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Hydrazone covalent adaptable networks modulate extracellular matrix deposition for cartilage tissue engineering

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Abstract

Cartilage tissue engineering strategies often rely on hydrogels with fixed covalent crosslinks for chondrocyte encapsulation; yet the resulting material properties are largely elastic and can impede matrix deposition. To address this limitation, hydrazone crosslinked poly(ethylene glycol) hydrogels were formulated to achieve tunable viscoelastic properties and to study how chondrocyte proliferation and matrix deposition vary with the time-dependent material properties of covalent adaptable networks. Hydrazone equilibrium differences were leveraged to produce average stress relaxation times from hours (4.01×10^3 s) to months (2.78×10^6 s) by varying the percentage of alkyl-hydrazone (aHz) to benzyl-hydrazone (bHz) crosslinks. Swelling behavior and degradation associated with adaptability was characterized to quantify temporal network changes that can influence the behavior of encapsulated chondrocytes. After four weeks, mass swelling ratios varied from 36 ± 3 to 17 ± 0.4 and polymer retention ranged from $46 \pm 4\%$ to $92 \pm 5\%$, with higher aHz content leading to loss of network connectivity with time. Hydrogels were formulated near the Flory-Stockmayer bHz percolation threshold (17% bHz) to investigate chondrocyte response to distinct levels of covalent architecture adaptability. Four weeks post-encapsulation, formulations with average relaxation times of 3 days (2.6×10^5 s) revealed increased cellularity and an interconnected articular cartilage-specific matrix. Chondrocytes embedded in this adaptable formulation (22% bHz) deposited $190 \pm 30\%$ more collagen and $140 \pm 20\%$ more sulfated glycosaminoglycans compared to the 100% bHz control, which constrained matrix deposition to pericellular space. Collectively, these findings indicate that incorporating highly adaptable aHz

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crosslinks enhanced regenerative outcomes. However, connected networks containing more stable bHz bonds were required to achieve the highest quality neocartilaginous tissue.

Graphical Abstract

Keywords

hydrogel; viscoelastic; hydrazone; covalent adaptable network; cartilage tissue engineering

1. Introduction

Millions of patients suffer debilitating pain from osteoarthritis caused by damage to articular cartilage in load-bearing joints.[1] Osteoarthritic degeneration of articular cartilage is exacerbated by poor innate healing capabilities, stemming from lack of vasculature and the low regenerative activity of resident chondrocytes.[2] Matrix-assisted autologous chondrocyte transplantation (MACT) has emerged as a promising tissue engineering strategy to enhance the ability of chondrocytes to repair critically sized cartilage defects.[3] This strategy often employs water-swollen polymer networks (hydrogels) as delivery vehicles to support chondrocytes and permit extracellular matrix (ECM) deposition.[4] Hydrogels used for cartilage tissue engineering are frequently covalently crosslinked to withstand compressive forces experienced in articulating joints, but this also renders them largely elastic in their response to mechanical forces. Densely crosslinked hydrogels and fixed elastic crosslinks have been linked to low rates of chondrocyte proliferation, as well as ECM deposition restricted to pericellular space.[5] This could help explain why MACT remains an ancillary clinical treatment for osteoarthritis and why robust articular cartilage regeneration remains elusive.[6,7]

To improve regenerative outcomes of MACT strategies, scaffolds used for articular cartilage regeneration can be designed to incorporate viscoelastic mechanical characteristics, making them more similar to native cartilage than fixed elastic hydrogels.[8] Biomechanical analysis of freshly isolated cartilage reveals viscoelastic behavior, such as stress relaxation and creep. However, few covalent hydrogels used as chondrocyte scaffolds exhibit stress relaxation and creep in response to mechanical deformation.[9,10] Moreover, the effects of viscous dissipation (e.g., stress relaxation) on cartilage regeneration remain largely understudied compared to the relationship between elastic stiffness and chondrocyte behavior (e.g., differentiation, proliferation, and matrix deposition).[11–13]

Most hydrogels that exhibit both viscous and elastic behavior (i.e., viscoelasticity) have relied on the physical or ionic association of naturally derived polymers, such as collagen,

hyaluronic acid or alginate.[14] The current study was motivated, in part, by seminal work using ionically crosslinked calcium-alginate hydrogels to investigate the effects of stress relaxation on cell behavior. Faster stress relaxation was shown to influence morphology, proliferation, differentiation, and secretory properties of mesenchymal stem cells (MSCs). [15–17] More recently, Lee *et al.* demonstrated increased ECM deposition by chondrocytes when stress relaxation times of calcium-alginate hydrogels were tuned from 2 hours to 1 minute.[18] While calcium-alginate hydrogels provide many benefits as biomaterial scaffolds, divalent metal ion crosslinking strategies (e.g., calcium-alginate crosslinks) can be disrupted by monovalent cation exchange in physiological environments, possibly limiting *in vivo* application within articulating joints.[19] Biologically derived hydrogels (e.g., collagen, hyaluronic acid, matrigel, and calcium-alginate) can also suffer batch-to-batch variability and present biochemical cues, confounding the effects of viscoelastic mechanical properties on chondrocyte secretory properties and tissue regeneration.[20–23]

Synthetic networks crosslinked with covalent adaptable bonds offer a complementary approach for cartilage tissue engineering and provide specific benefits. Ultimately, association interactions result in moduli that are much lower than those of equivalent networks that are covalently crosslinked.[24] In addition, covalent adaptable networks (CANs) are able to reorganize network connectivity to relieve local stresses, such as those caused by cell proliferation and the secretion of matrix molecules.[25] Despite these advantages, CANs have only recently begun to be explored as scaffolds for cell encapsulation and tissue engineering.[26,27] Prior research from our group has demonstrated that hydrazone CANs are able to be used for encapsulation of primary cells, due to mild formation conditions without catalyst.[28,29] Hydrazone bonds (R-HC=NH-NH-R) are formed by nucleophilic attack on a carbonyl electrophile followed by condensation (Figure 1A).[30] Hydrazone bonds (Hz) are susceptible to hydrolysis under physiologically relevant conditions (pH and temperature), regenerating the original nucleophile (Nu) and electrophile (El) and rendering the process reversible ($[El] + [Nu] \rightleftharpoons [Hz]$).[31] Chemical equilibria ($K_{eq} = k_1/k_{-1} = [Hz][Nu]^{-1}[El]^{-1}$) of hydrazone bonds govern network reorganization. This effect has been illustrated by small molecule kinetic studies that demonstrate close agreement between forward (k_1) and reverse (k_{-1}) rate constants and the mechanical properties of PEG CANs.[32]

Boehnke and coworkers used similar synthetic PEG hydrogels to culture mouse MSCs, demonstrating controlled degradation from 1 to 7 days by varying the ratio of hydrazone to oxime crosslinks.[33] Degradable hydrazone hydrogels were also used in a subcutaneous mouse model, showing ectopic ECM deposition by chondrocytes encapsulated within scaffolds formed by reacting adipic dihydrazide-modified poly(L-glutamic acid) with aldehyde-modified poly(L-glutamic acid).[34] More recently, hydrazone crosslinked elastin-like protein-hyaluronic acid hydrogels were used to illustrate the biochemical effects of hyaluronic acid, showing upregulation of chondrocyte specific genes with increasing hyaluronic acid concentration.[35] These studies demonstrate the versatile nature of hydrazone crosslinks, but the effects of distinct hydrazone equilibria and the resulting viscoelastic properties have yet to be studied in the context of cartilage tissue engineering.

Herein, we synthesized and characterized a range of hydrogel formulations crosslinked with hydrazone bonds as viscoelastic scaffolds to study the effects of covalent crosslink adaptability on chondrocyte functions for cartilage tissue engineering. Freshly isolated porcine chondrocytes were encapsulated in hydrogels that were designed to have average stress relaxation times varying from hours to months by changing the relative percentage of alkyl-hydrazone (aHz) and benzyl-hydrazone (bHz) crosslinks (Figure 1B). Chondrocyte proliferation and secretory properties were investigated as a function of hydrogel adaptability by monitoring the total collagen content, the sulfated glycosaminoglycan (sGAG) content, and the amount of double stranded DNA (dsDNA) in chondrocyte-laden CANs over the course of 4 weeks. The spatial distribution of deposited ECM molecules was also visualized by histological (sGAGs, total collagen) and immunohistochemical staining (aggrecans, collagen I, II and X) to assess the development of neocartilaginous tissue.

2. Materials and methods

2.1 Hydrazine and aldehyde macromer synthesis

All chemicals and solvents were analytical grade and acquired from commercial sources unless otherwise described. Alkyl PEG-aldehyde was synthesized by Dess-Martin oxidation of 8-arm PEG-OH ($M_n \sim 20,000$ g/mol) as previously described.[36] Briefly, PEG was dissolved with Dess-Martin periodinane (1.5 equiv. w.r.t. -OH groups) in minimal amount of dichloromethane (DCM) containing catalytic H_2O . The reaction was allowed to proceed for 3 hours at room temperature (23°C). PEG-hydrazine and PEG-benzaldehyde were synthesized by HATU (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium-3-oxidhexafluorophosphate) coupling to 8-arm PEG-NH₂ ($M_n \sim 20,000$ g/mol). [37] For each respective synthesis, either tri-boc-hydrazinoacetic acid or 4-formylbenzoic acid (2.2 equiv. w.r.t -NH₂ groups) were activated with HATU (2.0 equiv. w.r.t -NH₂ groups) and 4-methylmorpholine (5.0 equiv. w.r.t -NH₂ groups) in dimethylformamide (DMF) under argon for 10 minutes. In parallel, PEG amine was dissolved in DMF containing 4-methylmorpholine (5.0 equiv. w.r.t -NH₂ groups). The two solutions were mixed and the reactions were allowed to proceed overnight under argon at room temperature. Boc(tert-butyloxycarbonyl)-protected PEG-hydrazine was precipitated dropwise in cold (4°C) diethyl ether (Et₂O), then dissolved in a 50:50 mixture of trifluoroacetic acid (TFA) and DCM. The deprotection reaction was allowed to proceed in a vented flask for 3 hours prior to purification.

2.2 Macromer purification and characterization

Crude reaction mixtures were concentrated under reduced pressure and subsequently precipitated dropwise in cold Et₂O. Samples were centrifuged, decanted, and washed 3x with Et₂O before drying *en vacuo*. Dry products were dissolved in deionized H₂O and dialyzed in regenerated cellulose membranes (Spectra/Por) with a molecular weight cut-off of 8,000 g/mol for 48 hours at 4°. Polymers were then lyophilized and stored at -20°C. ¹H NMR spectra (Broker AV-III, 400 MHz, CDCl₃) were used to evaluate functionalization of PEG macromers by integrating the functional or protecting group peaks normalized to PEG protons. In each case the functionality was found to be 90%. Boc-PEG-NH-NH₂ $\delta=3.79-3.41$ (m, 227H, -O-CH₂-CH₂-O-), 5=1.57-1.42 (m, 27H, -O-C(CH₃)₃); PEG-NH-

NH₂ δ =3.77-3.54 (m, 227H, -O-CH₂-CH₂-O-); PEG-Ar-CHO δ =10.34-9.81 (s, H, CHO), δ =8.12-7.83 (m, 4H, -C₆H₄-), δ =4.09-3.15 (m, 227H, -O-CH₂-CH₂-O-); PEG-CHO δ =9.81-9.58 (s, H, CHO); δ =4.25-4.08 (s, 2H, -CH₂-CHO), δ =3.90-3.23 (m, 227H, -O-CH₂-CH₂-O-).

2.3 Hydrogel formation and rheological characterization

Functionalized PEGs were dissolved in phosphate buffered saline (PBS) and neutralized to pH 7.0 (10 w/w%). Hydrogels were formed with 5w/w% final polymer content on stoichiometry. *In situ* rheology was performed using parallel plates on a temperature-controlled Peltier plate (TA Instruments DH-R3). Shear rheology was performed at an angular frequency of 1 rad/s, which has been previously shown to be relevant for cell behavior.[38] aHz and bHz hydrogel formation was monitored by time sweep at 1% strain and 25°C. For stress relaxation experiments, a 10% shear strain was applied over a 10-second ramp at 37°C. Experimental conditions were formulated with bHz/aHz molar percentages of 0, 10, 20, 30, 40, 50, 70 and 100%. The experiment length (12 hours) was dictated by the amount of time for the 0% bHz condition to completely relax the applied stress. Mineral oil was applied to the air-hydrogel interface to prevent evaporation during experimentation. Theoretical models were fit using the curve fit application in MATLAB reporting 95% confidence intervals on fit parameters.

2.4 Acellular swelling and mass loss behavior

Hydrogels (75 μ L, 5 w/w%) were prepared off-stoichiometry ($r=[\text{El}]/[\text{Nu}]=0.8$) with bHz/aHz molar percentages of 0, 12.5, 25, 50, 75 and 100% with the same initial polymer mass (m_0). Hydrogels were swollen in chondrocyte growth media for 28 days. The wet mass (m_s) was measured every 7 days and normalized by the mass at the time of formation (m_f). After 28 days, hydrogels were frozen in liquid nitrogen (LN₂), lyophilized and weighed (m_d) to calculate the polymer retention (%retention = $m_0/m_d \times 100\%$) and equilibrium mass swelling ratios ($q=m_s/m_d$).

2.5 Chondrocyte isolation, encapsulation and cell culture

Primary chondrocytes were isolated from femoral condyles and the patellar groove of Yorkshire swine stifle joints ($n=6$) as detailed previously.[39] Freshly isolated porcine chondrocytes were encapsulated within 40 μ L hydrazone hydrogels formed in 1 mL syringe barrels at ~50 million cells/mL, as this density has been shown to produce high quality neotissue for *in vitro* encapsulation studies.[40] For chondrocyte encapsulation, three covalent adaptable formulations (12, 17 and 22 mol% bHz, 5 w/w%) were selected and compared to a primarily elastic and slow-relaxing 100% bHz control. Hydrogels were formulated off-stoichiometry ($r = [\text{El}]/[\text{Nu}] = 0.8$, 5 w/w%) to minimize potential cytotoxicity from pendant aldehyde groups. Cell viability was assessed by Live/Dead assay (Invitrogen) immediately after encapsulation to verify that the encapsulation process was cytocompatible (Figure S1). Chondrocyte-hydrogel constructs were cultured in chondrocyte growth medium composed of high-glucose DMEM (Gibco) containing 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin and fungizone (Gibco, Invitrogen), 50 mg/mL L-ascorbate-2-phosphate (Sigma-Aldrich), 40 mg/mL L-proline (Sigma-Aldrich), 100 mg/mL

non-essential amino acids (Gibco), 100 mg/mL HEPES buffer (Sigma-Aldrich) and 50 mg/mL gentamicin (Invitrogen). Medium was changed every other day, and cell-hydrogel constructs were maintained with 5% CO₂ at 37°C.

2.6 Biochemical analysis of cell-laden hydrazone CANs

Cell-laden hydrogels (n=4) were removed from culture conditions 1, 7, 14, 21 and 28 days post-encapsulation, snap frozen in LN₂ and stored at -70°C prior to analysis. Samples were then lyophilized before being added to digestion buffer composed of 125 µg/mL papain (Worthington Biochemical) and 10 mM cysteine (Sigma Aldrich). Samples were homogenized for 10 minutes with 5-mm steel beads shaking at 30 Hz (Qiagen TissueLyser). Digestion was allowed to proceed overnight at 60°C. Samples from each digest solution were hydrolyzed with an equal volume of 12 M HCl for 3 hours at 120°C. Total collagen content was analyzed by a hydroxyproline assay, where hydroxyproline was assumed to make up 13.4% of the amino acid content of collagen.[41,42] Remaining digest solutions were centrifuged and the supernatant was used for a DMMB assay, with results reported as chondroitin sulfate (ChS) equivalents. Supernatant was also used to quantify cell number using dsDNA from a PicoGreen assay (Life Technologies) assuming each chondrocyte accounts for 7.7 pg of dsDNA.[43]

2.7 Histological sectioning, staining and immunofluorescence

On day 28, constructs were fixed for 1 hour at room temperature in 4% paraformaldehyde. Hydrogels were rinsed with DPBS and then soaked in a 30% sucrose solution overnight at 4°C. Hydrogels were transferred to molds, embedded in optimal cutting temperature (OCT) compound and slowly frozen to -70°C overnight. Slides were prepared with 30-µm sections cut with a Leica Cryostat CM1850. Sections were stained with Safranin-O and Masson's Trichrome using a Leica Autostainer-XL. Cover slides were applied with PermOUNT (Fisher) and slides were imaged by bright field microscopy with a Nikon TE-2000 inverted microscope. For immunofluorescence staining, cell-hydrogel samples were harvested on day 28 and fixed in 10% formalin for 1 hour at room temperature. Hydrogels were frozen and cryosectioned as described above. Frozen sections were stored at -70°C. Antigen retrieval was performed on thawed slides with Retrieval (BD Biosciences). For collagen II and aggrecan immunostaining, samples were treated with C-ABC (100 mU/mL, Sigma) and keratanase (50 mU/mL, Sigma) for 1 hour at 37°C followed by hyaluronidase (2000 U/mL, Sigma) pretreatment under the same conditions. For collagen I and collagen X staining, samples were pretreated with pepsin (1 mg/mL, ~ 4000 U, pH 2.0, Sigma) for 1 hour at 37°C. Samples were permeabilized with 0.25 w/w% TritonX-100 and then blocked with 1 w/w% bovine serum albumin (BSA) for 1 hour at room temperature. Samples were then incubated with rabbit polyclonal antibodies for collagen type II or collagen X (1:200, Abcam) as well as mouse monoclonal antibodies for aggrecan or collagen I (1:200, Abcam) in 1% BSA overnight at 4°C. Samples were incubated with secondary antibodies, AlexaFluor-555 donkey-anti-mouse and AlexaFluor-647 goat-anti-rabbit (1:200, Abcam), in 1% BSA for 12 hours in a dark humidified chamber at 4°C. Samples were counterstained with DAPI for 30 minutes at room temperature in the dark. Coverslips were mounted onto slides with Fluoromount (Sigma) and sealed with nail polish. Stained samples were stored in the dark at 4°C until imaging. All samples were processed at the same time to minimize

sample-to-sample variation. Images were taken on a Zeiss LSM710 scanning confocal microscope using identical acquisition settings and post-processing for all samples.

2.8 Statistical analysis

Unless otherwise noted, graphical representations and error bars represent the mean $\pm$ standard deviations. Individual differences between sample means were analyzed by unpaired two-tailed t-tests with Welch's correction. Standard thresholds for significance were used throughout (e.g., $P < 0.05 = *$, $P < 0.01 = **$, $P < 0.001 = ***$, $P < 0.0001 = ****$). Comparisons of three or more independent groups were analyzed by ordinary 1-way or 2-way ANOVA with Dunnett's or Sidak's multiple comparison tests. Statistical analyses were performed with GraphPad Prism 6 software.

3. Results

3.1 Alkyl and benzyl-hydrazone hydrogel formation and shear moduli

To create hydrazone CANs with varied adaptability, three distinct 8-arm PEG macromers ($M_n \sim 20,000$ g/mol) were synthesized with complementary reactive groups. Hydrazone hydrogels were formed by reacting nucleophilic PEG-hydrazine with two different electrophilic PEG-aldehydes (alkyl and benzyl). Hydrazone hydrogels were compared by monitoring step-growth polymerization at the same macromer concentrations (Figure 2A). Non-significant differences between storage moduli (G') shown in Figure 2B resulted in similar complex shear moduli ($G^*_{aHz} = 20.3 \pm 0.3$ kPa vs. $G^*_{bHz} = 20.4 \pm 0.1$ kPa). In contrast, the final loss moduli (G'') were significantly different, resulting in differences between loss tangents ($\tan(\delta)$) depicted in Figure 1C. These findings indicate that aHz and bHz equilibria can be leveraged to crosslink hydrogels resulting in viscoelastic differences, without requiring differences in polymer content or elastic stiffness.

3.2 Two-element stretched exponential to model biphasic hydrazone stress relaxation

To more thoroughly investigate the viscoelastic differences between aHz and bHz hydrogels, we performed stress relaxation experiments by shear rheology (Figure 2D). The composition was graduated from 0% bHz crosslinks (i.e. 100% aHz crosslinks) to 100% bHz crosslinks in order to investigate intermediate levels of covalent adaptability. This resulted in precise incremental control over the stress relaxation characteristics of hydrazone hydrogels. Normalized stress relaxation data was fit with a two-element Kohlrausch-Williams-Watts function (Figure 2D).

$$\frac{\sigma}{\sigma_0} = X_a \exp\left(-\left(\frac{t}{\tau_a}\right)^{\beta_a}\right) + X_b \exp\left(-\left(\frac{t}{\tau_b}\right)^{\beta_b}\right) \quad (\text{Eq 1})$$

In is equation, the two elements are stretched exponential terms where the pre-exponential factors represent the mole fractions (X) of either alkyl (a) or benzyl (b) hydrazone crosslinks. Similarly, the relaxation time constants (τ) are characteristic of the reorganization of each type of covalent bond, with stretching parameters (β) to account for heterogeneity in

relaxation behavior. The model fit parameters with 95% confidence intervals are shown in Figure 2E and Figure 2F showing time constants and stretching parameters, respectively. The bHz relaxation time constants increased drastically with the percentage of bHz crosslinks, unlike the aHz relaxation time constants which remained relatively constant. bHz crosslinks also showed a constant level of highly heterogeneous stress relaxation behavior, likely due to larger contributions from alternative rearrangement mechanisms (e.g., transimination) compared to the aHz crosslinks, which remained dominated by rapid hydrolysis and reformation.[44] We next derived the equation for average relaxation time ($\langle\tau\rangle$) by integrating the model over its entire domain ($t=0$ to $t=\infty$).

$$\langle\tau\rangle = \frac{X_a\tau_a}{\beta_a}\Gamma\left(\frac{1}{\beta_a}\right) + \frac{X_b\tau_b}{\beta_b}\Gamma\left(\frac{1}{\beta_b}\right) \quad (\text{Eq 2})$$

Using fit parameters from stress relaxation data, single value outputs were calculated for each hydrogel formulation to illustrate the wide range of accessible viscoelastic properties (Figure 2G). This resulted in hydrazone hydrogel average relaxation times spanning three orders of magnitude ($\langle\tau_{0\%}\rangle\approx 4\times 10^3\text{s}$ to $\langle\tau_{100\%}\rangle\approx 3\times 10^6\text{s}$) corresponding to a range from roughly one hour to one month.

3.3 Acellular swelling and mass loss associated with the adaptability of the hydrazone crosslinks

Swelling experiments were performed for the length of the intended cell culture experiments to understand any temporal network changes or mass loss that may be associated with the adaptability of the hydrazone crosslinks. The swollen mass was measured every 7 days (Figure 3A). The two most adaptable hydrazone CANs (0 and 12.5% bHz) demonstrated increased swollen masses at early time points (days 1, 7, and 14). All other conditions (25, 50, 75 and 100% bHz) maintained constant swollen masses for the duration of the experiment. After the final time point, acellular hydrogels were lyophilized and the dry mass was used to calculate polymer retention. The hydrogels composed primarily of aHz crosslinks (0, 12.5 and 25% bHz) showed reduced polymer content when compared with 100% bHz hydrogels (Figure 3B). Similarly, on day 28, mass swelling ratios were calculated to illustrate changes observed over the course of the experiment (Figure 3C). Again, the conditions with the highest percentages of aHz crosslinks demonstrated significant differences when compared to the 100% bHz hydrogels.

3.4 Hydrogel formulations based on Flory-Stockmayer percolation threshold

Formulations used for chondrocyte cell culture were designed based on Flory-Stockmayer theory to create hydrogels with varied adaptability using two hydrazone equilibrium reactions (Figure 1 A).[45]

$$p_c = \frac{1}{\sqrt{r(f_{Nu}-1)(f_{El}-1)}} \quad (\text{Eq 3})$$

The percolation threshold (p_c) for step-growth polymerization off-stoichiometry, represents the minimum conversion required for hydrogel formation. Where f_{Nu} and f_{El} are the number of nucleophilic or electrophilic groups on each PEG macromer and r is the stoichiometric ratio ($r = [El]/[Nu]$). For cell culture, hydrogels were designed with the electrophilic species as the limiting reagent ($r = 1$). Assuming ideal network formation, the fraction of bHz crosslinks (p_b) must be greater than p_c for slow-relaxing bHz bonds to span the dimensions of the hydrogel (Figure 1B). For 8-arm macromers 0.17 (17%) is the calculated percolation threshold. We were particularly interested in studying chondrocyte behavior in viscoelastic adaptable networks above and below this threshold to test the effects of distinct levels of adaptability near this critical point. For this reason, 12, 17 and 22% bHz crosslinks, as well as a 100% bHz control, were used for further experiments with chondrocyte-laden hydrogels. These hydrogels are expected to produce approximate average relaxation times of $\langle\tau_{12\%}\rangle \approx 4.7 \times 10^4$ s, $\langle\tau_{17\%}\rangle \approx 6.3 \times 10^4$ s, $\langle\tau_{22\%}\rangle \approx 2.6 \times 10^5$ s, and $\langle\tau_{100\%}\rangle \approx 2.8 \times 10^6$ s based on the aforementioned results of rheological stress relaxation experiments.

3.4 CANs with intermediate covalent adaptability enhance cellularity

The dsDNA content of hydrazone hydrogels was assayed over the course of 28 days to characterize overall cellularity and proliferation with respect to covalent adaptability (Figure 4). The cellularity of hydrazone hydrogels increased initially in the most adaptable hydrogels (12% bHz), but over time was surpassed by conditions at intermediate levels of covalent adaptability (17 and 22% bHz). The 22% bHz hydrogels demonstrated nearly two-fold increase (1.68 ± 0.19) in the number of chondrocytes between day 1 and day 28. Conversely, chondrocyte populations were reduced in the 100% bHz hydrogels and demonstrated consistently lower cell populations over the time course of the experiment, despite the same initial seeding densities (day 0). These findings imply that dense networks of primarily elastic, fixed bHz crosslinks hinder chondrocyte proliferation and significantly reduce cellularity over extended culture times. However, cellularity can be rescued in hydrazone hydrogels by substituting a significant fraction of the bHz crosslinks with highly adaptable aHz crosslinks. Most interestingly, once the percentage of aHz crosslinks becomes 88% the network fails to retain proliferating cells and the cellularity of the resulting neotissue declines.

3.5 Interconnected sGAG matrix forms in viscoelastic CANs with intermediate adaptability

Histological sectioning and staining with Safranin O was used to visualize the spatial distribution of sGAGs in hydrazone CANs after 28 days of chondrocyte cell culture. Representative images are shown in Figure 5A, revealing maximal sGAG deposition in CANs formulated just above the percolation threshold for bHz crosslinks (22%). This is illustrated by comparison with the more diffuse, lighter sGAG staining in the 12 and 17% bHz hydrogels. Consistent with prior results using non-adaptable hydrogels, the slow-adapting 100% bHz hydrogels demonstrated sGAG deposition primarily in pericellular space with small dense stain areas observed around chondrocytes. Acellular hydrogels and articular cartilage sections were also stained for comparison, helping to highlight differences in neotissue quality across hydrazone formulations. To complement this qualitative analysis, sGAG secretion as a function of time was quantified by a DMMB assay (Figure 5B). sGAG

content increased with time in all four hydrazone hydrogel conditions. Notably, sGAG deposition by encapsulated chondrocytes in the 22% bHz hydrogels was significantly higher than all other hydrogel formulations at the final time point. In contrast, the most adaptable 12% bHz hydrogels showed reduced sGAG content after 28 days. These findings are consistent with previous work studying degradation in non-adaptable hydrogels, but also suggests that adaptable covalent bonds can be tuned to support matrix deposition without requiring irreversible degradation of crosslinks.[46]

3.6 Collagen deposition is significantly enhanced in CANs with intermediate adaptability

Masson's trichrome staining was performed to assess the development of collagen matrix by chondrocytes encapsulated within hydrazone CANs after 28 days (Figure 6A). Chondrocytes formed robust and interconnected collagen networks in the 22% bHz hydrogels with collagen staining that spanned large clusters of cells. This condition most closely resembled native articular cartilage compared to other experimental conditions (12, 17 and 100% bHz). The primarily elastic 100% bHz hydrogels with very slow relaxation times showed only pericellular ECM deposition, with staining isolated to the immediate vicinity of the chondrocytes. The 100% bHz control also demonstrated limited scaffold replacement, maintaining large areas closely resembling acellular hydrogels. Biochemical analysis revealed even larger differences in the collagen content than was observed for sGAGs (Figure 6B). In particular, the 22% bHz hydrogels resulted in significantly more collagen deposition by encapsulated chondrocytes, nearly 1 mg after 28 days, or almost twice as much as the 100% bHz hydrogels.

3.7 The relationship between average relaxation time and ECM deposition

To more closely examine the relationship between the viscoelastic properties of hydrazone CANs and the secretory properties of encapsulated chondrocytes, the amount of collagen and sGAGs deposited after 28 days was plotted as a function of average relaxation time (Figure 7). This analysis reveals information about the timescales of network adaptation that may be important for chondrocyte processes. The most significant ECM deposition was observed in the 22% bHz hydrogels, which were formulated with average relaxation times of approximately 3 days (2.6×10^5 s). This observation lends insight for chondrocyte metabolic processes and matrix synthesis rates. For example, relaxation time scales on the order of 12 hours (4.6×10^4 s) were too fast for chondrocytes to effectively generate neotissue, perhaps due to changes in mechanosensing, diffusion and mass loss. On the other hand, relaxation timescales of about 1 month (2.8×10^6 s) were too slow, clearly limiting matrix deposition to pericellular regions and reducing overall cellularity. These differences were further dependent on the size of the matrix molecules, with collagen having a stronger dependence on average relaxation time than sGAGs.

3.8 CANs support the elaboration of articular cartilage specific matrix molecules

Immunohistochemistry verified that the deposited ECM was articular cartilage-specific. Hydrazone CANs showed large amounts of collagen II and aggrecans, two primary components of articular cartilage (Figure 8). The distribution was consistent with results for total collagen and sGAGs, showing more interconnected collagen II and more aggrecan-rich matrix in hydrogels formulated to have intermediate levels of adaptability (17 and 22%

bHz). Staining for collagen I revealed some limited fibrocartilaginous character across all hydrazone hydrogel formulations. Similarly, small amounts of hypertrophic collagen X were observed in all hydrazone hydrogels; however, qualitatively both collagen type I and type X were minimal in hydrazone CANs (Figure 8) compared to deep zone cartilage (+C).

4. Discussion

Arthritis-attributable medical expenditures and earnings losses in the United States alone were recently estimated to be greater than \$300 billion annually.[47] This financial burden falls disproportionately on people who suffer advanced symptomatic osteoarthritis in load-bearing joints. In this work, we investigated the use of hydrazone CANs that are able to provide mechanical strength for MACT in load bearing joints through covalent linkages that are able to break, adapt, and reform, demonstrating viscoelastic properties and allowing improved ECM deposition by encapsulated chondrocytes. The highest performing constructs were identified using Flory-Stockmayer theory to formulate hydrogels above the percolation threshold for slowly adapting bHz crosslinks (22%), while maximizing the percentage of highly adaptable aHz crosslinks. The material properties of the resulting scaffolds were able to maintain cellularity, retain deposited neotissue, and preserve scaffold integrity simultaneously, something that is difficult to achieve with classically degradable hydrogels.

The material properties of hydrazone CANs are dominated by equilibrium kinetics, particularly differences in reverse reaction rates of aHz and bHz formation ($k_{-1}^{aHz} = (2.8 \pm 0.6) \times 10^{-4} \text{ s}^{-1}$ vs. $k_{-1}^{bHz} = (8.7 \pm 0.3) \times 10^{-6} \text{ s}^{-1}$). [28] Kinetic differences in crosslink equilibrium manifested as significant differences in viscoelastic properties. Importantly, hydrogels were formed at the same polymer content (w/w%) and resulted in similar initial storage moduli (G'), allowing differences in covalent adaptability to be studied by tuning the percentage of aHz and bHz crosslinks. For cell encapsulation experiments, we selected formulations maintaining large percentages (>78%) of fast adapting aHz crosslinks near the Flory-Stockmayer percolation threshold for slow adapting bHz crosslinks. We hypothesized that this regime (12%-22% bHz) would be most interesting for cartilage tissue engineering applications. We assumed that a relatively small percentage of bHz crosslinks could maintain the overall structural integrity of the hydrogel, while maximizing crosslink adaptation to facilitate cellular activity and the distribution of cell-secreted ECM. As discussed later, the results of ECM deposition by encapsulated chondrocytes supports this hypothesis, showing large differences within this narrow compositional space, as well as significantly increased ECM with respect to 100% bHz controls.

Prior to encapsulation studies, it was important to characterize temporal changes in network architecture resulting from differences in covalent adaptability. After 28 days, hydrazone hydrogels demonstrated swelling ratios from approximately 20 to 40, corresponding to 97.5 to 95% water content. Although higher than typically observed in native cartilage tissue (~80%), high water content in synthetic polymer scaffolds has been previously linked to improved cellular viability.[48] In addition, higher water content can help facilitate transport within networks for exogenous delivery of biochemical factors, such as small molecules and growth factors to promote regenerative behavior of encapsulated chondrocytes. Of equal interest, we wanted to understand the extent to which unbound PEG macromers would be

released from hydrazone hydrogels over time. When the aHz content was high, the rate of hydrolysis of the aHz bonds was faster than the adaptable reformation, thus resulting in significant polymer loss for hydrogels composed almost entirely of aHz crosslinks. This highlights the need to balance the bHz content relative to the aHz content, especially with respect to tissue engineering applications. Specifically, bHz crosslinks help to maintain bulk material properties and structural integrity, while the aHz crosslinks create microenvironments that can respond to local forces generated by cells when they proliferate and secrete matrix molecules during tissue regeneration.

After seeding chondrocytes in CANs with varied adaptability, we sought to better understand how stress relaxation of these materials would influence the proliferation and matrix deposition. While proliferation was not directly investigated, the cellularity of constructs was quantified over the course of 28 days. Due to the low metabolic activity of chondrocytes, the differences observed in cell density were relatively narrowly distributed, with hydrazone hydrogels demonstrating less than a single doubling over the course of 4 weeks. However, cell density did increase more significantly at intermediate levels of covalent adaptability. To further support these observations, it would be interesting to directly measure chondrocyte proliferation and track survival as a function of network adaptability, especially in the presence of mitogens or when stimulated by co-culture with MSCs.[40] In this work, a balance between fast and slow relaxing crosslinks was required for long term survival and proliferation of chondrocytes while maintaining cells within the confines of the scaffold. Average relaxation times between 6.3×10^4 s and 2.6×10^5 s facilitated increased chondrocyte populations, implying that this range may be particularly relevant for survival and proliferation of chondrocytes during *in vitro* cell culture. Increased cellularity in adaptable constructs correlated strongly with enhanced matrix deposition (Figure S2), implying that proliferation is one of the primary mechanisms by which adaptable networks improve regenerative outcomes. We next studied how matrix components were spatially distributed as a function of the relaxation properties to better understand chondrocyte behavior within CANs, showing that ECM deposition was biphasic as a function of the hydrazone covalent adaptability. The formulation that formed neotissue most closely resembling native articular cartilage had an average relaxation time of 2.6×10^5 s, suggesting a link to chondrocyte timescales for ECM deposition and material properties that change on the order of days. Work focused on measuring local mechanical properties (e.g., microrheology) could provide further insight for how changes in mechanics of the cellular microenvironment alter chondrocyte phenotype and secretory properties, especially compared to the bulk rheological measurements conducted here.

Immunofluorescence was used to verify that chondrocytes in hydrazone CANs secreted articular cartilage-specific matrix molecules. Chondrocytes encapsulated in all of the hydrazone hydrogel formulations demonstrated relatively low levels of collagen I and collagen X deposition. In contrast, other clinical treatments currently used to repair articular cartilage, such as microfracture surgery, result in primarily type I collagen, indicative of mechanically inferior fibrocartilage formation.[49] Similarly, the development of hypertrophic collagen type X typically precedes neotissue mineralization and osteoarthritic phenotypes.[50] Rather than displaying these markers of dedifferentiation, chondrocytes in hydrazone CANs deposited primarily collagen type II found in healthy articular cartilage.

After only 4 weeks, the large amount of ECM deposited by encapsulated chondrocytes in these hydrazone hydrogels compared well to previously published literature. The hydrazone condition composed of 22% bHz crosslinks produced $140 \pm 20 \mu\text{g}$ collagen / mg dry construct weight (Figure S2). This value falls well within the range for ECM deposition by chondrocytes previously cultured within degradable PEG hydrogels (hydrolytically and enzymatically) as well as chondrocytes in naturally derived polymer networks such as hyaluronic acid and calcium-alginate hydrogels (-50 - $300 \mu\text{g}$ collagen / mg dry construct weight).[18,35,40,50,51] This result is particularly promising considering that this study did not use juvenile chondrocytes, which produce more ECM than older chondrocytes from animals that have been alive for several months. This further encourages the use of CANs for MACT where chondrocytes are often isolated from elderly patients.

Alternatively, these CANs could be used to investigate the influence of non-linear scaffold mechanics on differentiation of MSCs for cartilage tissue engineering. This could augment the clinical significance of CANs for MACT due to higher availability and expansion potential of MSCs.[52] CANs also exhibit self-healing and injectable behaviors uniquely suited to clinical challenges facing cartilage tissue engineering and MACT.[53] For example, irregular defects could be press fit with self-healing CANs and invasive surgery requirements could be circumvented by injectable delivery schemes.[54,55] To simulate application in articulating joints, cell-laden hydrazone constructs could be cultured in a bioreactor to examine the effects of confined mechanical loading *in vitro*. Furthermore, hydrazone crosslinks have been previously used for *in vivo* studies suggesting facile translation for future animal studies.[34,56] Based on the results of this work, we postulate that covalent equilibria of hydrazone hydrogels could be tuned to provide mechanical support for encapsulated cells in load bearing joints, while adaptable crosslinks allow for improved proliferation, matrix deposition and cellular remodeling.

5. Conclusion

We demonstrated the use of synthetic hydrazone CANs with tunable viscoelastic properties that modulate behavior of encapsulated chondrocytes. Incorporation of highly adaptable aHz crosslinks improved cellularity by a factor of two over four weeks and enhanced regenerative outcomes such as proliferation and ECM deposition with respect to the 100% bHz control. However, weakly percolating networks of slowly relaxing bHz crosslinks were also required to achieve the highest quality neocartilaginous tissue and maintain scaffold integrity. The reported results reveal covalent networks with relaxation timescales applicable for cartilage tissue engineering (6.3×10^4 - 2.6×10^5 s) and provide insight for how chondrocytes respond to differences in covalent network adaptability. Dense chondrocyte populations and interconnected neotissue observed in hydrazone CANs with average relaxation times of ~ 3 days suggests that this timescale is particularly relevant for chondrocyte behavior in CANs. Additionally, these results point to cellularity and reorganization timescales as determinant factors for development of neocartilaginous tissue in viscoelastic scaffolds. Chondrocytes in these adaptable hydrogels produced significantly more collagen ($190 \pm 30\%$) and sGAGs ($140 \pm 20\%$) than chondrocytes in predominantly elastic hydrogels with slow average relaxation times of ~ 1 month. Adaptable hydrazone hydrogels also yielded largely articular cartilage-specific type II collagen and aggrecan

deposition, supporting future use of CANs as scaffolds to improve cartilage tissue engineering outcomes for MACT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Significance

Covalently crosslinked hydrogels provide robust mechanical support for cartilage tissue engineering applications in articulating joints. However, these materials traditionally demonstrate purely elastic responses to deformation despite the dynamic viscoelastic properties of native cartilage tissue. Here, we present hydrazone poly(ethylene glycol) hydrogels with tunable viscoelastic properties and study covalent adaptable networks for cartilage tissue engineering. Using hydrazone equilibrium and Flory-Stockmayer theory we identified average relaxation times leading to enhanced regenerative outcomes and showed that extracellular matrix deposition was biphasic as a function of the hydrazone covalent adaptability. We also showed that the incorporation of highly adaptable covalent crosslinks could improve cellularity of neotissue, but that a percolating network of more stable bonds was required to maintain scaffold integrity and form the highest quality neocartilaginous tissue.

Figure 1. Chemistry and hydrogel formulation.

A) Schematic representing chemical structures for the reaction of hydrazine with alkyl (a) and benzyl (b) aldehyde to form alkyl-hydrazone (aHz) and benzyl-hydrazone (bHz) bonds. B) For an interconnected polymer network of bHz bonds to span the complete volume, the percentage of bHz bonds (p_b) must be greater than the percolation threshold (p_c) assuming ideal step-growth. 2D slices illustrate network connectivity for each experimental condition used for cell culture (12, 17, 22 and 100%).

Figure 2. Rheological characterization of hydrazone hydrogels.

Hydrazone hydrogels (5w/w%) formed by the stoichiometric reaction of 8-arm 20kD PEG-hydrazine with 8-arm 20kD alkyl or benzyl PEG-aldehyde. A) Evolution of the shear storage (G') and loss (G'') modulus as a function of time during step-growth polymerization. B) The final storage and loss modulus and C) loss tangent ($\tan(\delta)=G''/G'$) for hydrogels with 100% alkyl-hydrazone (aHz) or 100% benzyl-hydrazone (bHz) crosslinks. In A-C, bars represent mean $\pm$ standard deviation. Significance represents results of unpaired two-tailed t-tests ($P < 0.05 = *$) with Welch's correction. D) Shear stress (σ/σ_0) as a function of time with solid lines representing model fits (Eq 1) for hydrazone hydrogels formulated with varied percentages of benzyl-hydrazone crosslinks (e.g., 30% bHz $\Rightarrow$ 70% aHz). Fitted values for the E) relaxation time constants (τ_a and τ_b) and F) stretching parameters (β_a and β_b) as a function of the hydrazone bond composition. Points represent fitted parameters $\pm$ 95% confidence bounds. G) Average relaxation times ($\langle \tau \rangle$) calculated (Eq 2) as a function of the percentage of bHz crosslinks in the hydrogel formulations calculated using fit parameters and 95% confidence bounds.

Figure 3. Swelling and mass loss of acellular hydrogels.

A) Swollen mass (m_s/m_f) normalized by the mass at the time of formation (m_f) for hydrogels with varied percentages of bHz crosslinks over time. B) The percentage of initial polymer mass retained ($\% = m_d/m_0 \times 100\%$) and C) the mass swelling ratio ($q = m_d/m_s$) as a function of benzyl-hydrazone content; calculated using the dry mass (m_d) of constructs at the end of the 28-day experiment and the initial polymer mass of formulated hydrogels (m_0). In each case four hydrogels were averaged for each condition ($n=4$) with significance representing 1-way or 2-way ANOVA or with Dunnett's multiple comparisons test. Significant differences with respect to the 100% bHz control are depicted with $P < 0.05 = *$, $P < 0.01 = **$, $P < 0.001 = ***$, $P < 0.0001 = ****$.

Figure 4. Cellularity and proliferation.

Quantification of the number of chondrocytes in hydrazone hydrogels over time as measured by dsDNA levels. Four hydrogels were averaged for each condition (n=4) with significance representing 2-way ANOVA with Dunnett's multiple comparisons test. Significant differences with respect to the 100% bHz control are depicted with $P < 0.05 = *$, $P < 0.01 = **$, $P < 0.001 = ***$, $P < 0.0001 = ****$.

Figure 5. sGAG content.

A) Brightfield microscopy images showing histological sections of cryosectioned constructs stained with Safranin O to visualize the spatial distribution of sGAGs deposited by chondrocytes after 28 days. sGAGs are represented by the red stained area with nuclei stained violet/black. Hydrazone hydrogels are labeled with the percentage of bHz crosslinks, and articular cartilage and acellular hydrogels are included as positive and negative controls, respectively. Scale bars represent 50 μm . B) Graphical depiction of the total sGAG content as a function of time from a DMMB assay. Four hydrogels were averaged for each condition ($n=4$) with significance representing 2-way ANOVA with Dunnett's multiple comparisons test, showing differences with respect to the 100% bHz hydrogel, $P < 0.05 = *$, $P < 0.01 = **$, $P < 0.001 = ***$, $P < 0.0001 = ****$ and with respect to the day 1 values, $P < 0.05 = +$, $P < 0.01 = ++$, $P < 0.001 = +++$, $P < 0.0001 = ++++$.

Figure 6. Collagen content.

A) Brightfield microscopy images showing histological sections stained with Masson's Trichrome to show the spatial distribution of collagen deposited by chondrocytes after 28 days. Collagen is represented by blue stain area with nuclei stained violet/black. Hydrazone hydrogels are labeled with the percentage of bHz crosslinks, with an articular cartilage positive control and an acellular hydrogel negative control. Scale bars represent 50 µm. B) The total collagen content as a function of time from a hydroxyproline assay. Four hydrogels were averaged for each condition (n=4) with significance representing 2-way ANOVA with Dunnett's multiple comparisons test. Differences with respect to the 100% bHz hydrogel are denoted by $P < 0.05 = *$, $P < 0.01 = **$, $P < 0.001 = ***$, $P < 0.0001 = ****$ and differences with respect to day 1 values are denoted by $P < 0.05 = +$, $P < 0.01 = ++$, $P < 0.001 = +++$, $P < 0.0001 = +++++$.

Figure 7. ECM deposition as a function of hydrogel average relaxation time.

The mass of ECM produced by encapsulated chondrocytes at the final time point (day 28) with respect to the average relaxation time ($\langle \tau \rangle$) of hydrazone hydrogels. Four hydrogels were averaged for each condition ($n=4$) with significance representing 2-way ANOVA with Sidak's multiple comparisons test showing differences with respect to the 100% bHz control, $P < 0.05 = *$, $P < 0.01 = **$, $P < 0.001 = ***$, $P < 0.0001 = ****$, and with respect to the most adaptable 12% bHz condition, $P < 0.05 = \#$, $P < 0.01 = \##$, $P < 0.001 = \###$, $P < 0.0001 = \####$.

Figure 8. ECM Immunostaining.

Immunohistochemical staining of chondrocyte laden-hydrazone hydrogels on day 28 to assess articular cartilage-specific ECM deposition. Images are maximum intensity projections of 30 μm sections imaged with a confocal microscope. Labels represent the percentage of bHz crosslinks, and porcine articular cartilage positive controls (+C). The first two rows show ECM specific to articular cartilage, collagen type II (red) and aggrecan (green). The second two rows show ECM indicative of chondrocyte dedifferentiation and fibrocartilage formation, collagen type X (red) and collagen type I (green). All images include a DAPI nuclear counterstain (blue) and scale bars represent 100 μm.