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Hydrolytically degradable poly(ethylene glycol) hydrogel scaffolds with tunable degradation and mechanical properties

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Abstract

The objective of this work was to create three-dimensional (3D) hydrogel matrices with defined mechanical properties, as well as tunable degradability for use in applications involving protein delivery and cell encapsulation. Thus, we report the synthesis and characterization of a novel hydrolytically degradable poly(ethylene glycol) (PEG) hydrogel composed of PEG vinyl sulfone (PEG-VS) cross-linked with PEG-diester-dithiol. Unlike previously reported degradable PEG-based hydrogels, these materials are homogeneous in structure, fully hydrophilic and have highly specific cross-linking chemistry. We characterized hydrogel degradation and associated trends in mechanical properties, i.e., storage modulus (G'), swelling ratio (Q_M), and mesh size (ξ). Degradation time and the monitored mechanical properties of the hydrogel correlated with cross-linker molecular weight, cross-linker functionality, and total polymer density; these properties changed predictably as degradation proceeded (G' decreased, whereas Q_M and ξ increased) until the gels reached complete degradation. Balb/3T3 fibroblast adhesion and proliferation within the 3D hydrogel matrices were also verified. In sum, these unique properties indicate that the reported degradable PEG hydrogels are well poised for specific applications in protein and cell delivery to repair soft tissue.

Keywords

Tissue engineering; PEG; Degradation; 3D scaffold; Cell viability

1. Introduction

Due to their high water content, hydrogel biomaterials can emulate the physical properties of soft tissues, making them highly suitable as scaffolds for tissue engineering.[1, 2] Hydrogels have also been extensively used as drug delivery carriers due to their excellent biocompatibility and their ability to improve the pharmacology of proteins without changing protein structure and drug efficacy.[3, 4] Degradable hydrogels in particular are desirable for a variety of applications; for example, tissue engineering scaffolds that can be degraded and remodeled as the cells migrate and synthesize new extracellular matrix are thought to allow more successful long-term tissue regeneration.[5] In cell-based therapies and protein delivery applications, hydrogel degradation can be used to control the release rate of the delivered component and also permit clearance of the device from the body when it is no longer needed.

One of the most studied and widely applied hydrogels is poly(ethylene glycol) (PEG).[6] PEG has been explored as cell scaffolds[5, 7, 8] as well as drug delivery devices.[9–14] However,

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PEG by itself is non-reactive and to create insoluble networks, it requires end-functionalization with cross-linking groups. A number of chemistries have been developed for the functionalization of PEG including the addition of acrylate, thiol, amine, maleimide or vinyl sulfone reactive groups.[15] As cross-linked networks, these materials are non-degradable under physiological conditions.

To facilitate degradation in PEG-based hydrogels, a number of strategies have been developed that utilize degradable block-copolymer components (e.g., poly(lactic acid) or PLA). One family of PEG-based degradable hydrogels is based on the copolymer PLA-*b*-PEG-*b*-PLA dimethacrylate.[12, 16–19] Despite its many successful applications, this type of hydrogel structure can be associated with drawbacks such as protein denaturation due to the PLA hydrophobicity and inflammation caused by acidic degradation byproducts of PLA such as lactic acid and poly(acrylic acid).[20] Additionally, the cross-linking reaction involves ultraviolet irradiation, which could be detrimental to cells.[21] Multi-arm PEG-amine cross-linked with an ester-containing amine-reactive PEG derivative has also been utilized as a hydrolytically degradable PEG scaffold for protein delivery.[22] This polymer is fully hydrophilic, but its application is restricted because the amine reaction allows covalent binding of encapsulated proteins to the polymer network during cross-linking. This issue had been circumvented by the use of PEG-multiacrylates and PEG-dithiols to form a fully hydrophilic hydrogels with selective cross-linking chemistry.[3] However, the cross-linking reaction for these hydrogels may take up to 1 h which could have a detrimental effect on specific cell types encapsulated prior to cross-linking. In addition, it has been shown that when cross-linking PEG-multiacrylates with PEG-dithiols, low acrylate concentrations favored intramolecular reactions which lead to cyclization and network non-ideality.[23]

Building on these works, Lutolf and Hubbell pioneered the use of PEG functionalized with vinyl sulfone (VS)[24] for tissue engineering applications. PEG-VS reacts specifically with free thiols, such as peptides terminated with cysteine residues.[25] Because free cysteines are rarely present on the exposed surfaces of cells and proteins, this cross-linking approach allows a high degree of control over reaction specificity and rate. Peptides can be selected to render biological activity to the otherwise inert PEG gels by adding cell-adhesive domains from extracellular matrix proteins and peptide substrates for matrix metalloproteinase degradation. Whereas such biological sophistication is required for complex tissue engineering applications, cases such as protein delivery may benefit from a simpler approach. Moreover, unique peptides are often expensive, available only in small quantities, and difficult to process and store when synthesized with terminal cysteines due to rapid disulfide bond formation.

In this work we adapted the PEG-VS approach to yield a fully hydrophilic and inert hydrogel with a rapid and highly specific cross-linking chemistry. Moreover, we developed an approach to render the PEG gels to be degradable via hydrolysis, a process that does not require the presence of specific biological compounds, such as proteases, which may vary in concentration due to the specific type or source of cells present. The products of hydrolysis were designed to be of sufficiently low molecular weight to allow clearance from the body (3.4-20 kDa).[26] This novel approach does not preclude the incorporation of peptides, as cysteine-terminated adhesive or matrix metalloproteinase-degradable sequences may be used in conjunction with the cross-linker described in this work.

Herein, we present the synthesis of a new class of PEG-VS cross-linkers, PEG-diester-dithiols, and the use of these polymers to form stable and biocompatible PEG hydrogels under physiological conditions. Based on prior works, we hypothesized that the hydrogel degradation would depend on several factors including: a) molecular weight of the polymer, [16, 18] b) polymer density, c) polymer hydrophilicity, d) number of linkages susceptible to

hydrolysis (e.g., ester bonds), and e) position and reactivity of linkages susceptible to hydrolysis.[27, 28] To test this hypothesis, we characterized the hydrolytic degradation of a series of PEG hydrogels as well as the impact of degradation on hydrogel swelling, mesh size and mechanical properties. Additionally, we modified the PEG hydrogels with the fibronectin-derived cell-adhesive peptide RGD and determined the impact of this change in structure to hydrogel mechanical properties, swelling, mesh size and viability of encapsulated fibroblasts in culture.

2. Materials and methods

All reagents were acquired from Fisher Scientific or Sigma Aldrich unless otherwise noted.

2.1. Synthesis of poly(ethylene glycol)-vinyl sulfone

The synthesis of 4-arm PEG-VS was adapted from a previous protocol[24] where 4-arm PEG-OH (10 kDa; Nektar, Huntsville, LA) was modified in the presence of excess divinyl sulfone. Briefly, PEG was dried by azeotropic distillation in toluene using a Dean-Stark trap and then dissolved in dry dichloromethane. Sodium hydride was added under Ar at a 5-fold molar excess over OH groups. After hydrogen evolution divinyl sulfone was added immediately at a 50-fold molar excess to OH groups. The solution was allowed to react for 3 d at room temperature under Ar and with constant stirring. The solution was then filtered and reduced in volume (~30 ml) by rotary evaporation. The polymer was recovered in ice-cold diethyl ether and dried under vacuum. The dry polymer was then dissolved in deionized water containing sodium chloride and extracted three times in dichloromethane. After drying with sodium carbonate, the polymer product was reduced in volume, precipitated, and dried as described above. The product was stored under Ar at −20°C until use. Derivatization was confirmed by ^{1}H NMR ($CDCL_3$): 3.6 ppm (982H, PEG backbone), 6.1 ppm (4H, 1H, $=CH_2$), 6.4 ppm (4H, 1H, $=CH_2$), 6.8 ppm (4H, 1H, $-SO_2CH=$). The typical yield from this procedure was 80 – 90% and the degree of end group conversion, as shown by NMR, was 93 – 98%.

2.2. Synthesis of poly(ethylene glycol)-diester-dithiol

PEG-diester-dithiol was synthesized by reacting PEG (3.4, 6, and 8 kDa; Spectrum, Gardena, CA) with thioglycolic acid (MP Biomedicals, Solon, OH) to give PEG-dithioglycolate (PEG-diester-dithiol with one methylene between the ester and thiol groups)[29] or 3-mercaptopropionic acid to give PEG-dithiopropionate (PEG-diester-dithiol with two methylenes between the ester and the thiol groups).[9] The general structure of these molecules and their abbreviated names are described in Figure 1 and Table 1. The general name PEG-diester-dithiol or the abbreviated names will be used further in the text.

The synthesis of all cross-linkers was similar; as an example, the reaction of PEG and 3-mercaptopropionic acid is described. Briefly, PEG was dried via azeotropic distillation in toluene and re-dissolved in toluene under Ar. Then, mercaptopropionic acid (20-fold molar excess to PEG OH groups), the catalyst *p*-toluenesulfonic acid (0.4 mmol) and the reducing agent dithiothreitol (1 mmol; to prevent the formation of disulfide bonds) were added and the reaction mixture was then refluxed with stirring for 24 h. Toluene was removed overnight under vacuum and the polymer was precipitated three times in ice-cold acetone. The product was recovered via filtration, dried under vacuum overnight and stored at −20°C until use. Derivatization was confirmed by ^{1}H NMR ($CDCl_3$): 4.27 ppm (4H, $-CH_2OC(O)-$, m), 3.74-3.50 ppm (726H, $-CH_2CH_2O-$, s), 2.80-2.69 ppm (8H, $-CH_2CH_2SH$, m). The typical yield from this procedure was 70% and the end-group conversion as shown by NMR was 95 – 98%.

2.3. Formation of PEG-based hydrogels

A schematic of the hydrogel formation is presented in Figure 1 (for detailed chemical structures of the components, the cross-linking reaction and the degradation products see Supporting Information). The hydrogels were formed by a Michael-type addition of PEG-diester-dithiol or non-degradable PEG-dithiol cross-linker onto 4-arm PEG-VS. Each polymer precursor was dissolved in a 0.3 M triethanolamine (TEA) solution of pH 8. To minimize weighing error, aliquots of 20% w/v PEG-VS in 0.3 M TEA were made in advance and stored at 4°C until use. The ratio of VS:SH was 1:1 for all hydrogel densities and types.[30] Immediately after mixing, the solution was quickly vortexed and then transferred to the center of a glass slide that was treated with RainX (Sopus Products, Houston, TX) to provide a hydrophobic surface. Silicone spacers (1- or 2.5-mm thick cut from CoverWell perfusion chambers, Grace Bio-Labs, Bend, OR) were placed at the ends of the glass slide and a second hydrophobic slide was placed on top. The two slides were clamped together over the spacers with binder clips. The slides were then transferred to a humidified incubator and allowed to gel at 37°C. Gelation occurred in several minutes but the hydrogels were left in the incubator for 1-2 h to achieve maximum cross-linking. A >90% conversion of reactive groups was assumed for all hydrogel types.[3, 23, 30]

2.4. Rheological measurements

To determine hydrogel mechanical properties, storage modulus (G') and loss modulus (G'') were measured with an AR 2000ex rheometer (TA Instruments) in parallel plate geometry with a 20-mm diameter acrylic upper plate, at 22°C, a frequency of 1-10 rad/s, and a constant 2% strain.[8] The hydrogel samples were prepared to yield discs of 20-mm diameter and 1-mm thick (thickness < diameter/4 per TA Instruments guide) following swelling in 10 mM phosphate buffer saline (PBS), pH 7.4 at 37°C. The water from the hydrogel surface was carefully blotted before measurement. G' was measured at regular intervals until the hydrogels had insufficient physical integrity to handle (~75% degradation). Rheological analysis was also used as an indirect measurement of the PEG hydrogel degradation.

2.5. Swelling experiments

The equilibrium degree of swelling for each hydrogel was acquired in order to estimate structural parameters such as molecular weight between cross-links, effective cross-link density and mesh size. Hydrogel swelling is a function of network structure, degree of cross-linking as well as hydrophilicity and was used to indirectly characterize hydrogel degradation. Hydrogels (50 μL) were incubated at 37°C in 10 mM PBS, pH 7.4. Hydrogel samples were collected at regular intervals and their mass after swelling (M_S) was measured. The hydrogels were then dried in an oven at 80°C for 24 h and their dry mass (M_D) was measured. Three hydrogels of each type were synthesized for each time point and incubation was continued until the hydrogel samples had insufficient physical integrity to handle (~75% degradation).

The swelling ratio based on hydrogel mass (Q_M) was calculated using Equation 1:[31]

$$Q_M = \frac{M_S}{M_D} \tag{1}$$

where M_S is the hydrogel mass after swelling and M_D is the dry hydrogel mass. Q_M was further used to calculate the volume swelling ratio (Q_V):

$$Q_V = 1 + \frac{\rho_p}{\rho_s}(Q_M - 1) \quad (2)$$

where ρ_p is the density of the dry hydrogel (1.12 g/cm^3 for PEG)[3] and ρ_s is the density of the solvent (1 g/cm^3 for water).

2.6. Flory-Rehner calculations for determining hydrogel mesh size

Flory-Rehner calculations were used to determine hydrogel mesh size (ξ). First the molecular weight between cross-links (M_c) was calculated by Equation 3:[12]

$$\frac{1}{\overline{M_c}} = \frac{2}{\overline{M_n}} - \frac{\frac{\bar{v}}{V_1}(\ln(1 - v_2) + v_2 + \chi_1 v_2^2)}{v_2^{1/3} - \frac{v_2}{2}} \quad (3)$$

where $\overline{M_n}$ is the number-average molecular weight of the un-cross-linked hydrogel (the molecular weight of the polymer), V_1 is the molar volume of the solvent (18 cm^3/mol for water), v_2 is the polymer volume fraction in the equilibrium swollen hydrogel, which is equal to the reciprocal of Q_V, $\bar{v}$ is the specific volume of the polymer (ρ_s / ρ_p), and χ_1 is the polymer-solvent interaction parameter (0.426 for PEG-water[12, 31] and assumed constant for our work because χ_1 has been found to be nearly independent of PEG v_2 for v_2 = 0.04–0.2).[32]

Mesh size was then determined as described by Canal and Peppas.[33] The root-mean-square end to end distance of the polymer chain in the unperturbed state ($(\overline{r_0^2})^{1/2}$) was calculated using the following equation:

$$(\overline{r_0^2})^{1/2} = l C_n^{1/2} n^{1/2} \quad (4)$$

where l is the average bond length (0.146 nm),[34, 35] C_n is the characteristic ratio of the polymer (typically 4.0 for PEG)[32, 35] and n is the number of bonds in the crosslink:[36]

$$n = 2\frac{\overline{M_c}}{M_r} \quad (5)$$

where M_r is the molecular weight of the repeat unit (44 for PEG). Mesh size was then calculated by

$$\xi = v_2^{-1/3}(\overline{r_0^2})^{1/2} \quad (6)$$

and was calculated for each time point during degradation.

2.7. Gelation time measurements

Gelation time was measured by the inverse tube method described by Prestwich *et al.*[37] Each hydrogel sample (10% w/v total polymer density,100 μL volume) was prepared at

room temperature in a microfuge tube, vortexed for 20 s, and monitored during repeated inversion of the tube. The gelation time was determined as the time when the hydrogel would no longer flow by the force of gravity. Effect of pH on gelation time was also determined.

2.8. Cytotoxicity studies

To determine possible polymer and cross-linked hydrogel cytotoxicity, fibroblast metabolic activity was characterized using a standard MTS assay (Promega, Madison, WI). All components were filter-sterilized with a 20-μm pore size syringe filter before use. Mouse fibroblasts (Balb/3T3, ATCC, Manassas, VA) were grown for 24 h under standard cell culture conditions in a 24-well plate at 50,000 cells/ml initial concentration in DMEM medium (HyClone Laboratories, Logan, UT), enriched with 10% bovine calf serum (Gibco Invitrogen, Carlsbad, CA), 50 U/mL penicillin/streptomycin (MP Biomedicals LLC, Solon, OH), and 40 mM L-glutamine (ATCC, Manassas, VA). The cells were cultured for an additional 24 h in medium containing 1 mM or 10 mM polymer. These concentrations correspond to the polymer concentrations used during the gelation process and represent concentrations to which encapsulated cells would be exposed. The medium was then replaced with fresh medium containing MTS according to the manufacturer's procedure. After 1-h incubation at 37°C, solution absorbance was measured at 492 nm (Cary 50 UV-Vis spectrophotometer, Varian Inc., Palo Alto, CA).

We also tested for possible toxicity of the cross-linked hydrogels and the products of hydrogel degradation. Cross-linked hydrogels were prepared in a Transwell insert (3.0 μm pore size, Millipore, Billerica, MA) and placed in indirect solution contact with the fibroblasts, which were seeded in the wells of 24-well plates.[31] This method allowed for toxicity testing of any soluble components as well as products of degradation that may leach from the hydrogel during culture. After 24 h, the hydrogels were discarded and cell metabolic activity was characterized using the MTS assay as described above. As a positive control cells were cultured in the presence of an empty Transwell insert. The results were reported as absorbance at 492 nm which correlates directly to levels of metabolic activity.

2.9. Cell viability in 3D PEG hydrogels

The LIVE/DEAD cell assay (Invitrogen, Eugene, OR) was used to assess cell viability according to the manufacturer's procedure. First, Balb/3T3 fibroblasts were incubated with a fluorescent green membrane stain ($DiOC_{18}$) for 24 h and then collected for the experiment. To encapsulate cells in 3D PEG hydrogels, 30 μl hydrogels were made as described in Section 2.3 with the following exception: the fibronectin-derived cell-adhesive peptide, RGDS, was covalently incorporated into the hydrogel structure to support fibroblast adhesion and viability following encapsulation. The peptide was terminated with the sequence GRCD, which contains a cysteine residue to allow for covalent attachment to PEG-VS,[38] thus, GRCD*RGDS*PD (CPC Scientific Inc., San Jose, CA) was added at a final concentration of 100 μM, which is a large stoichiometric deficit to the PEG-diester-dithiol SH groups. The solution containing PEG-VS and peptide was allowed to react for 15 min and then the cross-linker, pre-dissolved in 0.3 M TEA, was added to give a final total stoichiometric ratio of VS:SH of 1:1. Finally, $DiOC_{18}$-stained cells in 0.3 M TEA were added to bring the solution volume to 100 μL and cell concentration to 2500 cells/μL. The solution was mixed and used to dispense a 30-μL hydrogel per well of a 24-well plate and incubated for 30 min to allow complete gelation. Cells seeded on standard tissue culture polystyrene were used as a positive control. All the cells were grown for additional 10 or 24 h at which point the fluorescent red nucleolus stain (propidium iodide-PI; 7.5 μM) was added to each well to stain the dead cells. After incubation for 1 h, images of the cultures were captured under fluorescence microscopy (Olympus IX81, Center Valley, PA) and

image analysis was performed using NIH Image-J freeware. Cells were imaged over a 10 – 20 μm z-thickness, which is approximately the size of 1 cell body. De-convolution of the images and a high intensity threshold for the Image-J software were utilized in order to discard out-of-focus cells from the images prior to performing cell counts. Percent cell viability was calculated as the number of live cells divided by the total number of cells × 100%. The samples were not washed before or after adding propidium iodide; thus the analysis considers all cells that were present at the end of the culture period.

2.10. Influence of adhesive ligand on hydrogel mechanical properties

PEG hydrogels (10% w/v total polymer) were made with each of the degradable cross-linkers (i.e., PEG-SH 1 3.4, PEG-SH 2 3.4, PEG-SH 2 6, and PEG-SH 2 8), with and without GRCD*RGDS*PD adhesive ligand (100 μM); rheological and swelling experiments were performed as described above after 24 h of incubation in 10 mM PBS at 37oC. For hydrogels synthesized with PEG-SH 1 3.4 cross-linker, however, measurements were made after 6 h due to rapid degradation (~hours).

2.11. Statistical analysis

The results of all experiments are the mean values (± *SD*) of triplicate samples performed in three independent experiments. Comparisons between multiple samples were performed with single factor analysis of variance (ANOVA). Comparisons between two samples were performed with two-tailed Student's *t*-test and differences between two data sets were considered significant when $p < 0.05$.

3. Results

3.1. Hydrogel degradation monitored by rheology

The relationship between network composition and hydrogel properties (i.e., degradation and viscoelasticity) was examined by rheological measurements. Three experiments were carried out to determine the effects of PEG-diester-dithiol cross-linker molecular weight, the number of methylene groups between the ester and thiol moieties of the PEG-dieser-dithiol, and the rate of oscillatory strain on G' and G''.

In the first experiment, we varied the molecular weight of the cross-linker (3.4, 6, 8 kDa), while keeping all other parameters constant (Figure 2a). A non-degradable control of hydrogels made with PEG-SH 3.4 cross-linker was also used. The initial G' for all hydrogels was in the range of 1000-3000 Pa and G' decreased over the 5-d test period for all hydrogels synthesized with degradable cross-linkers. As expected, G' remained constant for the control hydrogels synthesized with non-degradable PEG-SH 3.4 cross-linker. When comparing the rate of change in G', it is apparent that hydrogels synthesized with PEG-SH 2 8 were associated with the fastest initial degradation rate (~900 Pa/d from day 1 to day 2) and PEG-SH 2 3.4 cross-linkers were associated with the slowest initial degradation (~500 Pa/d from day 1 to day 2). Also, the PEG-SH 2 8 hydrogels were fully degraded at 3 d, whereas the hydrogels made with PEG-SH 2 6 and PEG-SH 2 3.4 required 4 and 5 d, respectively, for complete degradation.

Next we determined the effect of the number of methylene groups between the ester and the thiol moieties in the PEG-diester-dithiol cross-linkers (Figure 2b). Note that data points for this graph were collected every 2 h (as opposed to every d) so that the trend in G' rate of change for the fast degrading gels made with PEG-SH 1 3.4 cross-linker could be followed closely. The PEG-SH 1 3.4 hydrogels exhibited an initial G' of 1400 Pa and were completely degraded after 12 h. Hydrogels synthesized with PEG-SH 1 3.4 degraded at an initial rate of

~100 Pa/h up to 10 h and then reached complete degradation between 10-12 h. Over the same time period, G' remained constant for PEG-SH 2 3.4 and PEG-SH 3.4 hydrogels.

Finally, we explored the dependence of G' and G'' on strain rate at a low angular frequency range of 1-10 rad/s. Both G' and G'' were independent of frequency for all hydrogel types for all time points reported in Figures 2a and 2b. An example of such data for PEG-SH 2 3.4 hydrogels is shown in Figure 2c.

3.2. Swelling experiments and mesh size calculations

We explored the relationship between network composition and the extent of hydrogel cross-linking during degradation by swelling experiments (Figure 3) and mesh size calculations (Table 2). We examined the effects of PEG-diester-dithiol cross-linker molecular weight, number of methylene groups between the ester and the thiol moieties, and the total polymer density.

As with the rheology studies, we first examined swelling ratio, Q_M, for hydrogels with cross-linkers of varying molecular weight and all other parameters constant (Figure 3a). For all hydrogels, Q_M remained constant over the first 2 d of degradation. After day 2, for all hydrogels containing degradable cross-linkers, swelling ratios increased by ~50% before degrading completely. Similar to the rheology results, hydrogels with the PEG-SH 2 3.4 kDa cross-linker remained stable for the longest time, 6 d, whereas the hydrogels with PEG-SH 6 and 8 kDa cross-linkers were completely degraded at 3-4 d; Q_M remained constant for the non-degradable PEG-SH 3.4 hydrogels. We also considered the overall change in Q_M (ΔQ_M) over the course of the experiment. ΔQ_M was defined as the difference in Q_M between the final time point (~75% gel degradation) and the initial time point of the experiment. ΔQ_M was found to be ~16.0 ± 5.5 and independent of the molecular weight of the cross-linker.

In the second experiment, we varied the number of methylene groups between the ester and the thiol of the 3.4 kDa PEG-diester-dithiol (Figure 3b). Again, a non-degradable PEG-SH 3.4 cross-linker was used as a control. Note that data points for this graph were collected every 2 h as well (as opposed to every d) so that the trend in ΔQ_M for the fast degrading gels made with PEG-SH 1 3.4 cross-linker could be followed closely. The degradation was followed for 16 hours shortly after which the hydrogels made with PEG-SH 1 3.4 cross-linker were completely degraded. The swelling ratio for these hydrogels increased linearly ~2-fold for the first 12 h, then stabilized prior to complete hydrogel disruption. At 16 h ΔQ_M was found to be 25.5 ± 3.9, which is greater than the hydrogels made with cross-linker of the same molecular weight but with two methylene groups between the ester and thiol moieties. As expected, Q_M did not change significantly for hydrogels made with PEG-SH 2 3.4 and PEG-SH 3.4 for the duration of the experiment.

Lastly, we determined whether polymer density before cross-linking (i.e., precursor concentration) of the hydrogel affects the swelling ratio and degradation rate. Hydrogels of 5%, 10%, and 15% w/v total polymer were synthesized with PEG-SH 2 3.4 cross-linker (Figure 3c). The most rapid increase in Q_M was exhibited by the 5% w/v hydrogel reaching final Q_M of 52.0 ± 1.6 at day 3, after which hydrogels of this density completely degraded. Both 10% and 15% w/v hydrogels demonstrated similar trends in Q_M with complete degradation occurring at 6-7 d. Furthermore, 15% w/v hydrogels exhibited the highest ΔQ_M of 33 ± 1.9, followed by 5% w/v hydrogels with ΔQ_M of 27.6 ± 0.3 and 10% w/v hydrogels with ΔQ_M of 16.3 ± 2.2.

As expected, mesh size changes followed similar trends as the swelling ratio with degradation. From Table 2 it can be seen that the initial mesh size increased with the increase in molecular weight of the cross-linker. However, at ~75% degradation the trend

was in reverse where the hydrogels made with PEG-SH 2 3.4 cross-linked reached the largest mesh size. The polymer density also showed an effect on the initial mesh size where the hydrogel of 15% polymer density exhibited the lowest mesh size. There was no significant difference in the mesh size at ~75% degradation between the hydrogels of different polymer density. Finally, both the initial and the mesh size at ~75% degradation were dependent on the number of methylene moieties between the thiol and the ester group. Hydrogels made with PEG-SH 1 3.4 had the lowest initial and the largest final (at ~75% degradation) mesh size.

3.3. Gelation time

We tested the effect of reaction pH and cross-linker (type, molecular weight) on PEG hydrogel gelation time (Figure 4). For all hydrogel types gelation time decreased with increase in pH; at pH 8 and above, all hydrogel chemistries had a gelation time of 5 min or less. At pH 6.9-7.5, significant differences in hydrogel chemistry yielded gelation times of up to 24 min and several trends were observed. First, incorporation of an ester bond in the chemical structure of the cross-linker decreased the gelation time as compared to a non-degradable (lack of ester moiety) cross-linker of the same molecular weight; second, the gelation time decreased with decrease in the number of methylene groups between the ester and thiol moieties of the degradable cross-linker; third, the most rapid gelation times were associated with PEG-SH 1 3.4 cross-linker; (Figure 4a). We also found that the gelation time correlated with the molecular weight of the cross-linker (Figure 4b).

3.4. Cytotoxicity experiments

The cytotoxicity experiments with hydrogels suspended in the cell medium in indirect contact with the cells showed that there was no significant change in Balb/3T3 fibroblast viability after 24 h of cell culture (Figure 5). As supported by the results described in Section 3.2, visual inspection of the cultures indicated that the hydrogels were at various stages of degradation during the cytotoxicity experiments. For example, the hydrogels made with PEG-SH 1 3.4 cross-linker were completely degraded by the end of the culture period, whereas the hydrogels made with the non-degradable cross-linkers remained intact.

Moreover, when incubated for 4 h with 1 mM or 10 mM of unreacted cross-linkers, cell viability was not significantly altered as compared to cells seeded in medium only (data not shown).

3.5. Cell viability in 3D PEG hydrogels

When the 3T3/balb fibroblasts were encapsulated within 3D PEG hydrogels, cell viability was retained at 90% or higher for all cross-linker types (Figure 6). The highest cell viability was associated with the PEG-SH 1 3.4 hydrogels. It is important to note that these particular hydrogels were completely degraded at 16 h and therefore the 10 h data point corresponds to the cells still encapsulated in the 3D hydrogel whereas at the 24 h time point the cells were already attached to the bottom of the plate and subjected to the hydrogel degradation products which were still present in the media.

3.6. Influence of adhesive ligand on hydrogel mechanical properties

To determine whether the addition of peptides in the hydrogel structure significantly altered hydrogel physical properties, we first examined the effect of incorporated adhesive ligand RGD on hydrogel G' (Figure 7a). The rheological measurement for the hydrogels made with PEG-SH 1 3.4 cross-linker were taken at 6 h due to rapid degradation (as seen in Figure 2b); for this cross-linker type we found that the hydrogels with RGD had G' 2.3× higher than the hydrogels with no ligand. For the other cross-linkers, the hydrogels were equilibrated for 24

h before analysis due to their relatively prolonged stability. As with the PEG-SH 1 3.4 hydrogels, there was a significant difference between the hydrogels made with and without ligand for the PEG-SH 2 3.4 cross-linker. For PEG 2 6 and PEG 2 8 gels, there was no significant difference in G' for hydrogels with and without RGD ligand.

We also tested the influence of incorporated RGD ligand on hydrogel swelling ratio (Figure 7b). Again the measurements for the hydrogels made with PEG-SH 1 3.4 cross-linker were taken at 6 h and it was noted that hydrogels with covalently attached ligand had lower swelling ratio than the hydrogels without ligand. This difference was statistically significant and also noted for PEG-SH 2 3.4 hydrogels. This effect was not observed for the hydrogels made with the higher molecular weight PEG-SH 2 6 and PEG-SH 2 8 cross-linkers.

4. Discussion

We present a novel series of hydrolytically-degradable PEG hydrogels. We have characterized the hydrogels' degradability, mechanical properties and potential utility as 3D cell scaffolds. Because the ultimate application of these hydrogels relies on predictable rates of cross-linking that occurs under conditions compatible for cell and protein encapsulation, we explored these concepts in depth to yield a scaffolding system with tunable physical properties and known rates of degradation. Below, we first discuss aspects critical to cross-linker and hydrogel synthesis. Then, characterization of the PEG hydrogel series is discussed in relation to variables of the hydrogel cross-linking reaction.

Though the cross-linker and hydrogel synthesis procedures are relatively straightforward, care must be taken to ensure hydrogels with reproducible properties. All synthesized materials were sensitive to humidity and oxygen and thus were stored under inert gas at −20°C at all times. The aliquot of PEG-VS in pH 8 TEA was found to be stable for over a year without reducing its reactivity. However, aliquots of PEG-diester-dithiol or non-degradable PEG-dithiol (also in TEA, pH 8) were found to have very limited stability (~min) and thus were used immediately upon preparation. This instability is due to the chemical structure of the PEG-diester-dithiols and the non-degradable PEG-dithiol. In basic solution, the ester bond rapidly hydrolyzes and the free thiols form disulfide bonds due to thiol deprotonation and conversion to a more reactive thiolate group.

In order to achieve tunable hydrogel properties, we designed cross-linkers to impart three mechanisms for manipulating hydrogel degradation time. First, to test the hypothesis that degradation time will be inversely proportional to cross-linker molecular weight, we designed cross-linkers based on PEG of three different molecular weights, 3.4, 6 and 8 kDa. Second, our cross-linkers were designed to contain hydrolytically degradable ester bonds and it is well-known that the environment local to the ester impacts the rate of hydrolysis. Thus, our second strategy was to vary the number of methylene moieties between the ester and the thiol group of the cross-linker, hypothesizing that this approach will provide greater control over hydrogel degradation rate. And lastly, we speculated that hydrogel degradation time will be proportional to polymer density and therefore tested total polymer densities of 5%, 10%, and 15% w/v. With these strategies, we have achieved hydrogel degradation times that span from several hours to several days. As shown by our experiments, the presented materials demonstrated degradation times comparable to other available PEG hydrogels. For instance, PEG-diacrylate hydrogels also formed by Michael-type addition via cross-linking with DTT had shown degradation times of up to 21 d[39] and PEG-PLA of increasing number of lactoyl repeat units had shown degradation times of 4 to 17 d respectively.[12]

We were aware that these cross-linker and hydrogel parameters will also affect certain physical properties of the hydrogel including but not limited to elasticity (G'), swelling ratio

and mesh size. Hence, we monitored hydrogel degradation by measuring these parameters at predetermined time intervals, which allowed further insight into the interplay between initial hydrogel structure, degradation kinetics, and hydrogel physical properties known to impact cell function and protein diffusivity. For example, hydrogel stiffness influences cell proliferation, motility and morphology.[40] Thus, hydrogel G' was an important parameter for characterization and also was used to indirectly measure the extent of hydrogel degradation over time. The values of initial G' for all cases described below was in the range of 1000 – 3000 Pa, which correlated with values reported in the literature for similar systems. For example, 10% w/v hydrogels made with 20 kDa 4-arm PEG-VS (as opposed to 10 kDa for our system) and MMP-2 sensitive peptides exhibited G' of 290 Pa[30, 36] while 40% w/v hydrogels made of 14.8 kDa 4-arm PEG-acrylate and 3.4 kDa PEG-dithiol (same molecular weight as some of our cross-linkers) exhibited a G' of 10 000 Pa.[3]

As shown by our experiments, the G' decreased for all hydrogel types as the degradation proceeded. This was an expected behavior since G' is directly related to cross-link density. (Degradation of the hydrogel leads to a lower cross-link density, resulting in a lower G'). Based on the non-linear dependence of G' on degradation time, we could speculate that the hydrogels undergo bulk degradation due to their high water content. From our swelling data, we calculated that all tested PEG hydrogels were highly hydrophilic and contained ~96% of water upon complete swelling. When exposed to water the PEG chains are subjected to random scission at the ester bonds and each ester bond has the same probability of being broken via hydrolysis. As the hydrogel degrades, the water content increases, which further promotes the rate of hydrolysis. Thus for equivalent chemical structures, the rate of hydrolysis should depend on the water content of the swollen network and number of hydrolyzable groups.

We found that cross-linker molecular weight was directly correlated with hydrogel degradation rate and hence inversely proportional to the rate of change in G' as well as initial G' (Figure 2a). These trends, also observed by others,[41] could be explained by the lower cross-linking density resulting from the higher molecular weight cross-linkers since all of the tested hydrogels were 10% w/v in total polymer density and a stoichiometric ratio of VS to SH groups. In addition, each cross-linker would have one ester at each end separated by a long PEG chain. Therefore, a lower cross-link density would correspond to a lower total concentration of ester bonds. The calculated theoretical concentration of ester groups for PEG-SH 2 3.4, PEG-SH 2 6 and PEG-SH 2 8 was 23.5 mM, 18.3 mM, and 15.5 mM respectively. Because fewer ester bonds are present in hydrogels synthesized with higher molecular weight cross-linkers, these hydrogels degrade faster and have greater rates of change in G'. Additionally, this trend could also be explained by the fact that in order for a cross-linker to be completely released from the hydrogel structure, two ester bonds need to be hydrolyzed. For the 4-arm PEG polymer to be released, four ester bonds need to be hydrolyzed. Therefore, a change in G' may only be measurable when sufficient ester bond hydrolysis had occurred and the rate of change in G' would increase further as sufficient PEG diffusion had occurred.

We also found that degradation and hence G' was strongly affected by the number of methylene units between the ester and the thiol moieties of the cross-linker (Figure 2b). The interplay of several factors can explain this phenomenon. It is well known that the hydrophobicity of the ester environment affects the rate of hydrolysis. By increasing the number of methylene groups, we effectively increased the hydrophobicity of the group adjacent to the ester making it less accessible to water. In addition, due to inductive effects, the carbonyl group of the glycolate (one methylene group) is more acidic (pKa, 7.68)[42] than that of the propionate (two methylene groups; pKa, 10.48)[43], rendering the glycolate more susceptible to nucleophilic attack and resulting in more rapid hydrolysis as compared to the

propionate. In conformity with our findings, it has been noted previously that increase in the number of carbons from 1 to 2 between a thiol and an ester in a thiol-acrylate polymers decreases the rate of hydrolysis 3-fold.[28] Schoenmakers *et. al.*[27] has also reported hydrolysis rate occurring as a function of the number of methylene groups present between a thiol and an ester. Comparing between three and four methylene groups, the probability of hydroxyl attack on the carbonyl group was attributed to the atomic charge on the carbon atom of the carbonyl group: as the number of methylene groups increased, the atomic charge and therefore the hydrolysis of the ester decreased. Through molecular modeling it was shown that the atomic charge on the carbonyl carbon decreased as the number of methylene groups increased rendering the ester less hydrolyzable. Therefore, we speculate that additional increase in the number of methylene groups between the thiol and ester groups in our in-house synthesized PEG-diester-dithiol cross-linkers would lead to further decrease in the degradation rate and thus further impacting the tunability of our materials.

We also showed that both G' (the elastic component) and G'' (the viscous component) were independent of frequency in the low frequency range studied (Figure 2c). This finding confirmed that the hydrogels were fully cross-linked and swollen at the time of the measurements. Further, the value of G' was 2 – 2.3 orders of magnitude larger than the value of G'', indicating that the PEG hydrogels remained intact and elastic during the experiment.

In addition, hydrogels synthesized with cross-linkers containing esters were correlated with decreased values of G' as compared to hydrogels made with the non-degradable PEG-dithiol (PEG-SH 3.4). This effect may be explained by the fact that the carbonyl oxygen of the ester is a strong hydrogen bond acceptor, which may increase the water content in the hydrogel network and result in increased initial swelling ratios. Further, G' is inversely proportional to the polymer volume fraction, v_2 and Q_M,[19] thus supporting the fact that hydrogels containing ester groups had lower initial values of G'.

We also monitored degradation indirectly by investigating the change in swelling ratio of the hydrogels. The initial values of Q_M were in the range of 18 – 30 for the various hydrogel systems studied, which correlated well with values reported in the literature for similar systems. For example, ~ 100% w/v hydrogels made of 10 kDa 8-arm PEG-VS and DTT (0.15 kDa) exhibited Q_M of 5[39] while 10% w/v hydrogels made of 4-arm PEG-acrylate and 3.1 kDa PEG-dithiol exhibited a Q_M of 19.[23]

Based on their inverse proportionality, the same factors discussed above that contributed to the decrease in G' would also contribute to the increase in Q_M. Moreover, swelling ratio is a measure of the hydrophilicity of the polymer and was also used to calculate the mesh size of the hydrogels. We found that hydrogels synthesized with cross-linkers of greater molecular weight (e.g., 6 and 8 kDa) were associated with a slight increase in initial swelling ratio, but this difference became more pronounced as the degradation proceeded (Figure 3a); this relationship is in agreement with the G' findings discussed above. It is interesting to note that varying cross-linker molecular weight from 3.4 to 8 kDa resulted in ~2× change both in the hydrogels' degradation times as well as in ΔQ_M (Figure 3a), but the effect of the number of methylene units had even more pronounced effect (Figure 3b): at 16 h, hydrogels made with PEG-SH 1 3.4 had a 2× greater swelling ratio and had degraded completely whereas the PEG-SH 2 3.4 hydrogels required 6 d to achieve the same change in properties. We also confirmed that polymer density could be exploited to control hydrogel degradation rate and Q_M. As expected,[44] both swelling ratio and degradation rate were greater in gels synthesized with greater polymer density (Figure 3c).

Cross-linker type not only altered the PEG hydrogel degradation profiles and mechanical properties but also affected gelation times. Acknowledging that Michael-type addition cross-

linking reaction is very susceptible to pH of the environment we determined gelation time at various pH for hydrogels made with the different cross-linkers (Figure 4). Our findings were in agreement with work carried out by Lutolf et al. where rheological measurements were used to establish that gelation time was inversely correlated with pH for gels made with PEG-VS and cysteine functionalized peptide cross-linkers.[24] For our PEG-VS hydrogels cross-linked with PEG-diester-dithiol, gelation time was also inversely correlated with pH (Figure 4a). Under acidic conditions (pH 6.9) the gelation time was on the order of minutes, and decreased to seconds under basic conditions (pH 10). All hydrogels behaved similarly except the hydrogels made with PEG-SH 1 3.4 cross-linker, which gelled in the matter of seconds at all pH conditions possibly due to inductive effects associated with the closer proximity of the ester to the thiol moiety within this cross-linker. We also observed that gelation time correlated with cross-linker molecular weight (Figure 4b); this trend could be explained by the increased entropy of activation due to the increased polymer chain flexibility and rotation. For our work, we found pH 7.4-8 to be optimal as these conditions are cytocompatible and result in gelation times of 10 min or less for all cross-linker types.

To evaluate the feasibility of future use of these hydrogels as tissue engineering scaffolds, we performed several cytotoxicity experiments. First, we cultured Balb/3T3 fibroblasts in indirect contact with the degradable hydrogels and found that there was not a significant difference in cell viability for any of the hydrogel types (Figure 5). It should be noted that during the 24-h culture period, the hydrogels underwent different rates of degradation and the hydrogels made with PEG-SH 1 3.4 cross-linker had completely degraded. The cell viability at the time of the measurement was 90% or above indicating that neither the products of degradation nor any byproducts of the synthesis were toxic to the cells. In a separate experiment we tested the toxicity of the unreacted cross-linkers in cell culture medium and found relevant concentrations of the cross-linkers to be non-toxic to cells as well.

Most of the hydrogel characterizations for this project were carried out for the simplest case when no biological factors were added to the gels. We chose this strategy to first unveil major structure-function trends and begin to identify favorable conditions for cell and protein encapsulation. However, we recognize that PEG is inert and therefore not fit to sustain the survival and proliferation of anchorage-dependent cells. Thus, to demonstrate that basic biological functionality can be incorporated into the hydrogels, we chose to implement one of the most commonly used polypeptide sequences RGD[45] to promote cell adhesion in the hydrogels. When encapsulated within the RGD-modified hydrogels synthesized with all types of degradable PEG-diester-dithiol cross-linkers, fibroblasts demonstrated >90% viability after 10 h or 24 h of culture (Figure 6) suggesting that all of the reported hydrogels are equally suitable as materials for tissue engineering scaffolds. We note that when adhesive ligand was omitted from the hydrogels, the cell viability dropped to 1.6% (data not shown) further supporting the assumption that PEG provided the structural backbone of the hydrogel scaffold but is completely devoid of biological activity, hence offering a potential for independent control of physical and biological properties of the system. Additionally, note that hydrogels made with PEG-SH 1 3.4 cross-linker were still present at 10 h but had completely degraded in 24 h and the cells had retained high viability. These results indicate that fibroblasts survived encapsulation and provide further evidence that the components released from degrading hydrogels are not toxic.

Lastly, we tested if the incorporated RGD ligand influences the hydrogel mechanical properties. We found that the effect of the ligand covalently bound to the hydrogel structure was closely related to the molecular weight of the hydrogel cross-linker. For the lower molecular weight cross-linker (3.4 kDa) the covalent addition of ligand affected hydrogel storage modulus (Figure 7a) and swelling ratio (Figure 7b); however, this effect was not

noticeable for hydrogels made with higher molecular weight cross-linkers (6 and 8 kDa). We hypothesize that the added electrostatic charge associated with the RGD ligand may be related to the altered hydrogel properties and further investigation has been undertaken[46] in order to confirm this hypothesis or determine other possible mechanisms that underlie this behavior.

5. Conclusions

We synthesized a degradable synthetic PEG hydrogel and demonstrated that these materials possess tunable rates of degradation and mechanical properties. By keeping the basic structure of the hydrogel repeat units and functional groups constant, but altering parameters such as molecular weight, polymer density, distance between thiol and ester group in the cross-linker, we can control the hydrogel properties while maintaining cross-linking and degradation conditions that are compatible for cell and protein encapsulation. Lastly, we showed that covalent addition of a cell-adhesive peptide ligand to our system supported cell viability in 3D culture, but in some cases also affected hydrogel mechanical properties. This report serves as the foundation for in-depth follow-up studies investigating the role of covalently bound ligands on gel mechanical properties as well as relationships between gel properties and cell responses related to soft tissue repair such as nerve regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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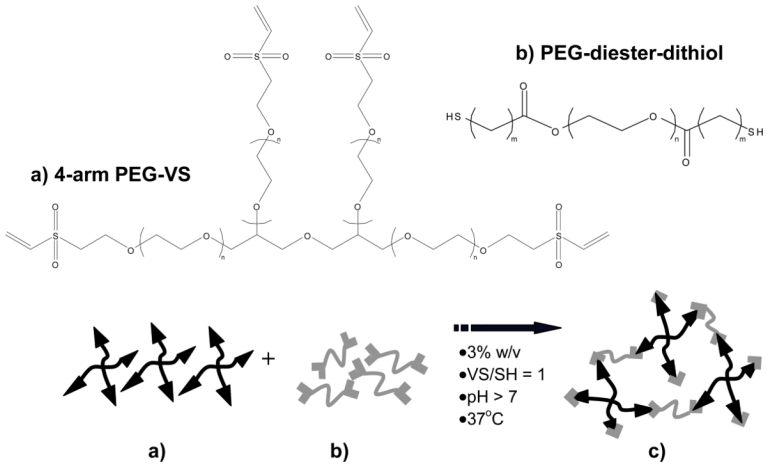

Figure 1.

Schematic for PEG hydrogel cross-linking reaction. a) a 4-arm PEG-VS precursor polymer solution is mixed with b) PEG-SH ester cross-linker at a VS to SH molar ratio of 1:1 to give c) a 3D hydrogel which is formed at physiological conditions. For 4-arm PEG-VS of molecular weight 10 kDa, the number of the repeat PEG unit (n) = 56. For PEG-dithioglycolate m = 1 and for PEG-dithiopropionate m = 2. Detailed chemical structures of the PEG components, cross-linking reaction and the degradation products are also provided in the Supporting Information.

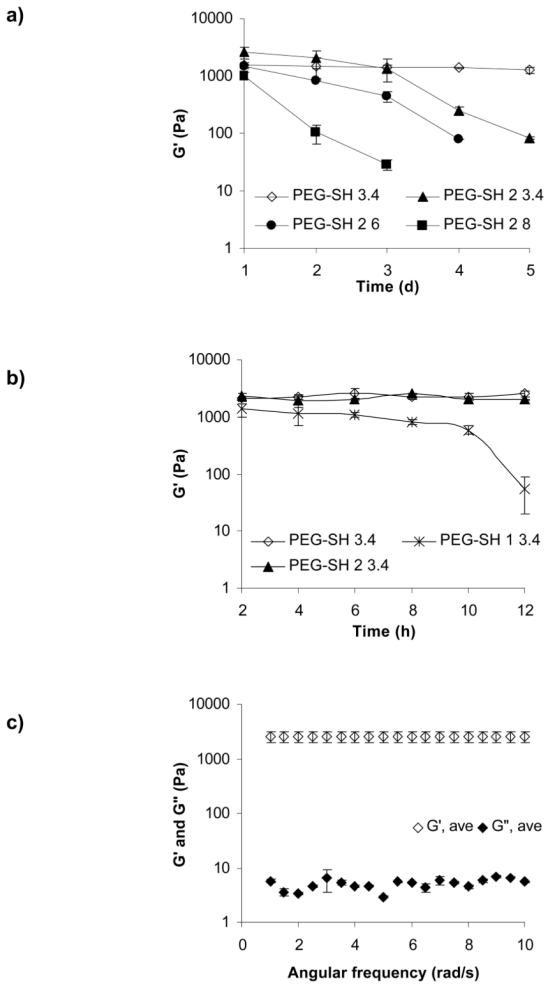

Figure 2.

Rheological assessment of PEG hydrogel degradation: Storage modulus (G′) monitored as a function of cross-linker molecular weight (a) and number of methylene moieties between the ester and the thiol group of the cross-linker (b). Abbreviations of PEG hydrogel names are given in Table 1; data shown was collected at 1 rad/s. Representative data for G′ and loss modulus (G″) as a function of angular frequency is shown for PEG-SH 2 3.4 hydrogels that were equilibrated in 10 mM PBS, pH 7.4 at 37°C for 24 h (c). Symbols represent the average ± standard deviation for n=6 samples; lines connecting data points are provided to guide the eye.

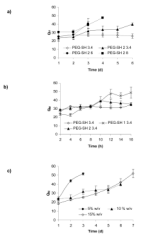

Figure 3.
Swelling measurements during PEG hydrogel degradation: Swelling ratio monitored as a function of cross-linker molecular weight (a) and number of methylene groups between the ester and the thiol group of the cross-linker (b). Swelling ratio was also examined as a function of total polymer density using PEG-SH 2 3.4 hydrogels (c). Symbols represent the average ± standard deviation for n=6 samples; lines connecting data points are provided to guide the eye.

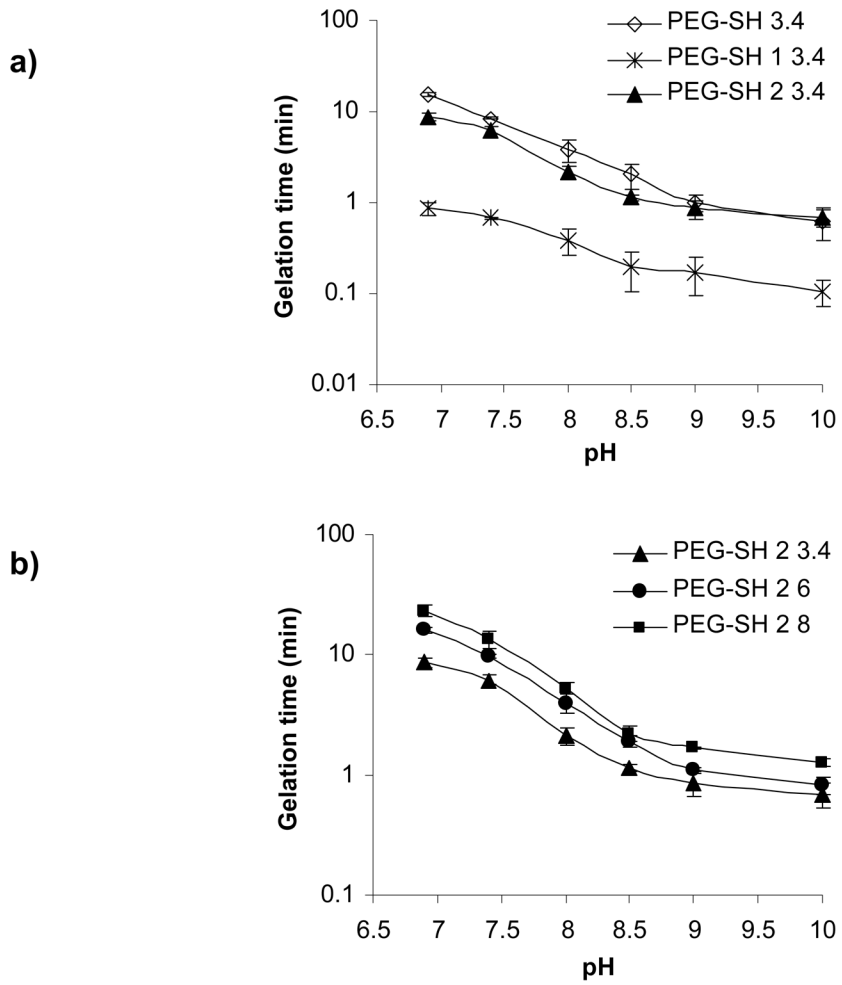

Figure 4.

Effect of reaction pH on gel transition time: gelation time was found to depend on the number of methylene groups between the thiol and the ester moiety of the cross-linker (a) as well as molecular weight of the cross-linker (b). Symbols represent the average ± standard deviation for n=3 samples; lines connecting data points are provided to guide the eye.

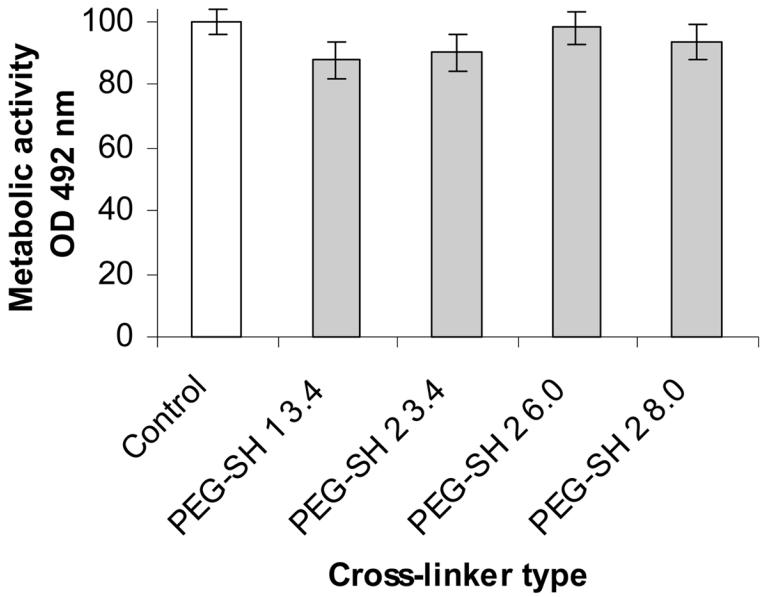

Figure 5.

Assessment of hydrogel cytotoxicity. Gels were cultured in indirect contact with 3T3/balb fibroblast monolayer culture for 24 h and then viability was assessed with a standard MTS assay. The OD at 492 nm which is representative of the cells' metabolic activity was normalized by the medium-only control. No significant differences were found between the cultures containing hydrogels and those containing medium alone. Bars represent the average ± standard deviation for n=6 samples.

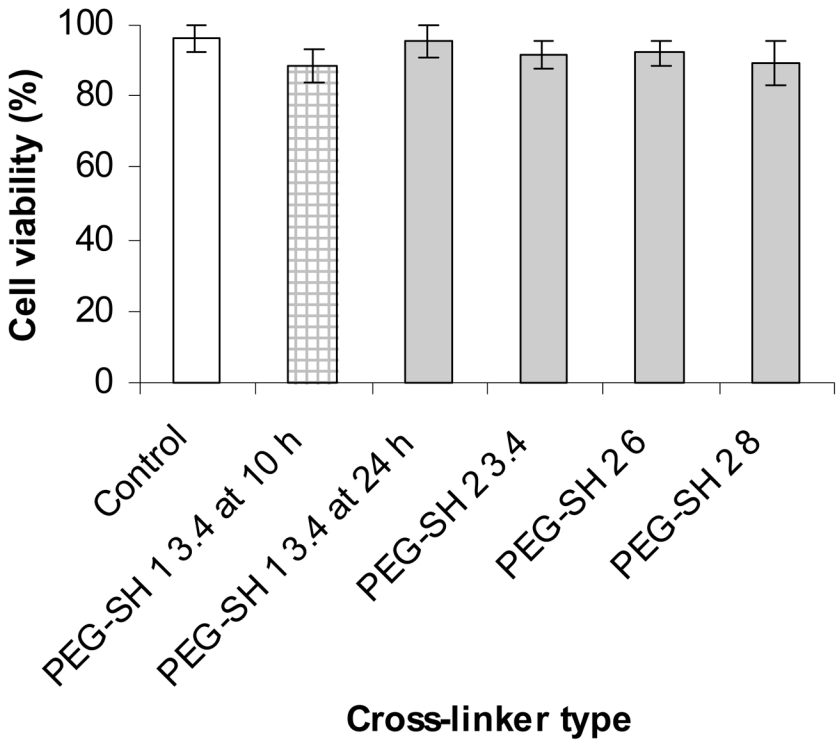

Figure 6.
Cell viability in degradable 3D PEG hydrogels containing 100 μM RGD. The control cells were seeded directly onto the bottom of the well plate. The LIVE/DEAD assay was performed after 24 h of cell culture. No significant differences were found between treatments. At 24 h, the PEG-SH 1 3.4 gels were completely degraded. Bars represent the average ± standard deviation for n=6 samples.

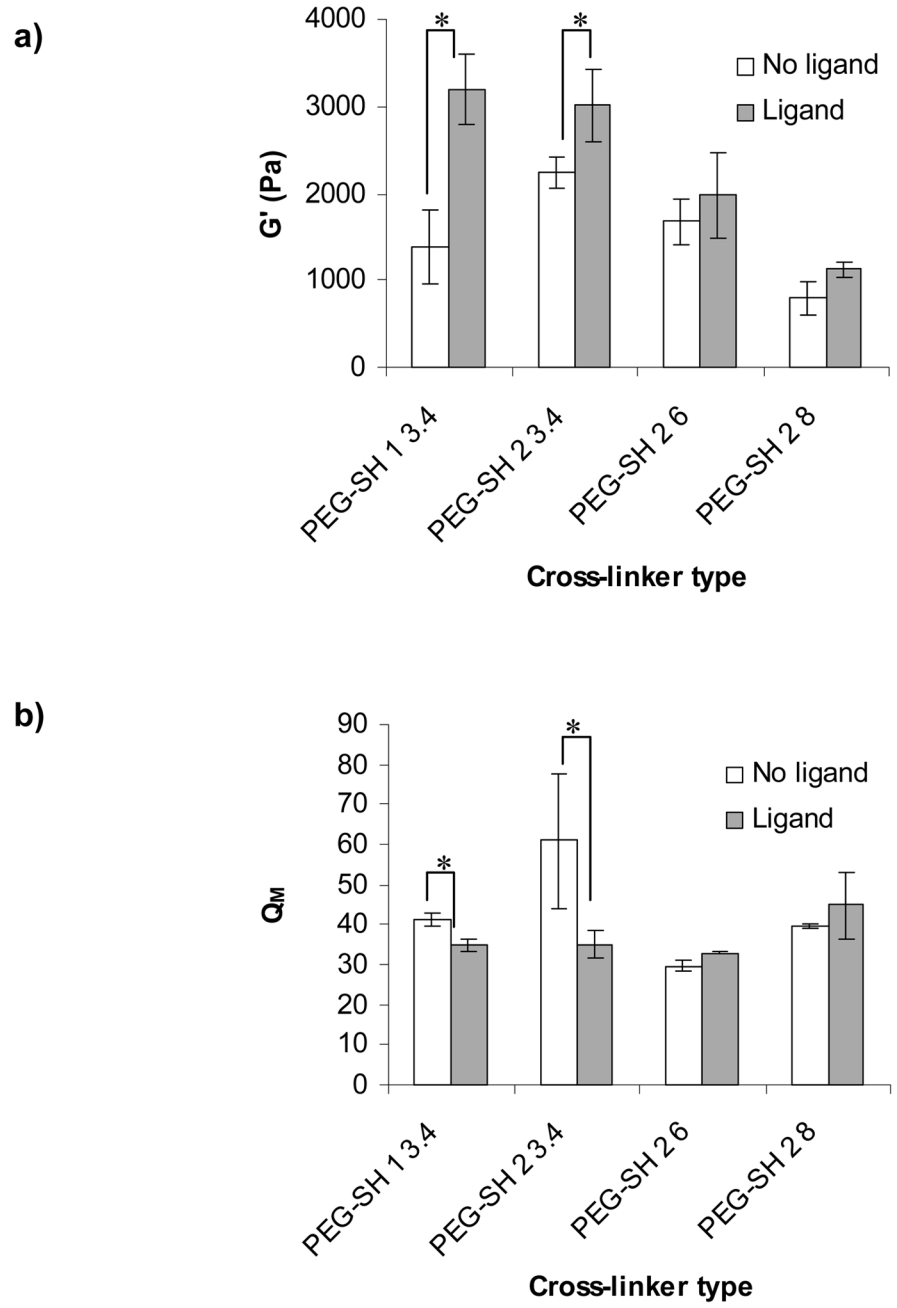

Figure 7.

Influence of covalently-incorporated peptide ligand on hydrogel physical properties. Hydrogels were synthesized with and without 100 μM RGD and were characterized for *G′* at 1 rad/s (a) and swelling ratio (b). Bars represent average ± standard deviation for n=9 samples. Asterisks note statistical differences between results.

Table 1

Summary of the abbreviations, characteristics and properties of the PEG-diester-dithiol and non-degradable PEG-dithiol cross-linkers examined. All of the degradable cross-linkers were synthesized in-house.

Cross-linker name	Molecular weight (kDa)	Number of methylene groups between thiol and ester moieties	Degradable
PEG-SH 3.4	3.4	0	no
PEG-SH 1 3.4	3.4	1	yes
PEG-SH 2 3.4	3.4	2	yes
PEG-SH 2 6	6	2	yes
PEG-SH 2 8	8	2	yes

Table 2

Summary of the calculated mesh size for all hydrogel types at an early degradation state of 2 or 24 h (noted with one asterisk - *) and at approximately 75% degradation (noted with two asterices - **). Mesh size was calculated from swelling ratio data presented in Figure 3.

Molecular weight of cross-linker (kDa)	**Initial mesh size (nm)***	**Mesh size (nm) at ~75% degradation****
3.4 (non-degradable control)	14.1 ± 0.1	14.2 ± 0.7
3.4	13.6 ± 0.8	21.2 ± 0.2
6	15.0 ± 1.8	18.8 ± 1.2
8	17.6 ± 0.4	17.2 ± 0.3
Polymer density (% w/v)		
5%	14.1 ± 0.3	19.1 ± 0.2
10%	14.0 ± 0.2	17.3 ± 0.9
15%	11.9 ± 0.3	19.1 ± 0.7
Number of methylene groups between thiol and ester		
0 -CH_2- groups (non-degradable)	14.4 ± 0.5	16.2 ± 0.3
1 -CH_2- group	13.5 ± 0.3	18.6 ± 0.7
2 -CH_2- groups	15.0 ± 0.1	16.4 ± 0.4