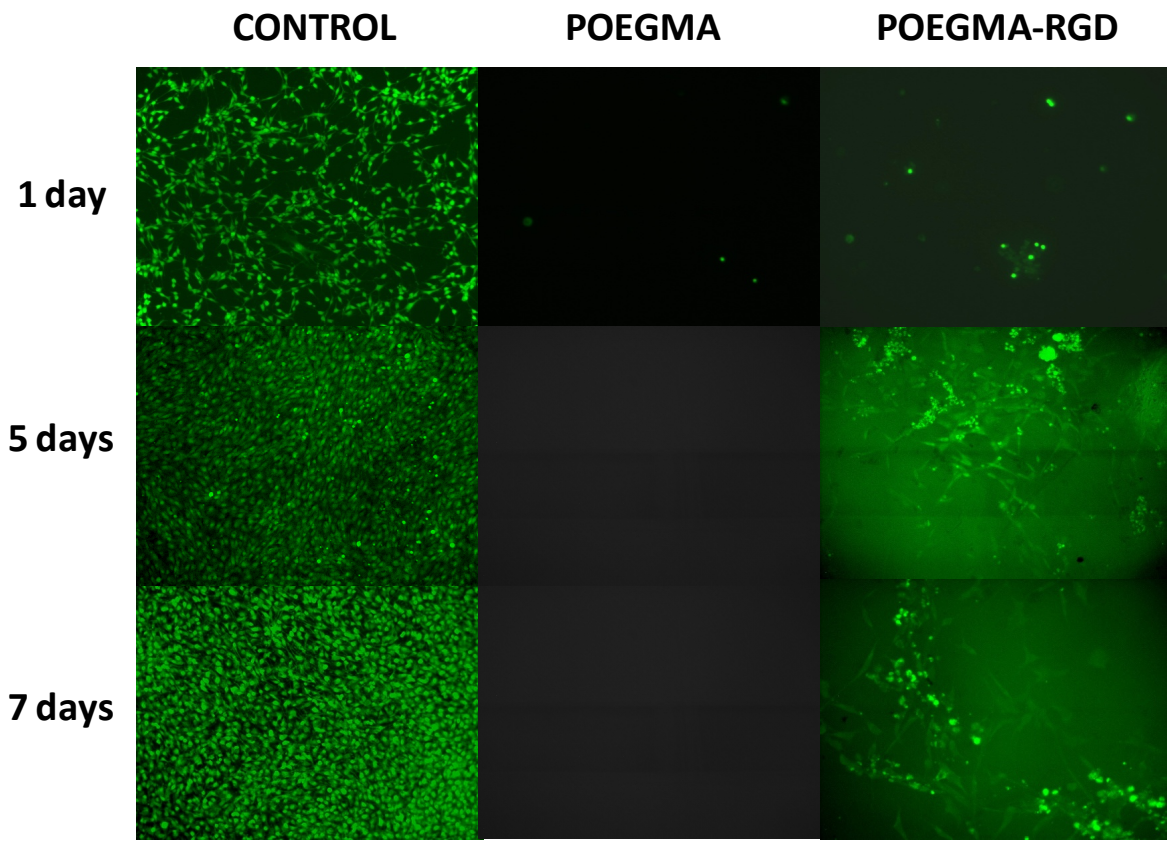


Figure S7. In-vitro fibroblast cell adhesion to a poly(styrene) control, POEGMA hydrogel and RGD-functionalized POEGMA hydrogel determined after 1, 5 and 7 days following cell incubation. No adhered cells are observed on the POEGMA hydrogel after 5 days, while cells cultured on RGD-functionalized POEGMA both proliferate and assume asymmetric geometries indicative of adhesion.

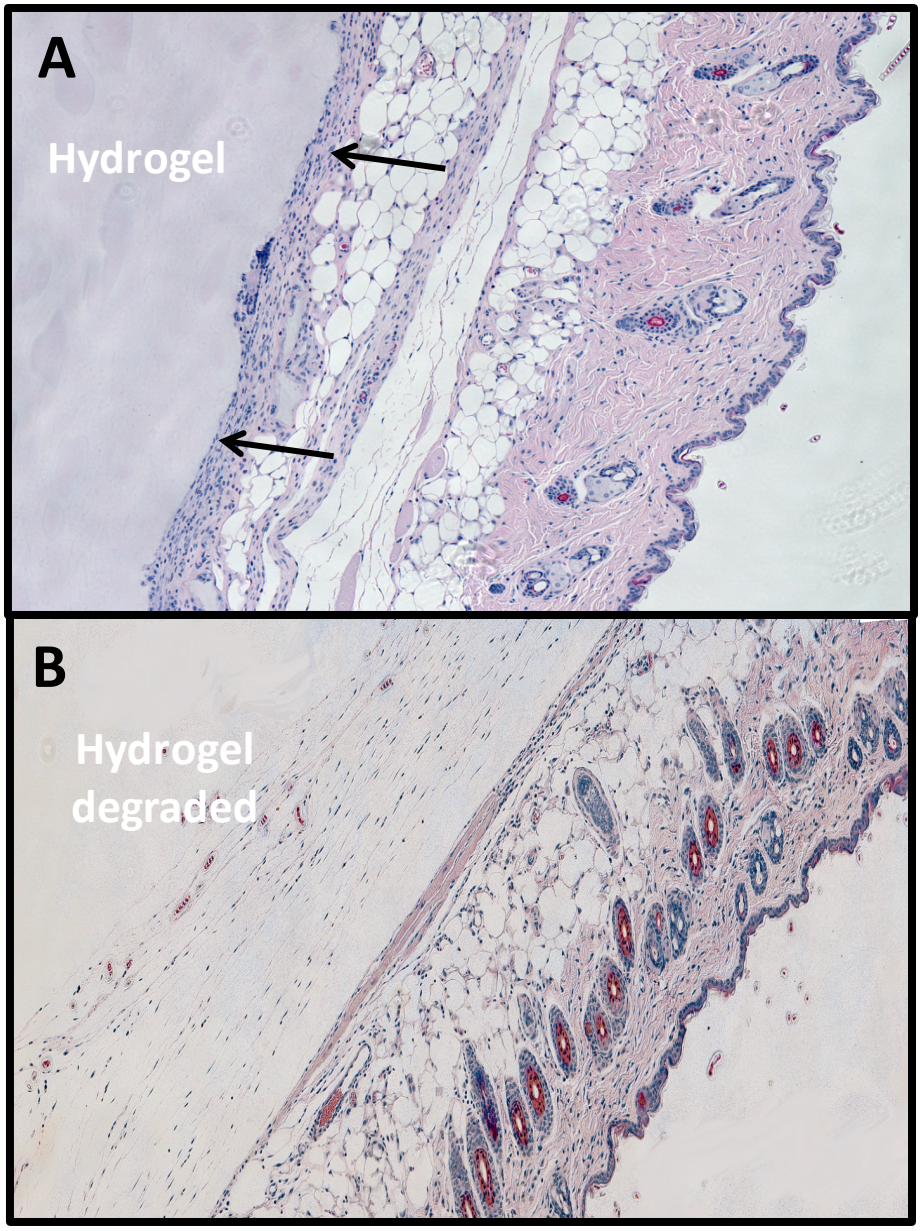


Figure S8 Enlarged images of Fig. 2F (A) and Fig. 2G (B) of the manuscript