Supplementary Information for

Viscoelasticity of hydrazone crosslinked poly(ethylene glycol) hydrogels directs chondrocyte morphology during mechanical deformation

Benjamin M. Richardson, Cierra J. Walker, Laura J. Macdougall, Jack W. Hoye, Mark A. Randolph, Stephanie J. Bryant, Kristi S. Anseth*

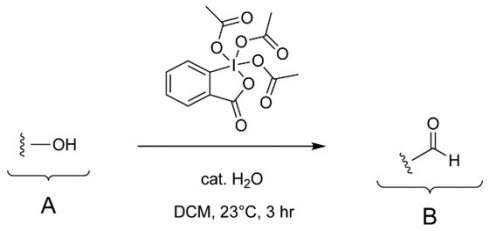
*Corresponding author. Email: Kristi.Anseth@colorado.edu

1. Materials and Methods

All chemicals and solvents were analytical grade and acquired from commercial sources unless otherwise described. Multi-arm polyethylene glycol (PEG) precursors were purchased from JenKem Technology USA.

1.1 alkyl PEG aldehyde synthesis (8a-PEG(TP)-10kD-CHO):

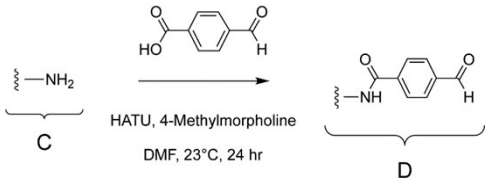
Alkyl-PEG-aldehyde was synthesized by Dess-Martin Oxidation of 8-arm PEG-OH (M_n ~10,000 g/mol) as previously described.[1] Briefly, PEG was dissolved with Dess-Martin Periodinane (1.5 equiv. w.r.t. OH groups) in a minimal amount of dichloromethane (DCM) containing catalytic H_2O. The reaction was allowed to proceed for 3 h at room temperature. (23 °C)



1.2 benzyl PEG aldehyde synthesis (8a-PEG(TP)-10kD-Ar-CHO):

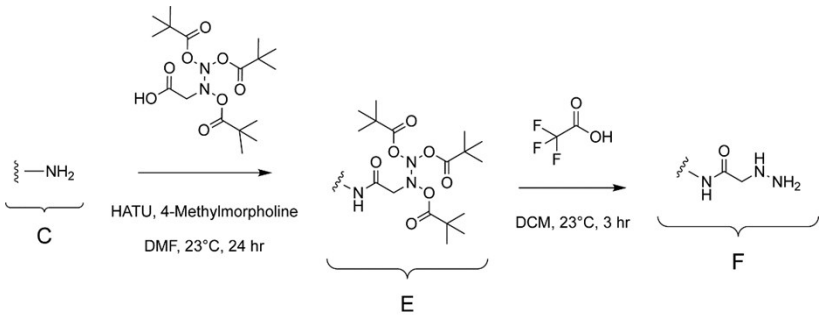
Benzyl-PEG-aldehyde was synthesized by HATU (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium3-oxidhexafluorophosphate) coupling to PEG-NH_2.[2] Briefly, 4-formylbenzoic acid (2.2 equiv. w.r.t NH_2 groups) was activated with HATU (2.0 equiv. w.r.t NH_2 groups) and 4-methylmorpholine (5.0

equiv. w.r.t NH_2 groups) in dimethylformamide (DMF) under argon for 10 min. In parallel, PEG-NH_2 was dissolved in DMF containing 4-methylmorpholine (5.0 equiv. w.r.t NH_2 groups). The two solutions were mixed and the reactions were allowed to proceed overnight under argon at room temperature. (23 °C)



1.3 PEG hydrazine synthesis (8a-PEG(TP)-10kD-NH-NH_2):

Hydrazine-PEG was synthesized by HATU coupling to PEG-NH_2 as described above using Tri-boc-hydrazinoacetic acid. Boc (tert-butyloxycarbonyl) protected hydrazine-PEG was precipitated dropwise in cold (4 °C) diethyl ether (Et_2O), then dissolved in a 50:50 mixture of trifluoroacetic acid in DCM. The deprotection reaction was allowed to proceed in a vented flask for 3 h prior to purification.



1.4 Macromer purification and characterization

Crude reaction mixtures were concentrated under reduced pressure, precipitated dropwise in cold Et_2O, centrifuged, and decanted. The products were washed similarly 3x and dried *en vacuo*. The products were then dissolved in deionized H_2O and dialyzed in regenerated cellulose membranes (Spectra/Por) with molecular weight cut-off of 8,000 g/mol for 96 h at 4 °C. Polymer solutions were then lyophilized, and stored dry at −20 °C. ^{1}H-NMR spectroscopy (Bruker AV-III, $CDCL_3$, 400 MHz) was used to evaluate the

functional efficiency of the oxidation and coupling reactions. (Fig S1) In each case the functionality was found to be >80%. (Table S2) Integrations were normalized by the setting PEG peaks to 113.5 as this represents the average number of PEG protons in a single polymer arm (for 8 arm 10 kD macromers). Under this normalization scheme, functional peak integrations should correspond to the number of protons within each functional unit. Therefore, the average percent functionalization for each macromer was calculated by dividing normalized functional peak integrations by the number of protons that should be present in each functional unit. (Eq. S1)

$$\%\, functionalization = \frac{\sum funtional\ peak\ integrations}{\#\ protons\ in\ functional\ unit} * 100\% \quad Eq.S1$$

1.5 Hydrogel formulation

Functionalized PEG macromers were dissolved in buffered saline (DPBS Gibco) and neutralized. (pH 7.2) Stock solutions were aliquoted for storage at -70°C prior to experimentation. Hydrogels were formed off stoichiometry (r = [El]/[Nu] = 0.8) to minimize the free aldehyde concentrations (~3wt%). Percolation thresholds were determined using Flory-Stockmayer theory (Eq. S2), where f_x represents the average number of reactive functional groups per macromer. (Table S2)

$$p_c = \frac{1}{\sqrt{r(f_{Nu}-1)(f_{El}-1)}} \quad Eq.S2$$

1.6 Shear rheometry and modeling

Rheological experimentation was performed between temperature controlled parallel plates on a controlled-stress rheometer. (TA Instruments DH-R3) Hydrogels were formed *in situ* with a mineral oil ring applied to the air-hydrogel interface to prevent evaporation during experimentation.

$$\tau = \eta/E \quad Eq.S3$$

Characteristic relaxation times (τ) represent the quotient of the spring constant (E) and viscosity (η) for a Maxwell Element, represented by a spring and dashpot in series. (Eq. S3) In this work, the Kohlrausch-Williams-Watts model was used to describe viscoelastic behavior and is represented by an infinite series of Maxwell Elements in parallel. (Fig. S2) Normalized stress relaxation data was fit with the Kohlrausch-Williams-Watts function. (Eq. S4) In this equation, relaxation time constants (τ) are characteristic of the reorganization of covalent bonds and stretching parameters (β) account for heterogeneous relaxation events.[4]

$$\frac{\sigma}{\sigma_0} = \exp\left(-\left(\frac{t}{\tau}\right)^{\beta}\right) \quad Eq.\,S4$$

Average relaxation times $\langle\tau\rangle$ represent the distribution of relaxation modes for the Kohlrausch-Williams-Watts function. (Eq. S5) Average relaxation times were calculated by integrating the stretched exponential model over its entire domain (t = 0 to t = infinity).

$$\langle\tau\rangle = \frac{\tau}{\beta}\Gamma\left(\frac{1}{\beta}\right) \quad Eq.\,S5$$

Average linear creep rates (1/<η>) represent inverse viscosities and are characteristic of material deformation rates. (Eq. S6) This parameter can be found as the slope of the line describing the creep compliance (J) as a function of time, excluding initial creep ringing.

$$J - J_0 = \frac{t}{\langle\eta\rangle} \quad Eq.\,S6$$

1.7 Chondrocyte isolation and cell culture

Primary chondrocytes were isolated from the stifle joints and the femoral patellar groove of Yorkshire swine as detailed previously.[5] Freshly isolated chondrocytes were frozen in 90% fetal bovine serum (FBS) with 10% dimethyl sulfoxide (DMSO) at 2 x 10^6 cells/mL and stored in LN_2 prior to use for experimentation. One week before encapsulation, chondrocytes were thawed and seeded onto T-75

tissue culture flasks. Expanded cell populations were encapsulated after reaching 80% confluence without passaging (P0). Chondrocytes were stained with CellTracker™ Orange and encapsulated at 5 x 10^6 cells / mL. Hydrogels (50 µl) were formed in rectangular molds (4x4x3 mm) for 30 minutes prior to submersion in chondrocyte growth medium. Medium was composed of high-glucose DMEM (Gibco) supplemented with 10% FBS (Gibco), 1% penicillin-streptomycin-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 acid (NEAA) (Gibco), 100 mg/mL HEPES buffer (Sigma-Aldrich) and 50 mg/mL gentamicin (Invitrogen). Medium was changed every other day and hydrogels were maintained at 37 °C and 5% CO_2.

1.8 *In situ* deformation microscopy

Cuboidal hydrogel constructs were loaded into a specially designed microscope mounted loading plate. Confocal images were acquired on a Nikon A1R laser scanning confocal microscope (40x objective) fitted with an environmental chamber to maintain humidity at 37°C and 5% CO_2. (Fig. S3) Image processing and analysis were completed using ImageJ (NIH) or Imaris (Oxford Instruments) for intensity thresholding, edge exclusion, counting and measurement. Data are shown as representative analysis from Z-stack maximum intensity projections, unless otherwise noted. Chondrocyte recovery is defined as the percentage of the initial deformation recovered by chondrocytes during 10 hours of static loading. (Eq. S7) The timeline for the *in situ* deformation experiments was defined by setting unstrained (0% strain) conditions as t = 0 hour, the initial deformed time point (20% strain) as t = 1 hour, and the final deformed time point (20% strain) as t = 11 hour. (Fig. S4) Reported values represent error propagation of standard deviations from three hydrogel (n=3) hydrogel replicates.

$$\%\, recovery = \frac{\left[\left(\frac{X}{Y}\right)_{0\%\, strain}^{0\, hour} - \left(\frac{X}{Y}\right)_{20\%\, strain}^{11\, hour}\right]}{\left[\left(\frac{X}{Y}\right)_{0\%\, strain}^{0\, hour} - \left(\frac{X}{Y}\right)_{20\%\, strain}^{1\, hour}\right]} * 100\% \qquad Eq.\, S7$$

1.9 Histological sectioning and staining

One week after encapsulation, chondrocyte-hydrogel constructs were fixed for 30 minutes at room temperature in 10% formalin. Hydrogels were rinsed in DPBS for 45 minutes then incubated in optimal cutting temperature (OCT) compound (Tissue-Tek) overnight at 4°C. Samples were transferred to molds with fresh OCT and snap frozen in LN_2 prior to storage at -70°C. Slides were prepared with 20 µm hydrogel sections (Leica Cryostat CM1850). Sections were stained with Safranin-O (Leica Autostainer-XL) and cover slides were applied with Permount (Fisher) to preserve staining. Slides were imaged by bright field microscopy on a Nikon TE-2000 inverted microscope (100x objective).

1.10 Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was used to quantify the mRNA expression levels for collagen type I (COL1A1) and collagen type II (COL2A1) relative to the reference gene GAPDH. Chondrocytes were cultured in hydrazone CANs for one week then subjected to 6 hours of uniaxial compressive loading (20%) after which samples were homogenized for 3 minutes with 5-mm steel beads shaking at 30 Hz (Qiagen TissueLyser). RNA was then isolated using a RNeasy Micro Kit (Qiagen). RNA quantity and purity were measured via spectrophotometry (ND-1000, NanoDrop). cDNA was synthesized from total RNA using the iScript Synthesis kit (Bio-Rad) and quantified via qRT-PCR using SYBR Green reagents (Bio-Rad) on an iCycler (Bio-Rad). Relative expression levels of Col1 and Col2 were quantified using the delta delta Cq method by normalizing to GAPDH for three replicates per condition.[6] Primer sequences are listed in Table S1.

Table S1	Forward primer (5'–3')	Reverse primer (5'–3')
GAPDH	ACACTCACTCTTCTACCTTTG	CAAATTCATTGTCGTACCAG
COL1A1	GGGCAAGACAGTGATTGAATACA	GGATGGAGGGAGTTTACAGGAA
COL2A1	CCTCAAGAAAGCCCTGCTCA	CCCCACTTACCGGTGTGTTT

1.11 Statistics

Unless otherwise noted, graphical representations and error bars represent the mean ± standard deviations. 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 multiple comparison tests. Statistical analyses were performed with GraphPad Prism 8 software.

2. Supplementary Figures

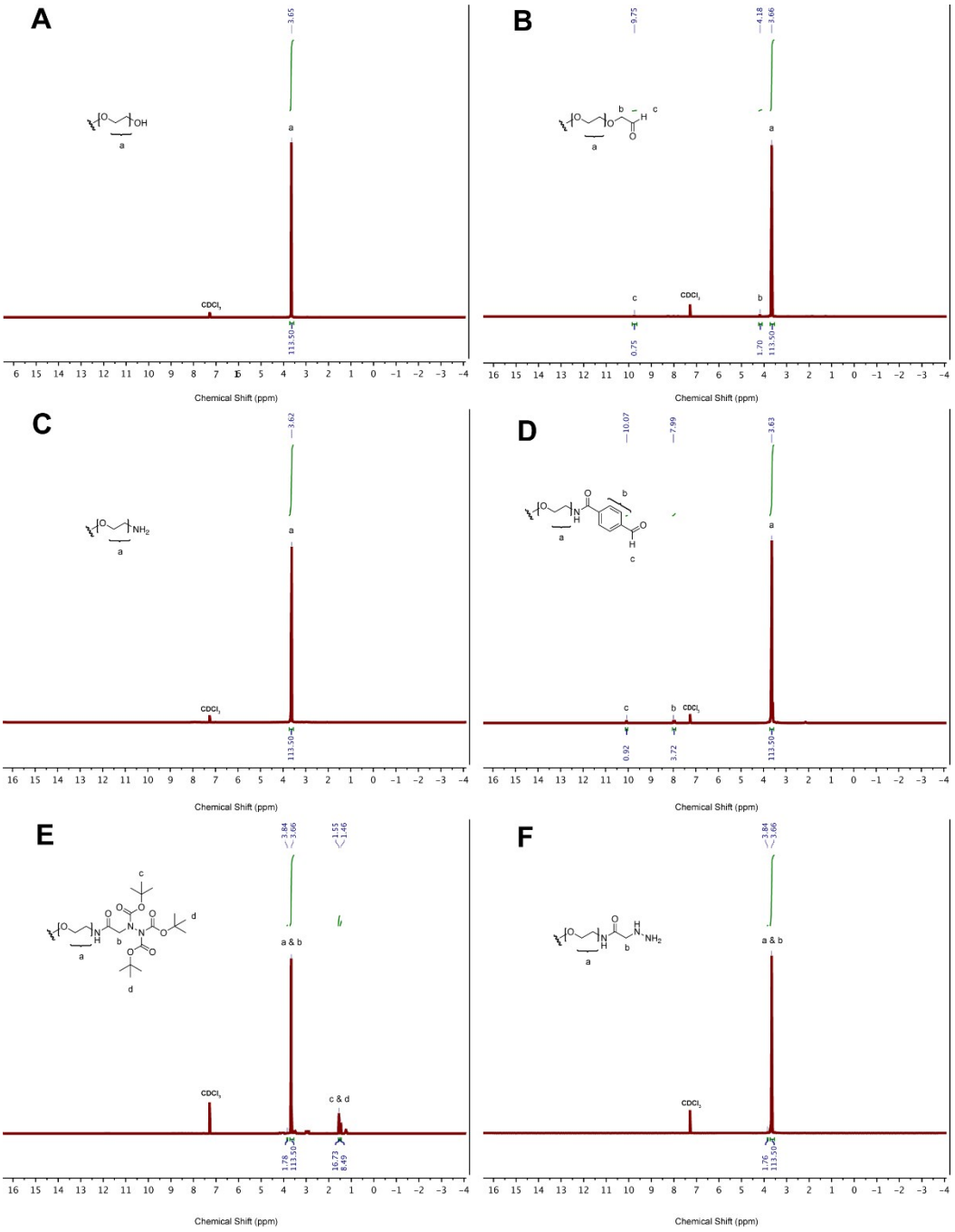


Fig. S1 H-NMR spectroscopy verifying functionalization of 8-arm 10kD PEG macromers. A) Unmodified PEG-OH, δ = 3.725-3.542 (m, 113.5H, -O-C**H**$_2$-C**H**$_2$-O-); B) Functionalized PEG-CHO, δ = 9.852-9.640 (s, H, -C**H**O), δ = 4.243-4.085 (s, 2H, -C**H**$_2$-CHO), δ = 3.725-3.542 (m, 113.5H, -O-C**H**$_2$-C**H**$_2$-O-); C) Unmodified PEG-NH$_2$ δ = 3.725-3.542 (m, 113.5H, -O-C**H**$_2$-C**H**$_2$-O-); D) Functionalized PEG-Ar-CHO, δ = 10.024-10.119 (s, H, -C**H**O), δ = 8.037-7.894 (m, 4H, -C$_6$**H**$_4$-), δ = 3.725-3.542 (m, 113.5H, -O-C**H**$_2$-C**H**$_2$-O-); E) Functionalized PEG-NBoc-NBoc$_2$, δ = 3.860-3.819 (s, 2H, -NH-CO-C**H**$_2$-), δ = 3.725-3.542 (m, 113.5H, -O-C**H**$_2$-C**H**$_2$-O-),

δ = 1.581-1.509 (d, 18H, -OC(C**H**$_3$)$_3$), δ = 1.505-1.441 (d, 9H, -OC(C**H**$_3$)$_3$); F) Functionalized PEG-NH-NH$_2$, δ = 3.860-3.823 (s, 2H, -NH-CO-C**H**$_2$-), (m, 113.5H, -O-C**H**$_2$-C**H**$_2$-O-).

Table S2 Tabulated variables related to synthesis reactions and hydrogel formulation

Table S2	hydrazine	alkyl-aldehyde	benzyl-aldehyde
Macromer structure	PEG-NH-NH2	PEG-CHO	PEG-Ar-CHO
Nucelophile (Nu) or electrophile (El)	Nu	El	El
PEG, M_W (g/mol)	10000	10000	10000
# of arms / PEG macromer	8	8	8
Functional Group M_W (g/mol)	71	-2.0	130
Approximate functionalization (%)	88%	82%	93%
Estimated macromer functionality (f_x)	7.0	6.6	7.4
Percolation threshold (p_c)	N/A (excess reagent)	0.19	0.18
Functionalized PEG, M_W (g/mol)	1.05 x 10^4	9.99 x 10^3	1.10 x 10^3

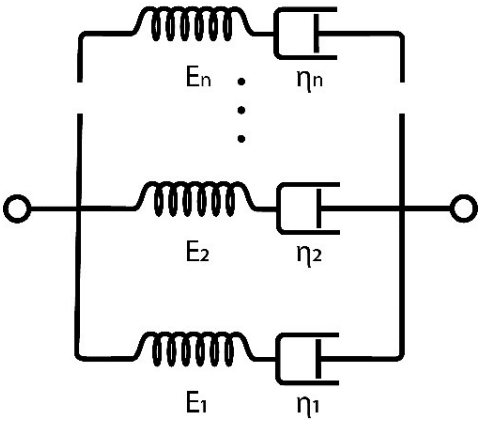


Fig. S2 Schematic representation of the Kohlrausch–Williams–Watts stretched exponential function as an infinite series of Maxwell Elements in parallel.

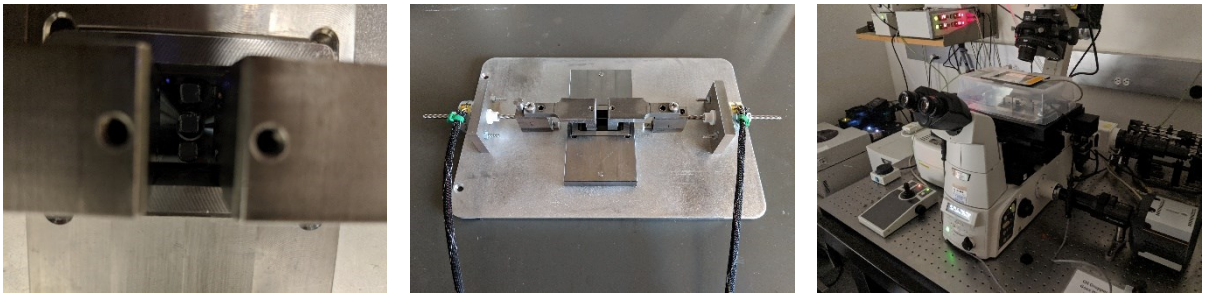


Fig. S3 Specially designed loading plate used for *in situ* deformation microscopy

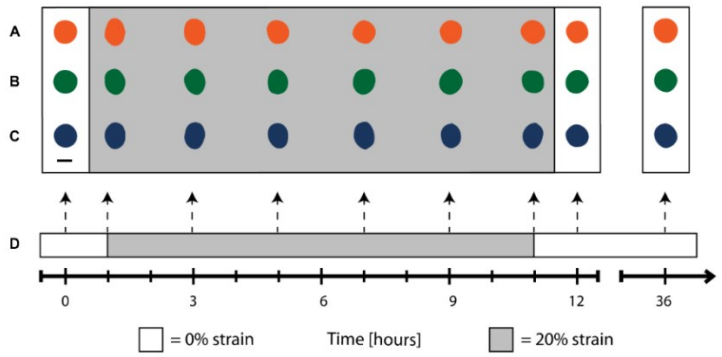


Fig. S4 The schematic above shows representative single chondrocytes at each time point during deformation. 2D cell masks were created from maximum intensity projections using Adobe Illustrator. A) Represents 0%, B) 22%, C) 100% and D) shows the experimental timeline for deformation experiments. Scale bar represents 10 µm.

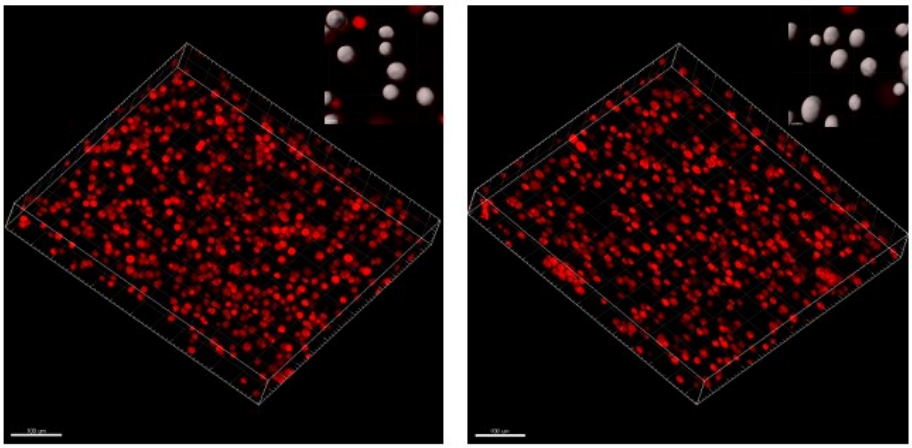


Fig. S5 3D reconstructions showing chondrocytes before (left) and during (right) application of a physiologically relevant compressive strain. Imaris Software was used to combine z-stacks of confocal microscopy images into 3D renderings. The software was similarly used to identify and count chondrocytes. Scale bars represent 400 µm.

3. References

1 S. D. Meyer and S. L. Schreiber, *J. Org. Chem.*, 1994, **59**, 7549–7552.

2 L. A. Carpino, *J. Am. Chem. Soc.*, 1993, **115**, 4397–4398.

3 K. S. Anseth, C. N. Bowman and L. Brannon-Peppas, *Biomaterials*, 1996, **17**, 1647–1657.

4 G. C. Berry and D. J. Plazek, *Rheol. Acta*, 1997, **36**, 320–329.

5 J. J. Yoo, D. A. Bichara, X. Zhao, M. A. Randolph and T. J. Gill, *J. Biomed. Mater. Res. A*, 2011, **99**, 102–8.

6 K. J. Livak and T. D. Schmittgen, *Methods*, 2001, **25**, 402–408.