Supplementary Information:

Control of Gelation, Degradation and Physical Properties of Polyethylene Glycol Hydrogels through the Chemical and Physical Identity of the Crosslinker

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Supplementary Materials

8-arm polyethylene glycol acrylate (PEGAc; 20 kDa) was obtained from Jenkem Technology USA Inc. (Plano, TX). 6-arm PEGAc (15 kDa) was obtained from Sunbio (Anyang-si Gyeonggi-do, South Korea), Dimethylsulfoxide (DMSO) was obtained from Fisher Scientific (Hampton, NH, USA).

Supplementary Methods

Gelation time, swelling ratio, storage modulus, *G'*, and degradation time of the hydrogels at different reaction pH and using different MW and number of arms PEGAc was monitored as described in the main manuscript. For all studies dithiothreitol (DTT) was used as the representative crosslinker. All hydrogels were 10% w/v polymer precursor concentration. For hydrogels made at different reaction pH, 0.3 M TEA buffer made in 1X PBS was adjusted to a pH of 7, 7.4 and 8.5. 4-arm PEGAc was used for all experiments involving change in reaction pH. For experiments involving the use of different MW and number of arms of PEGAc, 0.3 M TEA buffer (pH 7) was used.

Characterization of acrylate group conversion via ^1H NMR:

All gels were prepared using the same process as used for gel preparation for thiol consumption assay under section "Reaction Kinetics and Efficiency Measurements" as described in the main text. Briefly, all gel compositions consisted of 10% w/v polymer precursors with the same initial thiol and acrylate concentration. The dithiol crosslinkers were allowed to react with the 4-arm PEGAc with an Ac:SH ratio of 3:1. All reactions for all crosslinkers were carried out in 9% v/v DMSO (in 0.3 M TEA in 1X PBS) reaction buffer. At the end of 1 h the gel samples were diluted 10 times with deionized (DI) water to quench the reaction. The samples were then lyophilized and prepared for ^1H NMR by dissolution in $CDCl_3$. 4-arm PEGAc was processed in a similar manner as gel samples and used as control for measuring the initial number of acrylate groups before the start of the reaction. Consumption of acrylate groups in 4-arm PEGAc was confirmed using ^1H NMR (Avance II HDTM 700, Bruker). The conversion was evaluated by comparing the relative peaks of un-crosslinked and crosslinked ethylene protons using unmodified 4-arm PEGAc as control. Acrylate proton ^1H NMR ($CDCl_3$) peaks were obtained at ≈ 5.8, 6.1, 6.5 (3H, $HC=CH_2$). The peaks of the PEG repeat unit were at 3.5-3.7 ppm. The repeat unit of a PEG polymer has a molecular weight of 44 g mol^{-1}, with ~227 repeat units (4 hydrogen atoms per repeat unit) in a 10 kDa polymer. This was used to calculate signal-per-proton value. Similarly signal-per-proton value for acrylate and internal standard (tetramethylsilane (TMS)) was calculated. Since each measurement was carried out using a known amount of internal standard molar ratio of acrylate to TMS and PEG repeat unit to TMS were calculated. The degree of acylation for the unmodified 4-arm PEGAc was calculated to be 93.5%. Acrylate consumption was calculated by comparing the degree of acrylation in the control (unmodified 4-arm PEGAc polymer) to the number of remaining acrylate residues post-reaction with the crosslinker. A

~100% acrylate consumption was assumed if ^{1}H NMR results showed a % mol acrylate consumption ≥33.3% in respective reactions with dithiol crosslinkers (since the ratio of acrylate to thiol groups was 3:1)

Cytotoxicity of crosslinkers and hydrogel leachables

NIH 3T3 cells were seeded in a 96-well plate at a density of $5x10^4$ cells/ml and incubated overnight in DMEM media supplemented with 10% FBS and 1% pen/strep at 37 ºC and 5% CO_2. Cell media was then replaced by media containing 2 mM of each crosslinker and 1% v/v DMSO. The crosslinker concentrations was chosen based on the highest anticipated concentration of each crosslinker to which the cells may be exposed, if all crosslinker remains unreacted in the final hydrogel. DMSO was included as control as some crosslinkers were hydrophobic. Hence, those were dissolved in DMSO prior to dilution in media to a final DMSO concentration of 1% v/v. The cells were incubated for additional 48 h. The cells cultured in media only (0 mM crosslinkers) were used as controls. Cell viability was assessed using an MTS assay as per the manufacture's protocol. Absorbance was measured at 490 nm (SpectraMax i3, Molecular Devices, Sunnyvale, CA). Relative percent viability was expressed with respect to control cells cultured at 0 mM crosslinkers.

To assess the cytotoxicity of hydrogel leachables, 50 µl hydrogels were incubated in 500 µl DMEM media (supplemented with 10% v/v FBS and 1% v/v pen/strep) for 48 h. The media was collected at 24 h and 48 h from all hydrogels and stored at -20ºC until use. For fast-degrading hydrogels (hydrogels made with GDT and GDMA, which degrade in <24 h), media was collected at 5 h. NIH 3T3 cells were seeded in a 96 well plate at a density of $5x10^4$ cells/ml and incubated overnight. Cell media was replaced by media containing the hydrogel leachables and

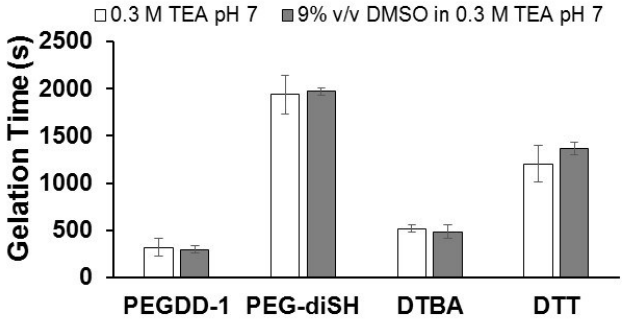
cells were incubated for additional 48 h. The cells cultured in fresh media and DMSO-containing media (equivalent to the maximum possible final concentration of 0.1% v/v in leachable byproducts) were used as controls. Cell viability was assessed as described above for direct cytotoxicity for the crosslinkers. Relative percent viability was expressed with respect to control cells cultured on TCP.

Supplementary Results and Discussion

S1. Effect of reaction pH, MW of multiarm PEGAc and solvent on hydrogel gelation time

S1.1 Effect of solvent on gelation time of hydrogels made with hydrophilic crosslinkers

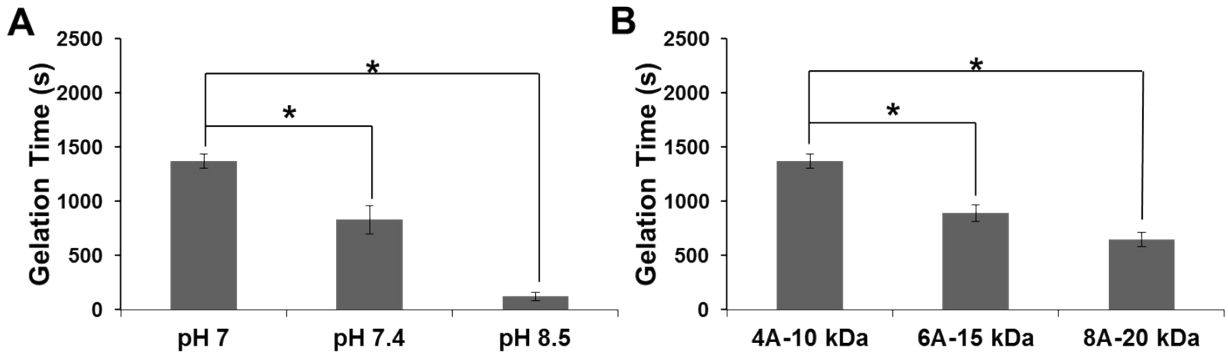
To account for differences in solvent for hydrophobic and hydrophilic crosslinkers, we compared the gelation times of hydrogels formed using water-soluble crosslinkers when made either in an all - aqueous buffer or a 9% v/v DMSO-buffer (0.3M TEA in 1X PBS) of pH 7. We did not find statistically significant differences in gelation times between hydrogels made in the two different buffers (**Supplementary Figure S1**).



Supplementary Figure S1: Comparison of gelation times for hydrogels made with water- soluble dithiol crosslinkers when made in either 9% v/v DMSO-buffer or in an all-aqueous buffer.

S1.2 Effect of reaction pH and MW of multiarm PEGAc on hydrogel gelation time

Gelation time was investigated as a function of reaction pH as well as PEGAc number of arms and MW. In all experiments 10% w/v hydrogels were made with DTT as the crosslinker. As expected, increase in both reaction pH and the number of arms of PEGAc led to a decrease in gelation time (**Supplementary Figure S2**).[1, 2] Reaction pH had the most profound effect on gelation time, leading to a 91% decrease for gels made at pH 8.5 as compared to gels made at pH 7.0. While less pronounced, the increase in number of arms of the PEGAc also led to a significant change in gelation time: it decreased by 32% from 4-arm PEGAc (10 kDa) to 6-arm PEGAc (15 kDa), and by an additional 22% when going from 6-arm PEGAc to 8-arm PEGAc (20 kDa). Note that while the MW of each PEGAc is different, the MW of each PEGAc arm was the same between the different macromers.



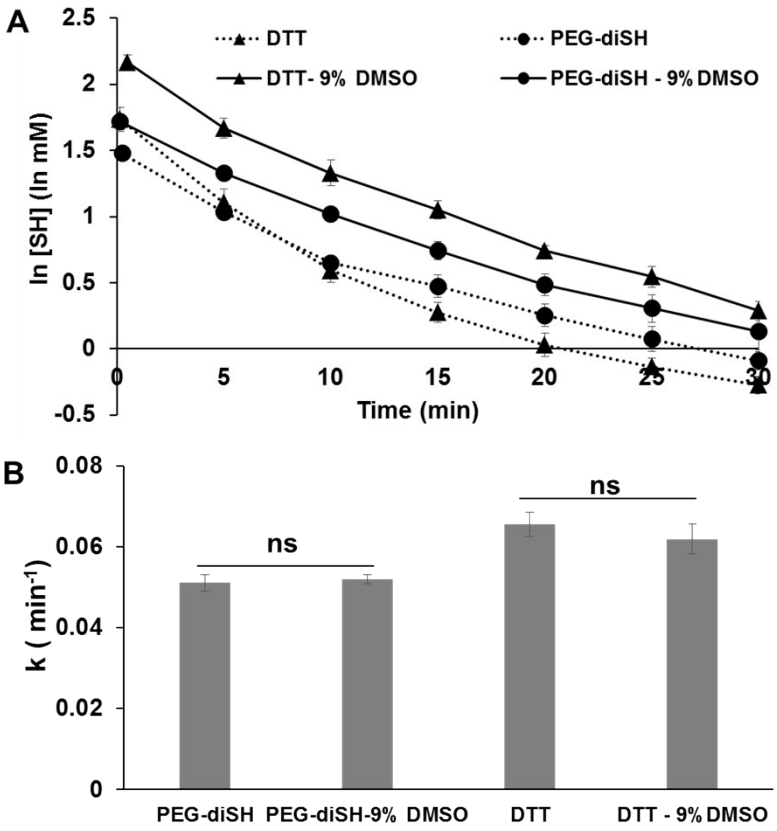
Supplementary Figure S2: Effect of reaction parameters on gelation time of hydrogels: **A)** effect of reaction pH and **B)** MW of multiarm PEGAc (where A stands for number of arms) on gelation time of hydrogels made with DTT. * indicates statistical significance ($p<0.005$) for n=3.

Michael-type addition reaction is base-catalyzed and, hence, it is governed by the catalyst base strength and the rapid protonation of the base, which was TEA in our case.[3, 4] A reason for

the faster gelation rates at higher pH could be the faster protonation of the catalyst TEA, which in turn can deprotonate the thiol and cause rapid formation of the active Michael's donor: the thiolate. Thus, formation of the thiolate is much favored at basic pH [3, 4]. Conversely, neutral reaction pH of 7 decreased the rate of thiolate ion formation by encumbering the protonation of the catalyst TEA, thus, leading to slower reaction times. Thus, gelation time for hydrogels formed by Michael-type addition reaction between a thiol and an acrylate could be modified by the reaction pH.

S2. Effect of solvent on thiol consumption kinetics of hydrophilic crosslinkers

To compare solvent effects on the hydrogel reaction kinetics, the same reaction for water-soluble crosslinkers was run in a 9% v/v DMSO-buffer of pH 7 and an all-aqueous buffer.



Supplementary Figure S3: Kinetics of thiol consumption of PEGAc hydrogels made with PEG-diSH and DTT in an all-aqueous buffer or in a 9% v/v DMSO buffer. **A)** Reaction kinetics of the dithiol crosslinkers as measured by following thiol consumption by Ellman's assay. **B)** First order rate constants for each of the crosslinkers in the two buffers. No statistical difference in the reaction kinetics or reaction rate constants between the two different buffers was noted ($p<0.05$, $n=3$).

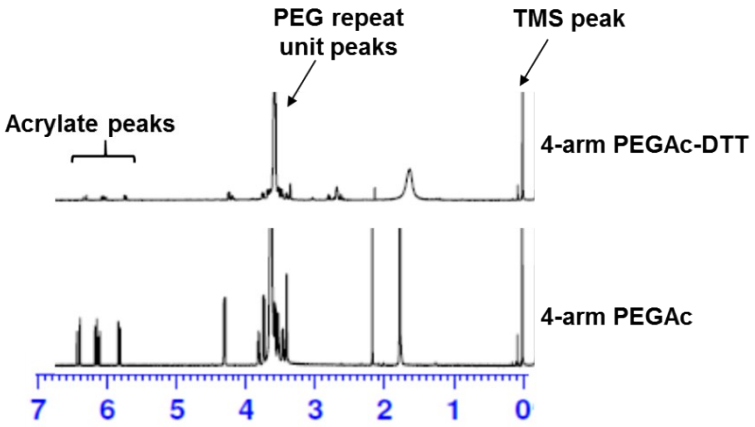
S4: Measurement of acrylate consumption in hydrogels via ^{1}H NMR

Consumption of acrylate groups in each of the hydrogels formed with different dithiol crosslinkers was confirmed by ^{1}H NMR. A ~100% acrylate consumption was assumed if the calculated %mol acrylate consumed was ≥33.3%, since the ratio of acrylate to thiol groups was 3:1. Representative ^{1}H NMR for 4-arm PEGAc and 4-arm PEGAc-DTT post-reaction is shown in **Figure S4**. The %mol acrylate consumed in each reaction was calculated and depicted in

Table S1. Gels formed with the croslsinkers DTT, EDDT, TEGDT, PEG-diSH and GDMP showed ~33-41 %mol of acrylate consumption. A slightly higher than expected acrylate consumption estimation during the reaction (i.e. higher than 33.3 %mol) may be caused by various factors such as intra-and intermolecular reaction of the acrylate[2, 5] or instrument sensitivity. Nonetheless, results from the ^{1}H NMR studies were in agreement with FTIR results (see main text) confirming complete acrylate consumption during hydrogel formation.

Supplementary Table S1: Calculated % mol of acrylate consumed in hydrogels formed using the indicated crosslinkers. Acrylate consumption was measured using ^{1}H NMR. The acrylate to thiol molar ratio was 3:1. A ~100% acrylate consumption was assumed if % mol acrylate consumption ≥33.3% in respective reactions.

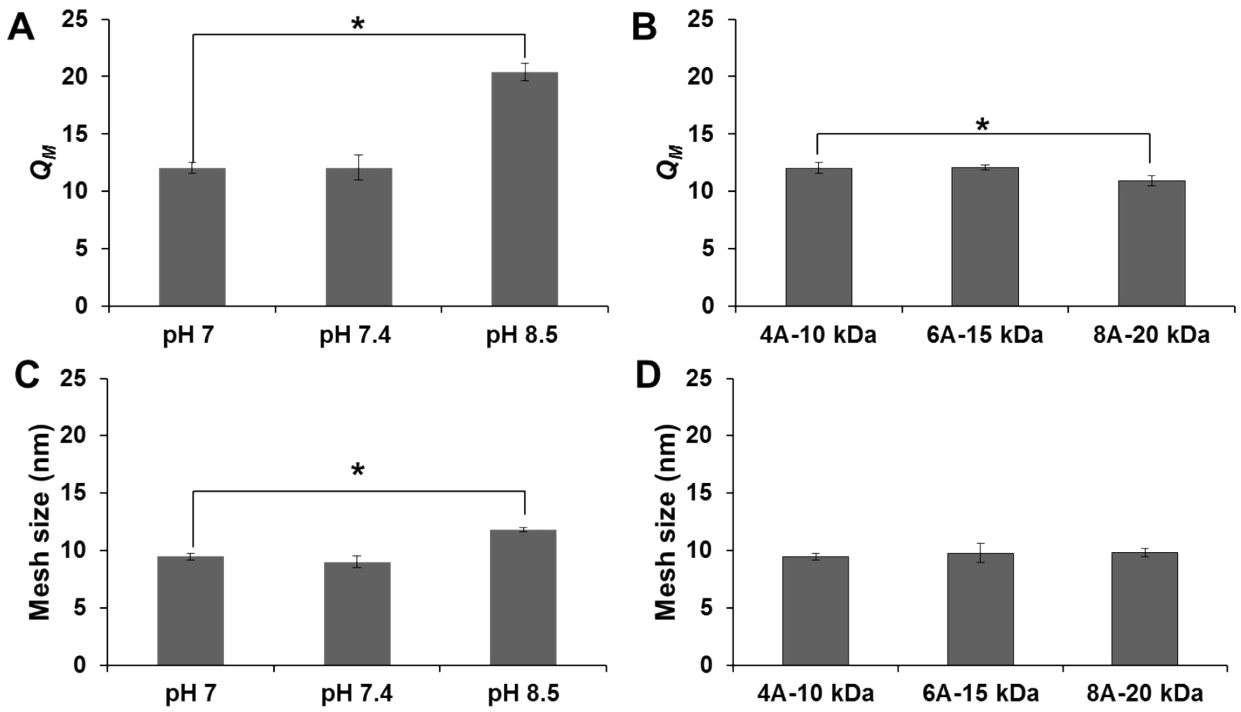
4-arm PEGAc hydrogel with	% mol Ac remaining after hydrogel formation	% mol Ac consumed in reaction
DTT	52.27	41.10
EDDT	53.41	39.96
TEGDT	58.00	35.37
PEG-diSH	60.18	33.19
GDMP	56.23	37.15



Supplementary Figure S4: Representative ^{1}H NMR spectra for 4-arm PEGAc and 4-arm PEGAc-DTT hydrogel (Ac:SH ration of 3:1).

S5. Effect of reaction pH and MW of multiarm PEGAc on hydrogel swelling ratio and mesh size

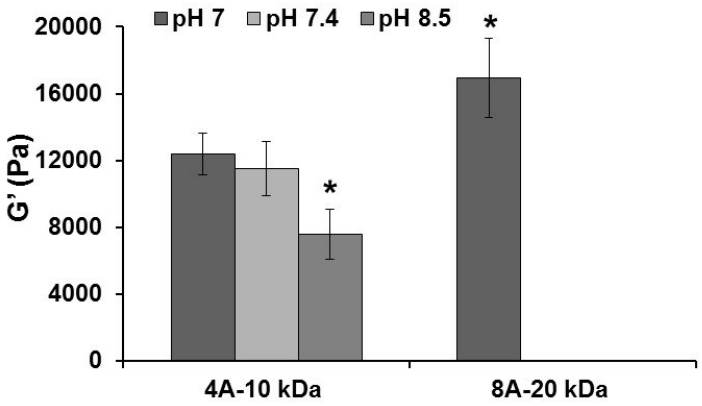
The reaction pH significantly influenced the initial swelling ratio, Q_M, and mesh size of the hydrogels (41% difference between hydrogels prepared at pH 7.0 and 8.5) (**Supplementary Figure S5A and S5C**). Increasing the number of PEGAc arms slightly decreased Q_M (**Supplementary Figure S5B**) but did not lead to change in mesh size of the resultant hydrogels (**Supplementary Figure S5D**).



Supplementary Figure S5: Effect of reaction parameters on hydrogel Q_M and mesh size. **A)** Q_M of PEG hydrogels made at different reaction pH and **B)** different MW of multiarm PEGAc. **C)** Mesh size of PEG hydrogels made at different reaction pH and **D)** different MW of multiarm PEGAc. * indicates statistical significance ($p<0.05$, n=3).

S6. Effect of reaction pH and MW of multiarm PEGAc on hydrogel storage modulus

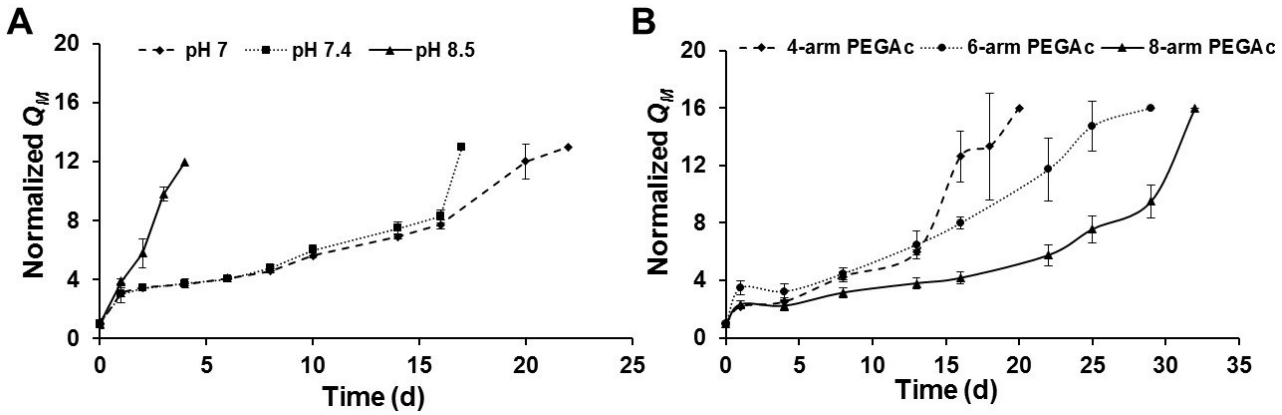
Initial *G'* was tested as a function of reaction pH, MW and number of PEGAc arms (**Supplementary Figure S6**). On increasing the reaction pH from 7 to 8.5, *G'* decreased by 32%. Increasing the number of arms for PEGAc from 4 to 8 (corresponding to MW from 10 to 20 kDa) increased *G'* by 28%.



Supplementary Figure S6: Effect of reaction parameters on *G'* of PEG hydrogels made at different reaction pH and multiarm PEGAc MW. * indicates $p<0.05$ when compared to *G'* of hydrogels made at pH 7 using 4-arm PEGAc.

S7. Effect of reaction pH and MW of PEGAc on hydrolytic degradation

The reaction pH strongly influenced degradation times of hydrogels. Hydrogels formed at pH 7, 7.4 and 8.5 degraded in ~22 d, ~15 d, and ~48 h, respectively (**Supplementary Figure S7A**). Reaction pH of 8.5 leads to rapid formation of the highly reactive thiolate ions [4] and a possible conversion to form disulfide bonds.[2, 4] For a thiol of pKa ~9.2 there is 0.1% thiolate ion at pH 7, while it increases to 1% at pH 8, thus, increasing the rate of disulfide formation 10 times.[6] This in turn impairs the equimolar stoichiometry of Ac:SH resulting in lower crosslink density. Since for our system an ester is only present at the crosslink site and the degradation kinetic is proportional to the number of liable ester bonds,[1, 7, 8] we noted increased degradation time with decrease in crosslink density. Increasing the number of arms of the PEGAc macromer slowed the degradation rate by 32 - 45% for 6-arm PEGAc and 8-arm PEGAc, respectively (**Supplementary Figure S7B**).

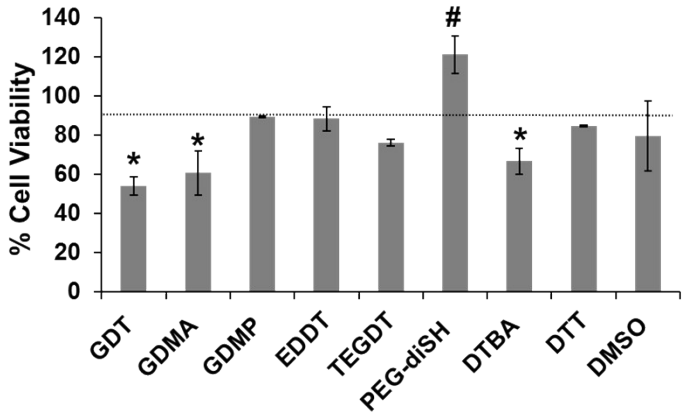


Supplementary Figure S7: Kinetics of PEG hydrogel degradation as followed by an increase in Q_M. **A)** Degradation of hydrogels made at different reaction pH. **B)** Degradation of hydrogels made with PEGAc macromers of different number of arms and corresponding MW. Q_M obtained at different time intervals was normalized to the Q_M of the unswollen network at time zero.

S8. Direct cytotoxicity of crosslinkers and hydrogel leachables

We examined crosslinker cytotoxicity on NIH 3T3 cell viability after a 48 h exposure. The cells showed a significant decrease in viability when crosslinkers were present in the cell media. The highest viability was found in presence of PEG-diSH crosslinker, which corroborates our earlier findings.[9] All other crosslinkers showed a decrease in cell viability: 84% for DTT and ~60% for GDT and GDMA (**Figure S8**). Compared to cell viability post-encapsulation in hydrogels, direct exposure of the cells to the crosslinkers showed decreased cell viability. This could be due to absence of a reactive group like acrylate (present in hydrogels) which can selectively quench the thiols and prevents significant side reactions with proteins in the media or on the cell surface. Some additional toxicity for the water-insoluble crosslinkers GDT, GDMA, EDDT, TEGDT and GDMP could also be due to the presence of DMSO used as a co-solvent. DMSO at 1% v/v led to decrease in cell viability to ~80%. Moreover, gelation time of the hydrogels described here was

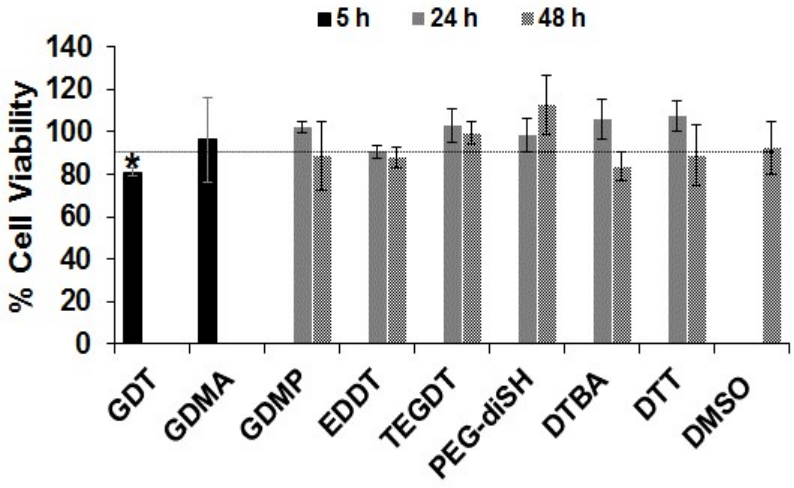
in seconds to minutes, hence, limiting exposure to the unmodified crosslinker during cell encapsulation.



Supplementary Figure S8: Percent cell viability of NIH 3T3 cells after 48 h exposure to the specified crosslinkers (2 mM). The hash line represents 90% cell viability. Characters #,* indicate statistical differences ($p<0.05$). Crosslinkers marked with the same character have no statistical difference in % cell viability but are statistically different from all other. Error bars represent ± SD for n=3.

To further ascertain the biocompatibility of the hydrogels made using the indicated crosslinkers, we tested the cytotoxicity of hydrogel leachables at 24 h and 48 h (**Figure S9**). In all cases cell viability was >80% after a 48 h exposure to media conditioned with the leachables. Cell viability was found to be lowest for cells incubated with media conditioned with leachable products from hydrogels made with GDT. GDT also showed the highest direct cytotoxicity toward cells (see **Figure S8**). Media collected at 48 h containing leachables from GDMP and DTBA hydrogels showed a slight but insignificant reduction in cell viability compared to media collected at 24 h. All other crosslinker leachables led to cell viability ≥90%. Note that hydrogels were not rinsed prior to testing. However, for drug encapsulation and delivery applications a pre-rinse could be

used to ensure removal of soluble un-crosslinked gel precursors and other leachables to further reduce possibility of toxicity. Further, we showed that the degradation products (**Figure 8B**, main text) did not show cytotoxicity, indicating that once the crosslinker was incorporated into the hydrogel it did not adversely influence cell viability.



Supplementary Figure S9: Percent cell viability of NIH 3T3 cells after 48 h exposure to media containing hydrogel leachables. The hash line represents 90% cell viability. Characters * indicate statistical differences ($p<0.05$). Error bars represent ± SD for n=3.

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