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Control of Mesh Size and Modulus by Kinetically Dependent Cross-linking in Hydrogels

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Graphical Abstract

Kinetically controlled cross-linking processes produce mechanically distinguishable hydrogels using identical precursor chemistry. The oxime ligation demonstrates tunable reaction kinetics with pH and buffer strength, which induces changes in the structural features of hydrogels and determines their mechanical properties. Small-angle neutron scattering and swelling studies provide insight into how structural properties correlate with mechanical properties for this hydrogel system.

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Keywords

hydrogels; tissue engineering; structure-property relationships; microstructures

Polyethylene glycol (PEG) based hydrogels are used widely for tissue engineering.^[1] The favorable properties of PEG, including its hydrophilic nature, ease of end group modification, and limited immunogenicity and antigenicity, contribute to its widespread use in biomedical applications.^[2–6] Many reports have shown that human mesenchymal stem cells (hMSCs) sense the rigidity of their microenvironment and respond by altering their genomic signaling as well as differentiation and proliferation processes.^[7–14] Consequently, there has been significant interest in controllably perturbing the mechanical properties of synthetic scaffolds to direct the maturation of hMSCs; in particular, hydrogel systems with tunable mechanical properties that mimic the properties of native tissues.^[14–19] However, these systems generally require chemical or structural modification in order to tune the moduli, which challenges the interpretation and control of cell studies. Strategies have included altering the precursor concentration or stoichiometry to modulate the effective cross-link density, adjusting precursor chain lengths to vary the molecular weight between cross-links, incorporating an additional or modified cross-linking agent, and changing the scaffold chemistry altogether.^[7,13–23] To the best of our knowledge, very few reports have demonstrated a covalently cross-linked hydrogel system that employs invariant precursor chemistry, concentration and stoichiometry to produce gels with tunable mechanical properties.^[14,24]

Herein, we report a PEG-based hydrogel system that employs a kinetically-controlled oxime reaction for cross-linking, and allows for the production of mechanically distinguishable hydrogels without any alteration in precursor chemistry or composition. The kinetics of network formation, which are influenced by pH and buffer strength, intrinsically regulate the degree of heterogeneity in the scaffold microstructure and produce the range of mechanical properties observed in this system. Prior research has shown that cross-linker reactivity greatly influences the degree of heterogeneity for *in situ* polymerized hydrogels.^[21] As

network defects (dangling ends and elastically ineffective loops) become more prevalent, they dictate the physical properties of the gels, *i.e.* modulus and mesh size. Likewise, the cross-linker reactivity is expected to influence the properties of hydrogels fabricated from methods that do *not* involve radical cross-linking reactions. For example, the gelation time for hydrogel systems with acid/base driven cross-linking is dependent on cross-linking kinetics under various pH conditions, but the resulting mechanical and structural properties were not explored.^[25,26] Here, we demonstrate how kinetically-controlled, oxime hydrogel systems can be used to modulate the mechanical properties and these property differences are directly linked to minor changes in structural features.

The oxime ligation involves a “click” condensation between aminooxy and carbonyl functional groups; the pH and buffer strength dictate the kinetics of oxime formation.^[26–28] This reaction offers a robust method for hydrogel formation and use in biomaterial applications, as no metal catalyst is required, water is produced as a by-product, and it can be performed under ambient to physiological temperatures. In addition, the oxime reaction is an orthogonal method, allowing for multiple “click” reactions to be carried out in the same system.^[24] In general, the oxime reaction is acid-catalyzed under mild conditions (pH 4–6). At lower pH, the conjugate acid formation of the aminooxy component impedes the reaction, while at higher pH the hydrolysis becomes the rate-limiting step.^[29] Interestingly, the kinetics of oxime formation are also dependent on buffer concentration as neutrality is approached, which is demonstrated by the non-monotonic variation in gelation times with buffer concentration for the pH range (pH 5.7 – 7.1) selected in this study. In order to demonstrate how the rate of oxime formation/cross-linking influences the network structure and mechanical properties of the hydrogels, small amplitude oscillatory shear (SAOS) rheology and small-angle neutron scattering (SANS) were used to measure the gelation kinetics, the storage modulus (G'), and phase correlation length (δ_p) as a function of both pH and buffer concentration. Equilibrium swelling studies enabled calculations of the mesh size (ξ_m) according to Flory-Rehner theory,^[30] providing additional evidence of these structural changes.

A series of hydrogels were prepared by mixing separate equimolar precursor solutions of levulinic acid-functionalized tetra-PEG (keto-PEG, 10 kDa) and 4-arm aminooxy cross-linker, each dissolved in phosphate-citrate buffer of the desired pH and buffer concentration. Figure 1 schematically outlines the basic synthetic route and structural differences of the hydrogels. A detailed description of the precursor synthesis and characterization is provided in the Supporting Information. The buffers were prepared by mixing sodium phosphate dibasic and citric acid stock solutions of the same buffer concentration, to obtain the desired pH (5.7, 6.8, 7.1) and buffer strength (10, 20, 50, 100 mM). The pH range and buffer concentrations selected were designed to alter the cross-linking reaction kinetics with increasing pH and buffer strength, yet remain within various physiological tissue conditions. For the rheology and swelling studies, the keto-PEG and 4-arm cross-linker solutions were mixed and subsequently cast into silicon molds. The molds were covered with a damp cloth and placed in a sealed container to minimize evaporation, and allowed to react for at least 8 hours at room temperature to ensure complete gelation.

The mechanical properties and gelation times were examined using an Ares G2 Rheometer equipped with 8 mm parallel plate geometry. Initially, a strain sweep (γ) was conducted to determine the linear viscoelastic region (LVR) followed by a frequency sweep (ω) at the fixed strain ($\gamma = 1\%$). All frequencies demonstrated linear behavior and $\omega = 10 \text{ rad s}^{-1}$ was selected for reporting G' (Figure 2A). Strain and frequency sweeps (Supporting Information, Figure S9) show the majority of the data is within the LVR and non-linear behavior is only observed at large strains and low frequencies. To illustrate the effect of pH and buffer strength on gelation kinetics for this oxime system, time sweeps were also conducted by rapidly mixing the precursors for 5 seconds and injecting 400 μL of the solution into the rheometer and measuring the response in parallel plate geometry. The time sweeps shown in Figure 2B and Figure 2C demonstrate the gelation kinetics dependence on pH and buffer strength, respectively. In general, the lower pH (5.7) hydrogels formed faster due to acid catalysis; however, the buffer strength significantly influenced cross-linking kinetics as neutrality was approached for high buffer concentration (100 mM) samples, which displayed much longer gelation times (Figure 2C). Since the kinetics of the oxime reaction are known to vary with pH and buffer concentration and all other variables were held constant, the variations in the resulting storage moduli of the hydrogels (Figure 2A) indicated that a kinetically controlled cross-linking reaction may be utilized to produce mechanically distinguishable hydrogels from identical precursors. To gain a deeper understanding of how the rate of cross-linking influences mechanical strength, a detailed investigation of the network microstructure was pursued.

Neutron scattering and swelling studies were used to quantify the structural properties of the hydrogels and establish a correlation between the mechanical properties and nanoscale structural features. The absolute scattering from SANS was fit using the Broad Peak model (Equation 1), where q_o is related to the phase correlation length by the relationship $q_o = 2\pi/\delta_p$, q is the scattering vector, n is the Porod exponent, m is the Lorentzian exponent, ξ_L is the Lorentzian screening length, and the remaining variables (A, B, C) are scaling constants. [31]

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

The Broad Peak model can be used to characterize amorphous soft materials with scattering length density correlations.^[32] Typically, the d-spacing (d_o) is provided by q_o for phase separated systems ($q_o = 2\pi/d_o$), but is interpreted here as the distance between scattering inhomogeneities related to cross-linking defects and denoted as the phase correlation length, δ_p . Figure 3A illustrates the scattering profiles for different buffer concentrations at pH 5.7. At low q ($0.001 - 0.01 \text{ \AA}^{-1}$) associated with large length scales, there are statistically significant differences in the scattering, however, the scattering is almost invariant with buffer concentration in the high q regime ($0.05 - 0.5 \text{ \AA}^{-1}$). This behavior is observed at each pH (Supporting Information). The differences seen at low q ($0.001 - 0.01 \text{ \AA}^{-1}$) suggests changes in the large-scale ($10^2 - 10^3 \text{ \AA}$) structural features, such as network defects and cross-link density fluctuations, while the similarities at high q ($0.05 - 0.5 \text{ \AA}^{-1}$) reveal that

the hydrogel structures remain the same on a molecular scale ($< 10^2$ Å). Figure 3B illustrates the high quality fits of these scattering data to the Broad Peak model (Equation 1). From the fits, δ_p provided an indication of the distance between scattering length density fluctuations, which can be related to the relative degree of inhomogeneity resulting from cross-link/network defects in the hydrogel structure. To further support the SANS data for structural characterization, swelling studies were performed on the hydrogel samples. The hydrogels were prepared as previously described and swollen in deionized water for 48 hours. The excess water was removed and the gels were weighed to obtain the swollen mass (M_s), then lyophilized for 48 hours to obtain the dry mass (M_d). After determining the swelling ratio (Q), Flory-Rehner theory was used to obtain ξ_m for the hydrogels at each pH and buffer concentration, as shown in Table 1.

The phase correlation length was compared to the mesh size, and their relationship with the resulting storage modulus was analyzed. Figure 4A shows the δ_p , ξ_m , and G' for hydrogels formed at pH 5.7, and indicates that δ_p and ξ_m follow a similar trajectory with an apparent inverse relationship to G' for these length scales. In general, when G' is maximized δ_p and ξ_m appear at a minimum, and when G' is at a minimum δ_p and ξ_m are maximized. This correlation can also be seen for pH 6.8 (Figure 4B) and pH 7.1 (Figure 4C), as δ_p and ξ_m appear to be nearly inverse that of G' with increasing buffer concentration. These results are intuitive because δ_p and ξ_m provide an indication of the relative size and distance between network defects, and it is understandable that a more flawed network (with larger δ_p and ξ_m) would produce an elastically weaker hydrogel. The ξ_m from swelling studies assumes a homogeneous network and provides a mesh size based on the molecular weight between cross-links; network defects increase the molecular weight between cross-links and yield an increased ξ_m , allowing for an interpretation of the relative size of defects in the network. The differences seen between δ_p and ξ_m are likely related to the homogeneous network assumption, as well as the difference between the distance of scattering length density fluctuations measured by δ_p and the actual size of the mesh. In addition, ξ_m follows the inverse relationship with G' more closely than δ_p since the swelling ratio is directly influenced by elastically effective chain entanglements, which are difficult to identify by SANS. The relevant physical quantities measured by rheology, SANS, and swelling studies are displayed in Table 1. Overall, the data indicates that smaller mesh sizes/correlation lengths are associated with higher moduli gels, whereas low moduli gels correspond with larger δ_p and ξ_m . This relationship is a direct consequence of decreased or increased structural heterogeneities within the hydrogel network, respectively.^[33,34] More importantly, the data suggests that nanoscale structural details can be influenced by reaction kinetics to induce changes in macroscopic mechanical properties.

In conclusion, it was shown that small changes in the structural features, resulting from variations in the kinetics of cross-linking, are capable of producing mechanically distinguishable hydrogels from chemically identical precursors. For an oxime cross-linking reaction, the kinetics of network formation are influenced by pH and buffer strength, and may be utilized to intrinsically control the degree of heterogeneity within the scaffold microstructure. The significance of this study, in comparison to existing hydrogel systems, is the use of kinetics for tuning the microstructure and modulus. This method provides tunability, yet maintains the chemical identity, concentration, and stoichiometry of the

hydrogel. Further developing and understanding this and other kinetically-driven systems should enable a facile route for precise control over the mechanical properties of hydrogels. Overall, these PEG-based hydrogels could provide definitive insight into the effect of substrate elasticity on human mesenchymal stem cell (hMSC) differentiation because the scaffolds employ the same precursor chemistry, yet exhibit a range of mechanical properties.

Experimental Section

Hydrogel Fabrication

The detailed description for precursor synthesis and characterization can be found in the Supporting Information. In general, hydrogels were fabricated using a precursor mixing method: the precursor solutions were prepared by dissolving keto-PEG (138.2 mg, 0.013 mmol) in the desired buffer (800 μ L), and 4-arm cross-linker (7.6 mg, 0.013 mmol) in the desired buffer (200 μ L). The precursor solutions were then mixed with sufficient initial shaking to provide a balanced stoichiometric solution of keto-PEG and 4-arm crosslinker and gelation ensued. For oscillatory shear measurements and swelling studies, the hydrogel precursor solutions were rapidly mixed and cast into silicone molds, then covered and placed in a reduced airflow environment to minimize evaporation. The gels were allowed to set for at least 8 hours to ensure complete gelation, and punched to 8 mm diameter ($\sim$ 2 mm thick) using a biopsy punch. For SANS measurements, the hydrogel precursor solutions were rapidly mixed and injected into a 0.5 mL titanium cell (25 mm diameter, 1 mm path length), and allowed to react for at least 8 hours. All hydrogels were fabricated at room temperature and were designed to produce 12 wt% PEG ($\phi = 0.135$) gels.

Swelling Studies

The hydrogels were cast in a silicon mold and punched to 8 mm diameter ($\sim$ 2 mm thick) using a biopsy punch, then swollen in a glass vial with deionized water (2.5 mL) for 48 hours. The excess water was gently removed by blotting with a soft tissue, and the samples were weighed using an analytical balance ($\pm$ 0.01 mg) to obtain the swollen mass (M_s). The hydrogel samples were lyophilized for 48 hours and weighed again to obtain the dry mass (M_d). After determining the swollen ratio (Q), the Flory-Rehner equations (Supporting Information, Equation S2 – Equation S5) were used to calculate the average mesh size (ξ_m) for hydrogels formed under each pH and buffer concentration condition ($n = 5$).^[30]

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. The hydrogel samples were centered on the test geometry and the gap height was set to $\sim$ 1.6 mm with a constant normal force of $\sim$ 2 N. Initially, a strain sweep was performed to determine the linear viscoelastic regime (LVR), and 1% strain was selected for frequency sweeps. The frequency sweeps were conducted from 100 rad s^{-1} to 0.1 rad s^{-1} ; all frequencies between 1 – 100 rad s^{-1} demonstrated linear behavior, and 10 rad s^{-1} was selected for reporting ($n = 3$). Time sweeps were performed at 1% strain and 1 rad s^{-1} to determine the gelation time. The precursor solutions were rapidly mixed for 5 seconds and 400 μ L of solution was injected

into the rheometer equipped with 25 mm parallel plate geometry. The gap was lowered to 0.80 mm and the measurement began.

Small Angle Neutron Scattering

SANS was used to investigate the structural features of the hydrogels. All measurements were taken at the National Institute of Standards and Technology Center for Neutron Research (NCNR). Using the instrument NGB30, the scattering intensity was measured as a function of the “wavevector” q , where $q = \left(\frac{4\pi}{\lambda}\right) * \sin\left(\frac{\theta}{2}\right)$, λ is neutron beam wavelength and θ is scattering angle. Three detector distances (13.2 m, 4 m, and 1.3 m), with lenses used at the 13.2 m detector distance to provide lower q , were examined to provide a measured q range of $0.001 \text{ \AA}^{-1}$ to $0.5 \text{ \AA}^{-1}$. The scattering results were circularly averaged over the 2D detector to obtain the 1D scattering of q versus intensity. The data were fit with the Broad Peak model in the q range of 0.001 to $0.5 \text{ \AA}^{-1}$, and the phase correlation length (δ_p) of the gel nanostructure was determined from q_0 .

Statistical Analysis

All experiments were conducted in triplicates ($n=3$). 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Tunable hydrogels were fabricated by mixing 4-arm crosslinker and keto-PEG precursors in phosphate-citrate buffer over a range of pH (5.7 – 7.1) and buffer concentrations (10 – 100 mM). The kinetics of network formation are influenced by pH and buffer strength, and can be exploited to control the structural properties of the hydrogel network, which in turn dictate the mechanical properties (G') for the resulting hydrogel. The cross-linking reaction involves oxime formation, which produces water as a by-product and does not require a metal catalyst.

Figure 2.

(A) The resulting G' for hydrogels formed at each pH (5.7 = ●, 6.8 = ■, 7.1 = ▲) were plotted against the various buffer concentrations (10, 20, 50, 100 mM) to show the production of mechanically distinguishable gels, ranging from $G' = 20.0 - 31.5$ kPa (+ indicates no statistical significance [$p > 0.05$] between different pH samples at a selected buffer concentration. ++ indicates no statistical significance between 10 and 50 mM samples at pH 5.7. +++ indicates no statistical significance between 10 and 100 mM samples at pH 6.8. All other samples were significant with a p value < 0.05). In addition, time sweeps were performed at 1% strain and 1 rad s^{-1} to monitor the gelation point for hydrogels formed at different pH (B) and also at different buffer concentration (C). The pH time sweeps shown were performed on hydrogel precursor solutions of 100 mM buffer concentration to demonstrate the effect of pH on cross-linking kinetics (* indicates p value < 0.05 between all samples, $n=3$). The buffer strength time sweeps were performed at pH = 5.7 to demonstrate the influence of buffer concentration on reaction kinetics (* indicates p value < 0.05 between 10 and 50 mM samples. ** indicates p value < 0.05 between 20 and 100 mM samples. *** indicates p value < 0.05 between 50 and 100 mM samples, $n=3$). All hydrogels were prepared either from the same batch, or different batches with weight variations 0.2 mg.

Standard conditions include precursors mixed under stoichiometric balance (1: 1, aminoxy: ketone) at room temperature, and 12 wt.% PEG (vol. fraction, $\phi = 0.135$).

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Figure 3.

(**A**) The absolute scattering profiles of hydrogels fabricated at pH 5.7 for a series of buffer concentrations exhibit differences at low q ($10^{-3} - 10^{-2} \text{\AA}^{-1}$), but are similar in the high q regime ($>10^{-1} \text{\AA}^{-1}$). The intensity variations seen at low q suggests changes in the large-scale structural features, such as network defects and cross-link density fluctuations, while the similarities at high q demonstrate that the structures are identical on a molecular level ($1 - 10 \text{\AA}$). (**B**) The scattering curves (pH 5.7) for each buffer concentration were fit using the Broad Peak model (solid lines) to determine the phase correlation lengths (δ_p). The scattering curves are offset vertically for clarity.

Figure 4.

Correlation between the mechanical properties ($G' = \blacksquare$) and structural properties of mesh size ($\xi_m = \bullet$), and phase correlation length ($\delta_p = \blacktriangle$). For hydrogels produced at pH 5.7 (A), δ_p from SANS trends similarly with ξ_m derived from swelling studies, and both are inversely related to the storage modulus of the hydrogels. These trends are also seen for pH 6.8 (B) and pH 7.1 (C), as ξ_m and δ_p demonstrate an approximately inverse relationship with storage modulus.

Table 1

Summary of the mechanical and structural properties determined for the kinetically-controlled hydrogels. The association between mechanical and structural properties generally reveals an inverse relationship; where G' is at a maximum, ξ_m and δ_p is at a minimum.

pH	[Buffer] (mM)	G' (kPa) ^{a)}	ξ_m (Å) ^{b)}	δ_p (Å) ^{c)}
5.7	10	22.24 (±0.02)	107.2 (±0.3)	144 (±2)
	20	27.9 (±0.3)	100.9 (±0.4)	129 (±1)
	50	23.1 (±0.1)	104.6 (±0.8)	157 (±4)
	100	20.1 (±0.8)	107.2 (±0.6)	239 (±17)
6.8	10	25.2 (±0.6)	103 (±1)	162 (±4)
	20	28.02 (±0.05)	99 (±1)	150 (±3)
	50	31.1 (±0.5)	95.9 (±0.4)	191 (±10)
	100	23.9 (±0.9)	104 (±3)	172 (±3)
7.1	10	21.6 (±0.4)	104.8 (±0.5)	178 (±7)
	20	26.0 (±0.2)	100.7 (±0.8)	149 (±3)
	50	31.55 (±0.03)	96.4 (±0.8)	146 (±2)
	100	29.9 (±0.4)	100 (±2)	187 (±6)

^{a)} Storage moduli (G') were obtained from SAOS frequency sweep data at 10 rad s⁻¹, and the averages are reported with one standard deviation (n = 3)

^{b)} Mesh sizes (ξ_m) were determined from swelling studies using the systems of equations described by Flory-Rehner theory. The averages are reported with one standard deviation (n = 5);

^{c)} Phase correlation length (δ_p) results were derived using the Broad Peak model used to fit the scattering curves produced by SANS. The error corresponds to the standard deviation resulting from general fit error.