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Tuning PEG-DA hydrogel properties *via* solvent-induced phase separation (SIPS)†

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Abstract

Poly(ethylene glycol) diacrylate (PEG-DA) hydrogels are widely utilized to probe cell-material interactions and ultimately for a material-guided approach to tissue regeneration. In this study, PEG-DA hydrogels were fabricated *via* solvent-induced phase separation (SIPS) to obtain hydrogels with a broader range of tunable physical properties including morphology (*e.g.* porosity), swelling and modulus (G′). In contrast to conventional PEG-DA hydrogels prepared from an aqueous precursor solution, the reported SIPS protocol utilized a dichloromethane (DCM) precursor solution which was sequentially photopolymerized, dried and hydrated. Physical properties were further tailored by varying the PEG-DA wt% concentration (5 wt%–25 wt%) and M_n (3.4k and 6k g mol $^{-1}$). SIPS produced PEG-DA hydrogels with a macroporous morphology as well as increased G′ values *versus* the corresponding conventional PEG-DA hydrogels. Notably, since the total swelling was not significantly changed *versus* the corresponding conventional PEG-DA hydrogels, pairs or series of hydrogels represent scaffolds in which morphology and hydration or G′ and hydration are uncoupled. In addition, PEG-DA hydrogels prepared *via* SIPS exhibited enhanced degradation rates.

Introduction

As with the extracellular matrix (ECM) in natural tissues, the physical properties of tissue engineering scaffolds mediate cell behaviour including regeneration.[1–5] Most notable are scaffold morphology (*e.g.* porosity)[6–12] and modulus.[13–16] Thus, regeneration of tissues which closely resemble native tissues may be accomplished through a material property-guided approach. Towards this goal, scaffolds whose physical properties can be precisely tuned over a broad range are essential.

Poly(ethylene glycol) diacrylate (PEG-DA) hydrogels have been widely utilized as scaffolds for the regeneration of diverse tissues.[17–23] They are particularly useful to probe cell-material interactions due to their resistance to protein and cell adhesion in the absence of the controlled introduction of adhesive ligands.[17,18,24–26] The limited range of physical properties which may be obtained for PEG-DA hydrogels restricts their utility to study material-guided cell behaviour. For instance, hydrogel mechanical properties may be tuned over a limited range by altering the weight % (wt%) concentration of PEG-DA in the precursor solution or the crosslink density as determined by PEG-DA number average

†Electronic Supplementary Information (ESI) available: Sol content values of hydrogels (Supplemental Table S1), LDH activity of PEG-DA hydrogels (Supplemental Figure S1) and degradation of conventional PEG-DA hydrogels (Supplemental Figure S2). See DOI: 10.1039/c1jm13943f

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molecular weight (M_n).[20,27] However, these changes simultaneously alter swelling behaviour and so limit the ability to uncouple modulus and swelling and thus the impact of each on the cellular response.[28] Hydrogel scaffold morphology, including porosity, is also known to influence cell behaviour.[6,29–32] Macroporous hydrogels have shown particular utility to enhance tissue engineering outcomes.[33–35] Furthermore, increased pore size has been shown to be useful to enhance the rate of degradation.[36,37] In order to increase their pore size range, several strategies have been used to produce PEG-DA hydrogels with macroporous morphologies, including: salt leaching,[38,39] cryogelation,[40,41] and gas foaming.[42] However, each method is associated with specific limitations such as difficulty leaching porogens (salt leaching), high temperatures or low pressures (gas foaming), and extremely low temperatures (cryogelation).[43,44]

To successfully produce functional tissues, it is highly desirable that the scaffold degrade at a rate which parallels regeneration.[44–46] Because of the stability of ether bonds and limited number of hydrolytically unstable ester crosslinks, PEG-DA hydrogels do not readily degrade under physiological conditions.[47] A variety of synthetic strategies have been reported to enhance degradation of PEG-DA hydrogels, including incorporation of polyester segments,[48–50] crosslinking to form labile bonds,[51–53] and introduction of enzymatically unstable peptides,[26,54] However, these alter the scaffold chemistry which is known to elicit changes in cell behaviour.[55–57]

Solvent-induced phase separation (SIPS) has been utilized to prepare macroporous poly(*N*-isopropylacrylamide) (PNIPAAm) hydrogels.[58,59] During SIPS, a solvent system is utilized which promotes phase separation of the growing polymer chain and network during cure. In this study, PEG-DA hydrogels were formed *via* SIPS by photocuring dichloromethane (DCM) precursor solutions rather than aqueous precursor solutions as is used to form conventional PEG-DA hydrogels. Precursor solutions were formed with DCM at various wt % concentrations (5 wt%–25 wt%) and M_n's (3.4k and 6k g mol $^{-1}$) of PEG-DA and subsequently dried and hydrated with water. These concentrations and M_n's represent those typically utilized to prepare PEG-DA scaffolds for tissue engineering studies. The resulting macroporous morphology, swelling behaviour, modulus, and degradation behaviour was related to composition and compared to analogous conventional PEG-DA hydrogels fabricated from aqueous precursor solutions.

Experimental

Materials

Allyl methacrylate, acryloyl chloride, triflic acid, 2,2-dimethyl-2-phenyl-acetophenone (DMAP), 1-vinyl-2-pyrrolidinone (NVP), triethylamine (Et_3N), $MgSO_4$, K_2CO_3, hexamethyldisilazane (HMDS) and solvents were obtained from Sigma Aldrich. HPLC grade toluene, CH_2Cl_2 and NMR grade $CDCl_3$ were dried over 4 Å molecular sieves. Poly(ethylene glycol) (PEG) [PEG-6000; MW = 5000–7000 g mol^{-1} and PEG-3400; MW = 3000–3700 g mol^{-1} per manufacturer's specifications] were obtained from BioChemika. The precise M_n's of PEG-3400 (3393 g mol^{-1}) and PEG-6000 (6143 g mol^{-1}) were back-calculated from ^{1}H NMR end-group analysis of the corresponding diacrylated products. Phosphate buffered solution (PBS, pH = 7.4, without calcium and magnesium), HEPES, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS),and PSA solution(10 U/mLpenicillin,10g L^{-1}streptomycin, and 10 g L^{-1} amphotericin) were obtained from Mediatech. Peptide RGDS was obtained from American Peptide. Acryloyl PEG-succinimidyl valerate (acryloyl-PEG-SVA, 3.4 kDa) was obtained from Laysan Bio Inc. Mouse smooth muscle precursor cells (10T1/2) were obtained from American Type Culture Collection (ATCC).

PEG-DA synthesis

PEG-DA (3.4k g mol^{-1} or 6k g mol^{-1}) were prepared as previously reported.[60] PEG-3400 (23.5 g, 7.0 mmol), Et_3N (1.95 mL, 14.0 mmol) and acryloyl chloride (2.27 mL, 28.0 mmol) were reacted to obtain PEG-DA (15.2 g, 63% yield). ^{1}H NMR (δ, ppm): 3.62 (s, 297H, –OCH_2CH_2), 5.81 (dd, 2H, J = 10.5 and 1.2 Hz, –CH=CH_2), 6.13 (dd, 2H, J = 17.4 and 10.5 Hz, –CH=CH_2), 6.40 (dd, 2H, J = 17.3 and 1.5 Hz, –CH=CH_2). By ^{1}H NMR end-group analysis, M_n of PEG-DA (3.4k g mol^{-1}) = 3393 g mol^{-1} (~3400 g mol^{-1}). PEG-6000 (24 g, 4.0 mmol), Et_3N (1.12 mL, 8.0 mmol) and acryloyl chloride (1.30 mL, 16.0 mmol) were reacted to obtain PEG-DA (31 g, 63% yield). ^{1}H NMR (δ, ppm): 3.61 (s, 547H, –OCH_2CH_2), 5.81 (dd, 2H, J = 10.4 and 1.5 Hz, –CH=CH_2), 6.13 (dd, 2H, J = 16.8 and 10.5 Hz, –CH=CH_2), 6.40 (dd, 2H, J = 17.3 and 1.5 Hz, –CH=CH_2). By ^{1}H NMR end-group analysis, M_n of PEG-DA (6k g mol^{-1}) = 6143 g mol^{-1} (~6000 g mol^{-1}).

NMR

^{1}H NMR spectra were obtained on a Mercury 300 300 MHz spectrometer operating in the Fourier transform mode. Five percent (w/v) $CDCl_3$ solutions were used to obtain spectra. Residual $CHCl_3$ served as an internal standard.

Hydrogel preparation

PEG-DA hydrogels formed *via* SIPS were prepared from DCM-based precursor solutions whereas conventional PEG-DA hydrogels were prepared from aqueous precursor solutions. Precursor solutions were prepared at concentrations of 5, 10, 15, 20, and 25 wt% PEG-DA (M_n = 3.4k or 6k g mol^{-1}) in DCM or deionized (DI) water. 10 μL of photoinitiator solution (30 wt% solution of DMAP in NVP) was added per one mL of the precursor solution. Solutions were vortexed for one minute following both the addition of PEG-DA and the subsequent addition of the photoinitiator solution. Planar hydrogel sheets (1.5mm thick) were prepared by pipetting the precursor solution between two clamped microscope slides (75 × 50 mm) separated by Teflon spacers and exposing the mold to longwave UV light (UV-Transilluminator, 6 mW cm^{-2}, 365 nm) for a total of 6 min with rotation to the alternate side after 3 min. After removal from the mold, the water-based hydrogel sheets were rinsed with DI water and soaked in a Petri dish containing DI water (60 mL) for 2 days with daily water changes to remove catalyst impurities. The DCM-based sheets were rinsed with DCM then air dried for 30 min to permit evaporation of DCM and subsequently placed in a Petri dish containing DI water (60 mL) to remove any remaining DCM and impurities. During the first hour of soaking, the water was changed every 15 min and thereafter daily for 2 days. All hydrogels were permitted to soak in DI water for a total of 72 h prior to testing.

Sol content

Three discs (13 mm diameter) were punched from a hydrogel sheet with a die. After air-drying (30 min), each disc was placed in an open scintillation vial and dried at room temperature (RT) in a vacuum oven (30 in. Hg, 24 h). Dried discs were weighed (W_{d1}), returned to the vial and 10 mL DCM added to each. Capped vials were placed on a rocker table (250 rpm) for 48 h to remove sol (*i.e.* uncrosslinked material). Next, discs were removed, air dried for 30 min, each placed in an open scintillation vial, dried in a vacuum oven (RT, 30 in. Hg, 24 h) and weighed (W_{d2}). Sol Content is defined as: sol content = $[(W_{d1} - W_{d2})/W_{d1}] \times 100$.

Cytocompatibility

The hydrogel cytocompatiblity was assessed by measuring lactate dehydrogenase (LDH) levels released by $10T1_{/2}$ SM progenitor cells 24 h following cell seeding. PEG-DA

hydrogels (3.4k g mol^{-1}, 10 wt%; 50 × 40 × 1 mm) were fabricated as above but with 1 μmol mL^{-1} (post-swelling) of acrylate-derivatized cell-adhesive peptide RGDS in the DCM or aqueous precursor solutions. Acryloyl-PEG-RGDS was prepared by reacting acryloyl-PEG-SVA (3.4 kDa) with RGDS.[61] The DCM-based sheets were air dried for 24 h. Both the water-based and dried DCM-based sheets were sterilized with two changes of ethanol/water (70/30; 24 h) and transferred into sterile Petri dishes where they were washed twice with sterile DI water (24 h) and finally rinsed twice with DPBS supplemented with 1% PSA (24 h). Four 8 mm diameter discs were punched from each sample and transferred into a 48 well plate. 10T $1_{/2}$ cells were seeded onto the hydrogel surfaces at 6000 cells cm^{-2}. After being maintained for 24 h at 37 °C with 5% CO_2 in DMEM (without phenol red) supplemented with 10% heat deactivated FBS and 1% PSG, the media surrounding each specimen was collected for LDH measurements following manufacturer (Roche) protocol.

Morphology

Water-swollen hydrogels discs (13 mm diameter) were flash frozen in liquid nitrogen for 1 min and immediately lyophilized for 24 h (Labconco Centri Vap Gel Dryer System). Specimen cross-sections were subjected to Pt/Pd-sputter coating and viewed with a field emission scanning electron microscope (FEI Quanta 600 FE-SEM) at an accelerated electron energy of 10 keV.

Equilibrium swelling

Three discs (13 mm diameter) were punched from a single sheet with a die. Each disc was placed in a sealed vial containing 20 mL DI water for 48 h at RT, removed, blotted with filter paper to remove surface water, and weighed (W_s). Equilibrium swelling ratio is defined as: swelling ratio = $(W_s - W_d)/W_d$, where W_s is the weight of the water-swollen hydrogel and W_d is the weight of the vacuum dried hydrogel (30 in. Hg, 60 °C, 24 h).

Dynamic mechanical analysis (DMA)

Three discs (13 mm diameter) were prepared as above. Storage modulus (G′) of each disc was measured in the compression mode with a dynamic mechanical analyzer (TA Instruments Q800) equipped with a parallel-plate compression clamp with a diameter of 40 mm (bottom) and 15 mm (top). A water-swollen disc (13 mm diameter) was blotted with a Kim Wipe, clamped between the parallel plates and silicone oil placed around the exposed hydrogel edge to prevent dehydration. Following equilibration at 25 °C (5 min), the samples were tested in a multi-frequency-strain mode (1 to 30 Hz).

Degradation

Six hydrogel discs (8 mm diameter) were prepared as above. After soaking in DI water for 3 h, an initial swollen weight was recorded. Three discs were each placed into a well plate (secured with Parafilm and covered with foil) containing 1 mL 0.05M NaOH and maintained at 37 °C on a rocker table at 50 rpm. The NaOH solution was exchanged every 12 h. Swollen weights were recorded at regular intervals (W_s) until the hydrogel exhibited a loss in weight with a corresponding loss in mechanical integrity. The time required for the disc to completely dissolve was also recorded. The remaining three hydrogel discs were vacuum dried (30 in. Hg, 60 °C, 24 h) and their weights recorded (W_d). Swelling ratio (SR) is defined as: swelling ratio = $(W_s - W_d)/W_d$. Results reported are based on the average of the three individual specimens.

Results and discussion

Both the hydrogels formed *via* SIPS and the conventional hydrogels were transparent (Fig. 1). However, the cross-section of PEG-DA hydrogels formed by SIPS displayed a sponge-

like or coarser texture. To verify the efficacy of photocrosslinking, sol content values of hydrogels were measured (Supplemental Table S1). Sol contents of PEG-DA hydrogels fabricated from DCM precursor solutions (~3–9%) and aqueous precursor solutions (~2–9%) were similarly low. To establish cytocompatibility, LDH assays were conducted on PEG-DA hydrogels (3.4k g mol^{-1}, 10 wt%) prepared with SIPS or from an aqueous precursor solution (Supplemental Figure S1). LDH is a soluble cytosolic enzyme that is released into the culture medium following membrane damage due to apoptosis or necrosis.[62] The LDH activity for the hydrogel produced *via* SIPS was essentially identical to that of the hydrogel prepared from an aqueous precursor solution.

Conventional PEG-DA hydrogels photopolymerized from aqueous precursor solutions at typical concentrations cannot be imaged *via* SEM as they collapse during the freezing/freeze-drying process.[33] In contrast, SEM images of PEG-DA hydrogels prepared *via* SIPS were obtained and revealed open porous structures (Fig. 2). During SIPS, phase separation of the growing polymer chains and network from the solvent leads to polymer rich as well as polymer lean domains (*i.e.* pores) which, upon hydration, fill with water. Depending on the extent as well as rate of phase separation prior to a final three-dimensional structure determined by significant crosslinking, the porosity will vary somewhat. A macroporous morphology was observed when PEG-DA hydrogels were prepared at concentrations between 10 and 25 wt%. Notably, for the hydrogel series based on 3.4k PEG-DA, higher wt % concentrations produced increasingly larger pores. At the same wt% concentration, hydrogels based on 6k PEG-DA exhibited a different morphology *versus* hydrogels based on 3.4k PEG-DA. Thus, SIPS is useful to achieve macroporous morphologies which may be tuned by PEG-DA wt% concentration as well as M_n.

PEG-DA hydrogels formed *via* SIPS generally exhibited similar swelling to the analogous conventional PEG-DA hydrogel (Fig. 3). The exceptions are hydrogels formed at very low (5 wt%) concentrations which produced substantially lower swelling for hydrogels prepared by SIPS. For a given PEG-DA M_n at a concentration between 10–25 wt%, rather similar and, in some cases, statistically similar swelling ratios were achieved when prepared *via* SIPS *versus* from an aqueous precursor solution. This was somewhat unexpected given the macroporous morphology of hydrogels prepared *via* SIPS (Fig. 2) which is typically associated with enhanced water uptake.[39,58] The lack of swelling increase for PEG-DA hydrogels prepared *via* SIPS may be attributed to reduced water uptake in the polymer-rich regions of the hydrogels. Thus, SIPS changes the distribution of water rather than the total water uptake in the resulting PEG-DA hydrogels. In addition, certain pairs of PEG-DA hydrogels prepared *via* SIPS at different concentrations also exhibit quite similar swelling ratios (*e.g.* 6k g mol^{-1} at 15 and 20 wt%). Hydrogel swelling (*i.e.* hydration) is an important scaffold property given its impact on cell behaviour in terms of local environment and diffusion of waste and nutrients.[46] Thus, the impact of scaffold morphology on cell behaviour decoupled from total hydration may be studied by utilizing series of PEG-DA scaffolds formed *via* SIPS and conventional PEG-DA hydrogels.

During DMA, hydrogel stiffness was measured in terms of the storage modulus (G') as a function of frequency of the applied strain in compression (Fig. 4). For a given PEG-DA M_n at a specific concentration (5–25 wt%), G' was substantially higher for hydrogels fabricated *via* SIPS compared to those fabricated from aqueous precursor solutions. As previously observed[20,27] for conventional PEG-DA hydrogels, G' increased with higher crosslink density (*i.e.* reducing PEG-DA M_n) and higher PEG-DA concentration. The same compositional changes to PEG-DA hydrogels fabricated *via* SIPS similarly produced an increase in G'. For conventional PEG-DA hydrogels, increased crosslink density or concentration produces a concomitant decrease in swelling such that the impact of G' on cell behaviour may not be uncoupled from the effect of hydration.[28] From the series of PEG-

DA hydrogels formed *via* SIPS and from aqueous precursor solutions, a pair or more of hydrogels with similar hydration (Fig. 3) but different G′ (Fig. 4) are available. Thus, PEG-DA hydrogels prepared *via* SIPS afford the opportunity to study the influence of modulus on cell behaviour decoupled from hydration.

Increased pore size has been related to faster degradation rates[36,37] and thus represents a strategy to tailor degradation without changing the chemical composition of the scaffold material. Because SIPS produces macroporous PEG-DA hydrogels at 10–25 wt% concentrations (Fig. 2), we anticipated that degradation rates would be faster than the corresponding conventional PEG-DA prepared from aqueous precursor solutions. PEG-DA hydrogels were subjected to hydrolytic degradation under accelerated (basic) conditions (Fig. 5). Degradation was measured in terms of the time to achieve maximum swelling before loss of mechanical integrity and also in terms of time to completely dissolve.[48] For solid aliphatic poly-ester films, hydrolytic degradation rate increases with film thickness due to an autocatalytic effect of more slowly diffusing acidic degradation products.[63] In the same way, the degradation rate of porous aliphatic polyester materials is increased with larger pore size and hence greater pore wall thickness.[64] In the case of PEG-DA hydrogels, hydrolysis of labile ester bonds releases poly(acrylic acid) (PAA) kinetic chains[65] which similarly may induce autoacceleration if diffusion is limited. Thus, the observed enhanced rate of degradation of PEG-DA hydrogels prepared *via* SIPS *versus* conventional hydrogels is attributed to the larger pore size and thicker pore wall of the former. As expected, for PEG-DA hydrogels prepared *via* SIPS, lower wt% concentrations led to shorter dissolution times. Also, at the same wt% concentration, the degradation rate was higher for hydro-gels fabricated from 6k g mol^{-1} PEG-DA M_n (*i.e.* lower hydrogel crosslink density) *versus* from 3.4k PEG-DA. The effect of concentration and M_n were similarly observed for conventional PEG-DA hydrogels (Supplemental Fig. S2). Given the limited susceptibility to hydrolysis of PEG-DA hydrogels, their fabrication by SIPS to produce enhanced degradation rates without altering the chemical nature is a useful tool to study cell behaviour and ultimately tissue regeneration.

Conclusions

PEG-DA hydrogels with broader, tunable physical properties which furthermore are uncoupled (*i.e.* not dependent on one another) would be extremely useful to study cell-material interactions and ultimately improve tissue regeneration. Towards this goal, PEG-DA hydrogels were formed *via* SIPS with 3.4k and 6k g mol^{-1} PEG-DA at various wt% concentrations (5–25 wt%). While conventional PEG-DA hydrogels are fabricated from an aqueous precursor solution, the reported SIPS protocol utilized a DCM precursor solution followed by drying and hydration after photopolymerization. When prepared *via* SIPS, PEG-DA hydrogels displayed a macroporous morphology (10–25 wt%) but did not exhibit increased total swelling *versus* the corresponding conventional hydrogel. Thus, certain series of hydro-gels represent scaffolds in which morphology and hydration are uncoupled. In addition, PEG-DA hydrogels prepared *via* SIPS exhibited a substantial increase in modulus (G′) *versus* the corresponding conventional hydrogel. In this case, a particular series of hydrogels represent scaffolds in which hydration and modulus are uncoupled. Lastly, because of the larger pore size, PEG-DA hydrogels fabricated *via* SIPS exhibited an increased degradation rate under accelerated conditions. Thus, PEG-DA hydrogels formed *via* SIPS, particularly when combined with conventional PEG-DA hydrogels, form a useful library of scaffolds to study the influence of physical properties on cell behaviour and ultimately regenerate tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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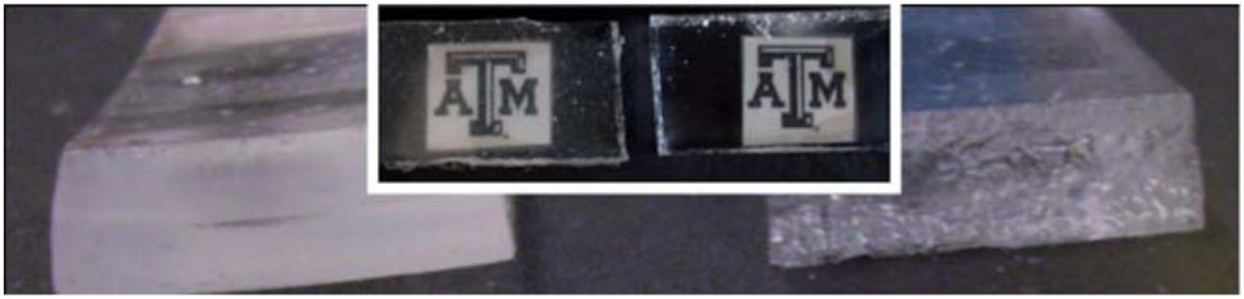

Fig. 1.

Macroscopic images of conventional PEG-DA hydrogels fabricated from an aqueous precursor solution **[left]** and PEG-DA hydrogels fabricated *via* SIPS with a DCM precursor solution (and subsequently dried and hydrated) **[right]**. Inset top view of each corresponding $^{-1}$; 10 wt% concentration). hydrogel. (PEG-DA: M_n = 6k g mol

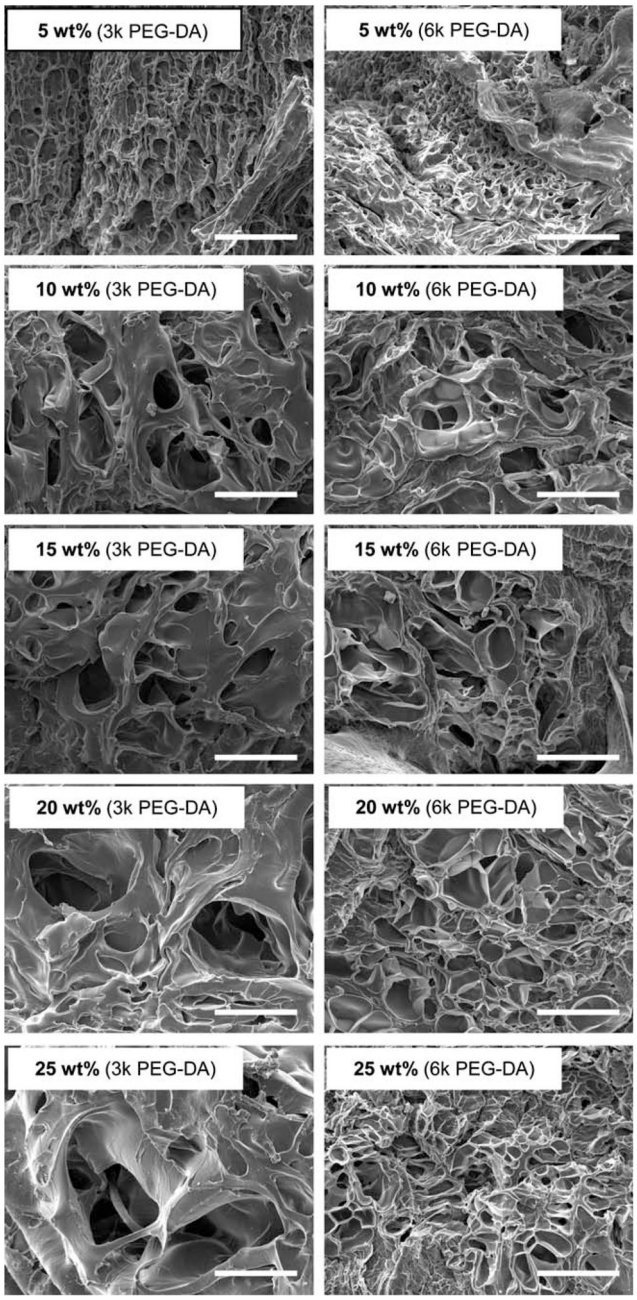

Fig. 2.
SEM images of PEG-DA hydrogels fabricated *via* SIPS (scale bars = 100 μm).

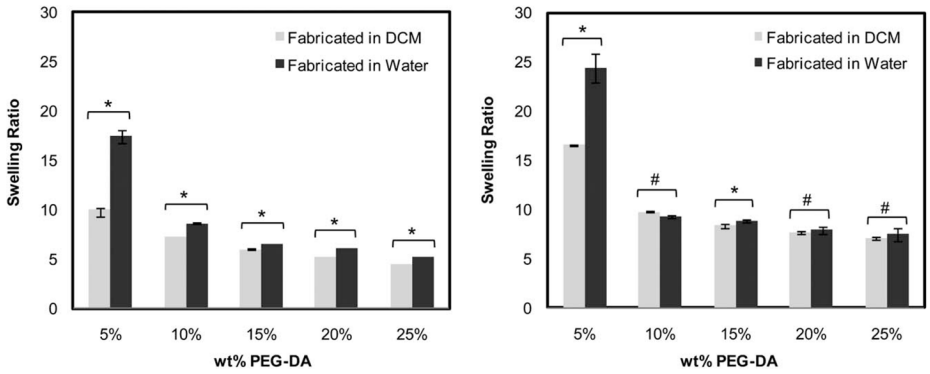

Fig. 3.

Swelling ratio of PEG-DA hydrogels fabricated with 3.4k g mol^{-1} (left) and 6k g mol^{-1} (right) PEG-DA from a DCM precursor solution (*i.e. via* SIPS) or from an aqueous precursor solution. Statistical significance was determined by student's *t*-test where (*): $p < 0.05$ and (#): $p > 0.05$.

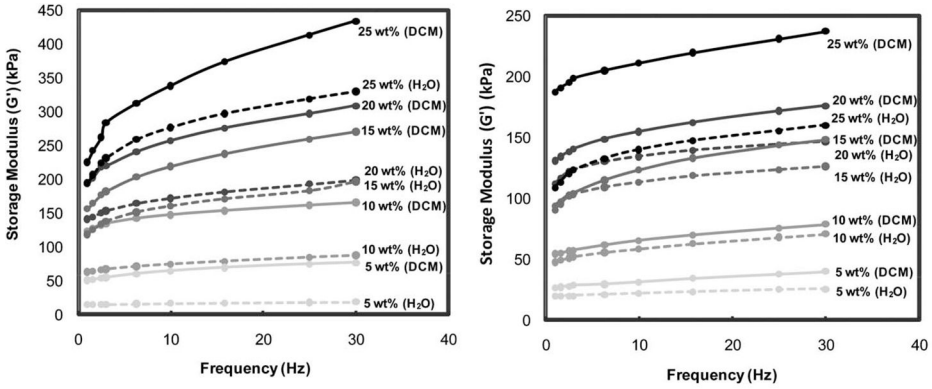

Fig. 4.

Storage modulus (G′) of PEG-DA hydrogels fabricated with 3.4k g mol^{-1} (left) and 6k g mol^{-1} (right) PEG-DA from a DCM precursor solution (*i.e. via* SIPS) [solid lines] or from an aqueous precursor solution [dashed lines].

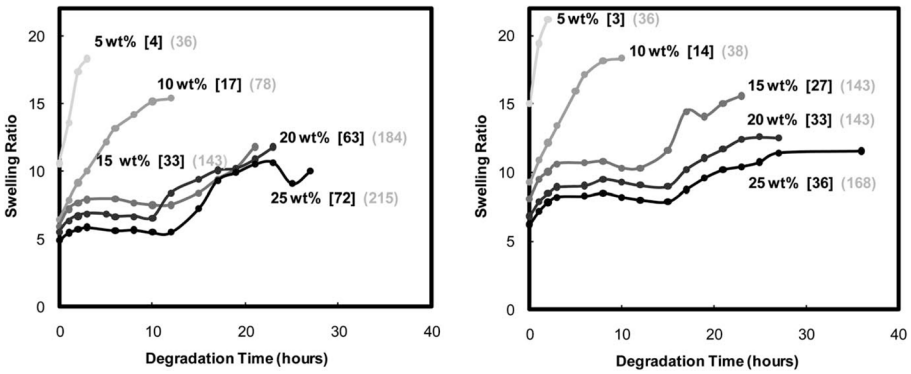

Fig. 5.

Swelling ratio as a function of degradation time under basic conditions (0.05 M NaOH) of PEG-DA hydrogels fabricated with 3.4k g mol^{-1} (left) and PEG-DA 6k g mol^{-1} (right) *via* SIPS at various wt% concentrations. **[]** = hours to complete dissolution. **()** = hours to complete dissolution for analogous conventional hydrogels (*i.e.* prepared from aqueous precursor solutions).