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Mechanically tunable, human mesenchymal stem cell viable poly(ethylene glycol)-oxime hydrogels with invariant precursor composition, concentration, and stoichiometry

Rodger A. Dilla[#], Yanyi Xu[#], Zachary K. Zander[#], Neil Bernard[#], Clinton G. Wiener[†], Bryan D. Vogt[†], Matthew L. Becker[#]

[#]The University of Akron, Department of Polymer Science, 44325, USA

[†]The University of Akron, Department of Polymer Engineering, 44325, USA

Abstract

Hydrogels are used widely for exploratory tissue engineering studies. However, currently no hydrogel systems have been reported that exhibit a wide range of elastic modulus without changing precursor concentration, identity, or stoichiometry. Herein, ester and amide-based PEG-oxime hydrogels with tunable moduli (~5–30 kPa) were synthesized with identical precursor mass fraction, stoichiometry, and concentration by varying the pH and buffer concentration of the gelation solution, exploiting the kinetics of oxime bond formation. The observed modulus range can be attributed to increasing amounts of network defects in slower forming gels, as confirmed by equilibrium swelling and small angle neutron scattering (SANS) experiments. Finally, *h*MSC viability was confirmed in these materials in a 24 h assay. While only an initial demonstration of the potential utility, the controlled variation in defect density and modulus is an important step forward in isolating system variables for hypothesis-driven biological investigations.

Graphical Abstract

Corresponding Author: Matt Becker: becker@uakron.edu, Bryan Vogt: vogt@uakron.edu.

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Supporting Information

The Supporting Information is available at DOI: xx. USANS data, NMR and mass spectra of precursors, experimental details for synthesis, gel swelling, neutron scattering, and rheology, frequency and amplitude sweeps.

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Keywords

hydrogels; tissue engineering; peptide; microstructures; hMSC

Introduction

Tissue engineering requires the use of a degradable scaffold that mimics natural physiological architectures and properties while simultaneously directing and promoting stem cell proliferation and differentiation.^[1] Poly(ethylene glycol)-based (PEG) hydrogels have been used widely for these efforts and have incorporated a number of reactive end groups to facilitate crosslinking and network formation including photo-crosslinked acrylates,^[2] azide/alkyne “click” reactions,^[3] and thiol-ene Michael additions.^[4] The control over structure, porosity, ease of chemical functionalization and tunable mechanical properties enable the mimicking the extracellular matrix (ECM) within soft tissues.^[5–10] Furthermore, PEG hydrogels have been used to culture a number of human cell lineages including human mesenchymal stem cells (hMSCs).^[11–14] hMSCs are undifferentiated stem cells that have been shown to vary their lineage commitment depending on the local mechanical environment they are cultured on.^[15–20] Engler *et al.* showed that hMSC differentiation was tightly correlated to the elastic modulus of a series of polyacrylamide hydrogel with different crosslink densities.^[21] Khetan *et al.* demonstrated that hMSCs encapsulated in degradable hydrogels exhibited cell spreading dependency on the local cell-mediated gel degradation and thereby influenced lineage commitment.^[22] However, these changes in modulus tend to be accompanied by changes in the chemical composition, which creates challenges in understanding the exact influence of modulus on hMSC differentiation.

We recently demonstrated one route to tune the mechanical properties of a PEG hydrogel over a narrow range without compositional changes by exploiting a kinetically-controlled oxime crosslinking reaction.^[23] The storage modulus could be tuned (20–30 kPa) by varying the pH and buffer concentration of the gelation solution. The change in modulus was directly correlated to microstructure defects (elastically-ineffective loops and dangling ends) within the hydrogel. These defects are intrinsically governed by the kinetics of oxime bond

formation and the diffusion of the chain ends within the network. While the variation in modulus was small, to the best of our knowledge, this hydrogel system was the first of its kind. Herein, we report the expansion of the modulus variation to a translationally relevant range using this kinetically controlled gelation process. We further examine the structure-property relationship of this gel system with an emphasis on the swollen gels, both mechanically and through neutron scattering experiments, as well as kinetics of oxime bond formation and *h*MSC cytocompatibility.

Materials and Methods

Materials.

The 4-arm, 10K polyethylene glycol was purchased from Creative PEGWorks (Chapel Hill, NC). All other commercial reagents and solvents were purchased from Sigma-Aldrich or Fisher Scientific and used as received unless noted otherwise. All reactions were performed under nitrogen unless noted otherwise.

Instrumentation.

Proton ^1H -NMR (300 MHz) and ^{13}C carbon (75 MHz) spectra were obtained using a Varian NMRS 300 MHz spectrometer. All chemical shifts are reported in ppm (δ), and referenced to the chemical shifts of residual solvent resonances (^1H -NMR, CDCl_3 7.26 ppm or $\text{DMSO}-\text{D}_6$ 2.49 ppm). Abbreviations for multiplicities are listed as following: s = singlet, d = doublet, t = triplet, br = broad singlet, m = multiplet.

Mass spectrometry for the 4-arm keto-PEG was performed using a Bruker UltraFlex III MALDI tandem time-of-flight (TOF/TOF) mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with a Nd:YAG laser emitting at 355 nm. The matrix and cationization salt were DCTB (2-[(2E)-3-(4-tert-butylphenyl)-2-methylprop-2-enylidene]malonitrile) and sodium trifluoroacetate, respectively. Solutions of the matrix (20 mg/mL) and cationizing salt (10 mg/mL) were prepared in THF and the polymer sample (10 mg/mL) was prepared in water. The matrix and cationizing agent solutions were mixed in 10:1 (v/v) ratio and applied to the target. After drying, a spot of the sample was applied, followed by an additional drop of the matrix/cationizing agent. For the 10K 4-arm PEG-aminooxy species, the spectrum was collected using a Bruker HCTultra II quadrupole ion trap (QIT) mass spectrometer (Billerica, MA) equipped with an ESI source.

The pH values of buffers were tested using an Orion® 350 PerpHecT® benchtop pH meter with an Orion® ROSS® Sure-Flow pH electrode at room temperature.

Synthesis of ω -4-oxopentanoate-star-poly(ethylene glycol)(keto-PEG_{ester}).

In a dry, nitrogen-purged 250 mL round bottom flask, 4-arm, 10K PEG-OH (8.01 g, 0.801 mmol, 1.00 eq.) of was dissolved in 15 mL of CH_2Cl_2 , followed by the addition of levulinic acid (0.4645 g, 4.00 mmol, 5.00 eq.) and 4-(dimethylamino)pyridinium 4-toluene sulfonate (DPTS) (0.110 g, 0.374 mmol, 0.500 eq.). The solution was stirred vigorously until the reagents were dissolved and was subsequently cooled to 0 °C in an ice bath for 15 minutes, followed by the dropwise addition of N,N'-diisopropyl carbodiimide (DIC) (0.62 mL, 4.00

mmol, 5.00 eq., 0.815 g/mL). The reaction was allowed to warm to ambient temperature with stirring and nitrogen gas over 24 h. Following completion of the reaction, the solution was gravity filtered with CH_2Cl_2 and precipitated into cold methanol, followed by centrifugation at 5,000 rpm for 3 minutes. The pellet was then dried, re-dissolved in CH_2Cl_2 and precipitated two additional times in methanol to remove any urea by-product. The product was then precipitated in diethyl ether an additional three times and was dried under high vacuum to obtain a fluffy, white solid (6.61 g, 79 % yield). Figure S2: ^1H NMR (300 MHz, CDCl_3 , 298 K): δ = 2.19 (s, 3H, $-\text{COCH}_3$), 2.60 (t, $^3J_{\text{H-H}} = 22$ Hz, 2H, $-\text{O}_2\text{CCH}_2\text{CH}_2\text{COCH}_3$), 2.75 (t, $^3J_{\text{H-H}} = 22$ Hz, 2H, $-\text{O}_2\text{CCH}_2\text{CH}_2\text{COCH}_3$), 3.41 (t, $^3J_{\text{H-H}} = 6$ Hz, 2H, $-\text{CH}_2\text{O}_2\text{CCH}_2$) and 3.64 ppm (s, 224H, $[\text{OCH}_2\text{CH}_2]_{56}$).

Synthesis of ω -O-(carboxylatemethyl)hydroxylamine-star-poly(ethylene glycol)(PEG_{Gester}-aminooxy).

In a dry, nitrogen-purged 250 mL round bottom flask, 4-arm, 10K PEG-OH (5.00 g, 0.500 mmol, 1.00 eq) of were dissolved in 15 mL of CH_2Cl_2 , followed by the addition of (bocaminooxy) acetic acid (0.48 g, 2.5 mmol, 5.00 eq) and DPTS (0.074 g, 0.251 mmol, 0.5 eq). The solution was stirred vigorously until all reagents were dissolved and was subsequently cooled to 0 °C in an ice bath for 15 minutes, followed by the dropwise addition of 0.315 g (2.49 mmol, 5 eq, 0.815 g/mL) of DIC. The reaction was allowed to warm to room temperature with stirring and nitrogen gas over 24 h. Following the completion of the reaction the solution was gravity filtered with CH_2Cl_2 . The solution was then cooled in a -20 °C freezer overnight to precipitate the urea-byproduct and was gravity filtered before precipitating into methanol and then ether. The precipitate was then dried under reduced pressure to give a white, fluffy intermediate product. Figure S3: ^1H NMR (300 MHz, CDCl_3 , 298 K): δ = 1.54 (s, 9H, $\text{COC}(\text{CH}_3)_3$), 3.62 (s, 224H, $[\text{OCH}_2\text{CH}_2]_{56}$), 4.38 (t, $^3J_{\text{H-H}} = 16$ Hz, 2H, $-\text{CH}_2\text{CH}_2\text{O}_2\text{C}$), 4.91 (s, 2H, $-\text{O}_2\text{CCH}_2\text{ONH}$). The intermediate product was then dissolved in ~12 mL of 4M HCl/Dioxane under nitrogen for 24 h to remove the boc protecting group and was subsequently precipitated into cold diethyl ether, centrifuged, and dried to give the fluffy white solid product (3.86 g, 74.9 % yield). Figure S4: ^1H NMR (500 MHz, CDCl_3 , 298 K): δ = 3.62 (s, 224H, $[\text{OCH}_2\text{CH}_2]_{56}$), 4.38 (t, $^3J_{\text{H-H}} = 6$ Hz, 2H, $-\text{CH}_2\text{CH}_2\text{O}_2\text{C}$), 4.91 (s, 2H, $-\text{O}_2\text{CCH}_2\text{ONH}_2$) Figure S5: ESI-MS m/z : M_n for the functionalized species was 10026.76 Da, a mass difference of 419.11 Da from the precursor with M_n of 9607.643 Da. This difference corresponds to the mass of the $\text{C}_5\text{H}_8\text{O}_4$ pentaerythritol core + 4 $\text{C}_2\text{H}_4\text{O}_3\text{N}$ aminooxy functionalities – 4* H_2O .

Synthesis of ω -4-oxopentanamide-star-poly(ethylene glycol)(keto-PEG_{amide}).

In a dry, nitrogen-purged 250 mL round bottom flask, 5.01 g (0.49 mmol, 1 eq.) of 4-arm, 10K PEG-NH₂ were dissolved in 25 mL of CH_2Cl_2 , followed by the addition of 0.348 g (3 mmol, 6 eq.) of levulinic acid and 0.4323 g (3 mmol, 6 eq.) of HOBt. The solution was stirred vigorously until all reagents were dissolved and was subsequently cooled to 0 °C in an ice bath for 15 minutes, followed by the dropwise addition of 0.379 g (3 mmol, 6 eq.) of DIC. The reaction was allowed to warm to room temperature with stirring and nitrogen gas over 24 h. Following completion of the reaction, the solution was gravity filtered with CH_2Cl_2 and dried under reduced pressure. The dry product was dissolved in 10 mL solution of $\text{H}_2\text{O}:\text{MeOH}$ and dialyzed in a 1K dialysis bag against water for 2 days. The product was

then freeze dried to produce a fluffy, white solid. (4.11 g, 79 % yield). Figure S7: ^1H NMR (300 MHz, CDCl_3 , 298 K): δ = 2.18 (s, 3H, $-\text{COC}\underline{\text{H}}_3$), 2.44 (t, $^3\text{J}_{\text{H-H}} = 22$ Hz, 2H, $-\text{ONHCC}\underline{\text{H}}_2\text{CH}_2\text{COCH}_3$), 2.79 (t, $^3\text{J}_{\text{H-H}} = 22$ Hz, 2H, $-\text{ONHCC}\underline{\text{H}}_2\text{CH}_2\text{COCH}_3$), and 3.64 ppm (s, 224H, $[\text{OC}\underline{\text{H}}_2\text{CH}_2]_{56}$).

ω -O-(carboxylamidemethyl)hydroxylamine-star-poly(ethylene glycol)(PEG_{amide-aminooxy}):

In a dry, nitrogen-purged 250 mL round bottom flask, 5.0 g (0.49 mmol, 1 eq) of 4-arm, 10K PEG-NH₂ were dissolved in 25 mL of CH_2Cl_2 , followed by the addition of 0.574 g (3 mmol, 6 eq) of (boc aminooxy) acetic acid and 0.432 g (3 mmol, 6 eq) of HOBt. The solution was stirred vigorously until all reagents were dissolved and was subsequently cooled to 0 °C in an ice bath for 15 minutes, followed by the dropwise addition of 0.379 g (3 mmol, 6 eq.) of DIC. The reaction was allowed to warm to room temperature with stirring and nitrogen gas over 24 h. Following the completion of the reaction the solution was gravity filtered with CH_2Cl_2 and dried under reduced pressure overnight. The dry product was then dissolved in a 10 mL solution of 80:20 H₂O:MeOH and dialyzed against water for 2 days before freeze drying to give the white intermediate. Figure S8: ^1H NMR (300 MHz, CDCl_3 , 298 K): δ = 1.48 (s, 9H, $\text{COC}(\underline{\text{C}}\text{H}_3)$) 3.64 (s, 224H, $[\text{OC}\underline{\text{H}}_2\text{CH}_2]_{56}$), 4.33 (s, 2H, $-\text{ONHCC}\underline{\text{H}}_2\text{ONH}$). The intermediate product was then dissolved in ~12 mL of 4M HCl/Dioxane under nitrogen for 24 h to remove the boc protecting group and was subsequently precipitated into cold diethyl ether, centrifuged, and dried to give the fluffy white solid product (3.58 g, 70 % yield). Figure S9: ^1H NMR (500 MHz, DMSO, 298 K): δ = 3.60 (s, 227H, $[\text{OC}\underline{\text{H}}_2\text{CH}_2]_{56}$), 4.40 (s, 2H, $-\text{O}_2\text{CC}\underline{\text{H}}_2\text{ONH}_2$).

Hydrogel Fabrication.

Hydrogels (12 wt%) were formed by mixing solutions of the two precursors together in a consistent manner to ensure repeatable results and to maintain the weight percent of the gels. Hydrogels (1 mL volume) were prepared by separately dissolving keto-PEG (69.1 mg, 0.007 mmol) and PEG-aminooxy (68.1 mg, 0.007 mmol) in the desired buffer (500 μL). The hydrogel solutions were then mixed and vortexed to ensure an even stoichiometric distribution. Gels were then allowed to sit at room temperature until gelation occurred (1–12 h).

Rheology.

The moduli of the hydrogels were determined using an ARES G2 Rheometer (TA Instruments, New Castle, DE) equipped with 8 mm parallel plate geometry. For rheology experiments, hydrogels (1 mL) were prepared in petri dishes and were allowed to set at ambient temperature for 24 h prior to testing to ensure complete gelation. The gels were stored in a moist, reduced airflow environment to minimize evaporation. Samples were punched to 8 mm diameter disks about 2 mm thick using a biopsy punch. Strain sweeps were performed to determine the linear viscoelastic region (LVE) (Figure S6). As cast hydrogels for SANS and USANS experiments were prepared by injecting the mixed precursor solution into 0.5 mL titanium cells (25 mm diameter, 1 mm path length) and allowing to gel for at least 16 h prior to testing, while swollen gels were formed in a petri dish for 24 h, cut out to 25 mm in diameter, swollen for an additional 48 h, and then cut to

25 mm diameters once more. Hydrogel samples for USANS measurement were additionally degassed under reduced pressure at room temperature for 30 min to remove any air bubbles.

Neutron Scattering.

Hydrogel structural features were probed by SANS and USANS at the NIST Center for Neutron Research. All measurements were performed at the National Institute of Standards and Technology Center for Neutron Research (NCNR). SANS measurements were performed on NGB-30 m SANS with a beam wavelength of 6.27 Å. Three detector distances (13.2 m, 4 m, and 1.3 m) were examined to provide a measured q range of 0.004 Å⁻¹ to 0.4 Å⁻¹. USANS measurements were performed on BT5. Measurement conditions for USANS used 6 theta slice ranges (θ) with a defined step size for each slice and count time per step listed for each slice as follows (θ Min, θ Max, θ /step, sec) θ_1 Slice: (-1, 0.6, 0.5, 20); θ_2 Slice: (0.7, 0.9, 0.1, 100); θ_3 Slice: (2, 4, 8, 0.2, 600); θ_4 Slice: (5, 9.5, 0.5, 1200); θ_5 Slice: (10, 19, 1.5, 600); and θ_6 Slice: (20, 44, 4, 1000). The q range covered was 40 Å⁻¹ to 2.5×10^{-3} Å⁻¹. The two SANS measurements (USANS plus 30 m SANS) were combined to show the fitted data. All data were reduced and fit using standard software protocols. By fitting the absolute scattering to the Broad Peak Model (Equation 1), scattering density length correlations can be determined for soft amorphous materials, where q_0 is related to the phase correlation length by:

$$I(q) = \frac{A}{q^n} + \frac{C}{1 + (|q - q_0| \xi_L)^m} + B \quad (1)$$

$q_0 = \frac{2\pi}{d_0}$, q is the scattering vector, n is the Porod exponent, m is the Lorentzian exponent, ξ_L is the Lorentzian screening length, and A , B , and C are scaling constants.^[24,25] Typically, q_0 is related to the d-spacing of phase separated systems by the relationship $q_0 = 2\pi/d_0$, which is interpreted in this study as the scattering distance between cross-linking inhomogeneities, holes or clusters in the network and is denoted as the phase correlation length Φ_p . The USANS data was also fit independently with a power law model and together with the 30 m SANS data to show similar power law behavior in the large scale regime (low q).

Statistical Analysis.

All experiments were conducted in triplicates (n=3), excluding SANS as a consequence of limited beamtime. A one-way analysis of variance (ANOVA) with Tukey post-hoc analysis was conducted when applicable. A significance value of $p > 0.05$ was set for all statistical analysis. All quantitative data are presented as the average $\pm$ standard deviation.

Results and Discussion

It was hypothesized that by increasing the molecular mass and arm length of the complementary tetra-PEG precursors, the distance between cross-links and consequently the mesh size would be increased. As prior work has demonstrated an apparent correlation between increased mesh size and decreased modulus, this larger PEG precursor should lead to a decrease in the modulus.^[6,26,27] Specifically, both precursors were made from the same 4-arm, 10 kDa PEG-star to make a hydrogel from symmetrically-sized components as

opposed to our previous work, in which one component was a small molecule and therefore produced a hydrogel from asymmetric component sizes (Figure 1). The results confirmed this hypothesis, dramatically increasing the accessible compositionally invariant modulus range (~5–30 kPa).

Summary of the Synthesis.

Keto-PEG_{ester} stars were synthesized by a DIC coupling between the free alcohol chain end of the PEG and levulinic acid, followed by simple precipitation washes to purify (Scheme 1). The PEG_{ester}-aminoxy also utilized a DIC coupling with a boc-protected hydroxylamine species which was then cleaved in HCl/dioxane to yield the final product. A similar methodology was used to functionalize the free amine end of PEG_{amide} stars, using dialysis rather than precipitations to purify. Hydrogels were synthesized by dissolving each precursor in a citric acid/phosphate buffer at the desired concentration and pH, then mixing and letting sit at room temperature.

Kinetics of Equilibrium Formation.

Differences in microstructure arising from only differences in pH and buffer concentration of the gelation solution indicate a strong dependence on the kinetics of the oxime reaction. This is in contrast to our previous study in which the two precursors had different sizes and diffusion constants. In this work, by making the precursors similar molecular weight the role of pH and buffer strength are enhanced relative to the diffusional effects on gel microstructure. Therefore, it was hypothesized that the equilibrium constant for each pH and buffer condition could provide insight into the kinetics of gelation and the observed G' . The oxime reaction proceeds in two steps: nitrogenous base addition of the hydroxylamine to a ketone followed by subsequent dehydration to form the oxime bond. While the dehydration step is considered largely irreversible, the formation of the carbinolamine intermediate is in equilibrium with the starting materials, as outlined by Jencks (Scheme 2).^[28] Table 1 summarizes the equilibrium constants of a model reaction as determined by UV-Vis spectroscopy, following the disappearance of the ketone absorption peak.. While no correlation between Keq and G' is evident, the low value for the pH 7.1, 100×10^{-3} M condition certainly indicates a high degree of reversibility and suggests the network can rearrange for quite some time before actually forming the oxime bond. This is observed in the gelation time for this condition (pH 7.1, 100×10^{-3} M) which can take in excess of 16 h to gel (Figure 3A). While the measured Keq for pH 5.7 and 6.8 at 100×10^{-3} M are very similar, differences in microstructure may arise from the much faster dehydration step and consequent faster gelation at pH 5.7. From these differences in Keq it stands to reason that the equilibrium constant of carbinolamine formation plays a significant role in the rate of gelation and therefore the network microstructure.

Mechanical Properties.

The shear moduli of swollen 12 wt% PEG-oxime hydrogels were measured as a function of varying pH (5.7, 6.8, 7.1) and buffer concentrations ($10, 100, 200 \times 10^{-3}$ M) using small-angle oscillatory shear rheology (SAOS). Oxime functionality was introduced by both ester (PEG_{ester}) and amide (PEG_{amide}) linkages to examine how bond identity would affect gel formation, while the selected pH and buffer concentrations were chosen to reflect conditions

relevant to the cellular environment.^[29] The modulus values for the PEG_{ester} gels show little variation at 10×10^{-3} M buffer concentration, however striking differences appear at 100×10^{-3} M buffer concentration across the measured pH values, producing a highly reproducible working modulus range of $G' \sim 5\text{--}30$ kPa (Figure S1). For equilibrium swollen PEG_{ester} gels this range drops about an order of magnitude to $G' \sim 0.5\text{--}11$ kPa (Figure 2A). A very similar trend is observed for equilibrium swollen PEG_{amide} gels, which is expected as the oxime bond is unchanged. However, the amide bond shifted the buffer concentration at which the modulus was most severely affected up to 200×10^{-3} M, as only minor differences in modulus were observed at 100×10^{-3} M.

Mesh Size Analysis.

As multiple reports in the literature have demonstrated an inverse trend of mesh size (ξ_m) with G' , hydrogel swelling experiments were performed to calculate the average mesh size at each pH and buffer condition (Figure 2B). Each gel was swollen for 48 h, weighed, and then freeze dried for 48 h before weighing. The mesh size was calculated using the Flory-Rehner equations (SI Equ. 1–4) with the modifications suggested by Canal and Peppas.^[30] The mesh size of PEG_{ester} gels are similar at 10×10^{-3} M buffer concentration but vary considerably at 100×10^{-3} M buffer concentration, in which higher mesh size values were obtained for lower G' . This trend was also observed in PEG_{amide} gels, for which the mesh size values were similar at 100×10^{-3} M buffer concentration but were varied at 200×10^{-3} M buffer in an inverse trend with G' .

Neutron Scattering.

Small angle neutron scattering (SANS) was used to quantify the structural-mechanical relationship on the length scale of 1–150 nm for both cast (non-equilibrium) and equilibrium swollen PEG_{ester} hydrogels. Over this length scale, the cross-linked structure and polymer chain network defects can be probed and quantified in terms of parameters (Porod exponent, n , Lorentzian screening length, ξ_L , and phase correlation length, Φ_p) associated with the Broad Peak model used to fit the SANS data. n describes the relative mass fractal density fluctuations in the low q regime (~ 100 nm) and is nearly invariant with reaction conditions for the hydrogel (Figure 4A). The Porod exponent decreases from $n \sim 2.3\text{--}2.4$ for the as cast hydrogels to $n \sim 1.8\text{--}1.9$ for the equilibrium swollen gels, which suggests that the density fluctuations in the hydrogel become more diffuse as the coils are expanded from swelling. As n does not vary significantly between gels with different moduli, the differences in G' are not related to large scale density fluctuations in the network. Ultra Small Angle Neutron Scattering (USANS) experiments were also carried out to probe the length scale of 0.1–20 μm (Figure 3C) and revealed no features other than the gels appear as a fractal network and are indistinguishable in their scattering features, indicating the gels are identical on length scales larger than the 150 nm regime.

The Lorentzian screening length was also found for each tested condition (Figure 4B). This parameter is proportional to the excluded volume of the polymer chains and therefore coil size.^[31] Minimal differences in screening length were observed for the as cast gels, but upon swelling this parameter increased for the pH 6.8 and 7.1 gels compared to the pH 5.7 gels. These data support the hypothesis that differences in bond connectivity are responsible for

the changes in G' : coil sizes in the as cast state vary only from 13 to 16 Å for all pH conditions as variations in bond connectivity impart minimal changes in size for a collapsed coil (i.e. chain entanglement and crosslinking are nearly indistinguishable in a non-dilute sample).^[32] However, upon swelling the consequent stretching of the chains and their different bonding features (e.g. loops from inefficient crosslinking) create differences in coil expansion. As less bond connectivity permits increased water uptake and increased average distance between crosslink points, an increased coil size is observed from the fitted scattering results.

These conclusions are further supported by the measured phase correlation lengths (Φ_p) derived from the Broad Peak Model (Figure 4C). This characteristic length is interpreted to be the average size of defects and was found to be proportional to mesh size in the system explored by our previous work which suggested monomer exclusion from the network produced holes that inflated the mesh size. However, no correlation between Φ_p and G' was observed in this symmetrical, two-component system. When considered with the Porod exponents, USANS data, and Lorentzian screening lengths this suggests that differences in precursor bonding are responsible for the changes in modulus, rather than monomer exclusion from the network. The Porod and USANS data demonstrated that the differences in microstructure must arise from features < 150 nm in size, while the Lorentzian screening lengths indicate that the coils swell much more for the lower modulus gels. As there is no correlation between the phase correlation length data and mesh size data it is reasonable to assume that the gel precursors are all being incorporated into the network. Therefore, the differences in microstructure must be occurring from variations in bond connectivity, of which there are three possible outcomes: uniform bonding at each functional end group, dangling end groups from incomplete conversion, and loops (i.e. two bonds formed between two precursors). Therefore, we conclude that the decrease in modulus must be a result of increased loops and dangling ends as the pH increases.

***h*MSC Viability Assay.**

Following this understanding of the length scales that affect the mechanical properties of the hydrogels was to assess *h*MSC viability on these materials. A 24 h *h*MSC proliferation assay was performed to determine encapsulated cell viability for PEG_{ester} gels formed at two buffer concentrations and all three pH values for a total of six conditions (Figure 5). Cell proliferation was quantified using a CyQuant® kit and cell viability was expressed as a percentage of live cells 24 h after encapsulation as compared to those in a control gel, in which the cells were encapsulated and quantified within 2 h of seeding. The control gel was set in a solution of pH 7.1, 100×10^{-3} M buffer concentration in order to minimize cell death as a consequence of lower acidity, and to normalize the cellular viability to the highest buffer concentration. A one-way ANOVA statistical test was done between the two buffer conditions (10 and 100×10^{-3} M) at each pH value (5.7, 6.1, 7.1) which suggested a statistically significant increase in cell viability for cells cultured in 100×10^{-3} M buffers. These results were promising even for cell cultured in pH 5.7 buffers, indicating further proliferation and differentiation assays should be possible in this gel system. For PEG_{amide} gels the pH was varied at a single buffer concentration of 200×10^{-3} M for three samples (Figure S10). While viability was lower than the PEG_{ester} gels, this can most likely be

attributed to the longer gelation time which for the pH 6.8 and 7.1 conditions of PEG_{amide} gels took over 5 h to form at 37 °C. As *h*MSCs are subject to apoptosis if they are unable to attach to a solid substrate quickly (i.e. < 2 h), this reduced the viability closer to that of the highly acidic pH 5.7 condition.^[33] Furthermore, this hypothesis is supported by the gelation time of the PEG_{ester} gels, which all formed a solid gel within < 2 h at 37 °C.

Conclusion

In conclusion, we have demonstrated a much larger range of G' (5–30 kPa) and provided further insight into the structure-property relationships inherent in the oxime hydrogel system explored by Zander *et al.* Through extensive neutron scattering analysis we have demonstrated that the differences in observed moduli among the gels arise from structural differences on the order of nanometers, and the gels are identical within error in the 0.1–20 μ m range. This provides chemically identical scaffolds with varying modulus for discerning the true effect of modulus on cell lineage commitment. Furthermore, we have provided further evidence into the kinetic control of this system, which coupled with promising *h*MSC viability data confirms that this system holds much promise for the further examination of the mechanical influence on *h*MSC proliferation and differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

PEG-oxime hydrogels were prepared at 12 wt% concentration citric acid/phosphate solutions of varying pH (5.7–7.1) and buffer concentration (10–100 mM). (*Top*) In our previous work, asymmetrical precursor sizes showed decreasing modulus with lower pH (shorter gelation times) as a consequence of kinetically-trapped defects. (*Bottom*) The current study features symmetrical precursor sizes for which the modulus decreases with higher pH (longer gelation times), an inverse trend of the previous study.

Figure 2.

A) Storage moduli (G') of swollen PEG-oxime hydrogels as a function of buffer concentration at various pH for both ester-linked and amide-linked oxime functionality; B) Mesh size values as measured by equilibrium gel swelling experiments.

Fig. 3.

(A) Inverted test tube experiments demonstrating increasing gelation time with increasing pH (*left to right*). (B) Reduced SANS scattering profiles of PEG_{ester}-oxime gels at 100×10^{-3} M buffer concentration showing only small variations at high q values. (C) USANS data demonstrate no large-scale (0.1–20 μm) defects in the networks, indicating homogenous structure over changing length scales.

Figure 4.

A) Model fitting of SANS scattering curves yielded Porod exponents, B) the respective Lorentzian screening lengths from cast and swollen (sw) gels, and C) and phase correlation lengths obtained from the Broad Peak Model.

Figure 5.

A.) Encapsulated *h*MSC viability as a percentage of control survival for various buffer and pH conditions. Asterisks (*) denote statistically significant differences in cell viability between the two buffer conditions at each pH value. B.) Reconstructed confocal microscopy image of survived cells after 24 h.

Scheme 1.

Synthesis of the various hydrogel precursors was accomplished using a diisopropylcarbodiimide (DIC) mediated esterification/amidation of 10 kDa, 4-arm PEG stars. Ketone functionality was introduced by the addition of levulinic acid to produce Keto-PEG_{ester/amide}, while a *tert*-boc-protected hydroxylamine species was attached and then cleaved to produce PEG_{ester/amide}-aminoxy.

Scheme 2.

Formation of oxime bond through a carbinolamine intermediate.

Table 1.

Summary of the measured equilibrium constants of oxime intermediate formation.

pH	[Buffer] ^a	K _{eq} ^b
5.7	10	1.5 (±0.4)
	20	9.4 (±0.9)
	50	11.7 (±0.4)
	100	15.1 (±0.4)
6.8	10	18.5 (±0.2)
	20	5.3 (±0.6)
	50	4.6 (±0.5)
	100	18.4 (±2.0)
7.1	10	10.5 (±0.3)
	20	20.3 (±0.4)
	50	6.1 (±0.3)
	100	1.9 (±0.1)

^aValue × 10⁻³ M^bMeasured by UV-Vis spectroscopy