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Sequential modes of crosslinking tune viscoelasticity of cell-instructive hydrogels

Kyle H. Vining, Alexander Stafford, and David J. Mooney*

Wyss Institute for Biologically Inspired Engineering and John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138, USA

Abstract

Materials that can mimic the fibrillar architecture of native extracellular matrix (ECM) while allowing for independent regulation of viscoelastic properties may serve as ideal, artificial ECM (aECM) to regulate cell functions. Here we describe an interpenetrating network of click-functionalized alginate, crosslinked with a combination of ionic and covalent crosslinking, and fibrillar collagen type I. Varying the mode and magnitude of crosslinking enables tunable stiffness and viscoelasticity, while altering neither the hydrogel's microscale architecture nor diffusional transport of molecules with molecular weight relevant to typical nutrients. Further, appropriately timing sequential ionic and covalent crosslinking permits self-assembly of collagen into fibrillar structures within the network. Culture of human mesenchymal stem cells (MSCs) in this mechanically-tunable ECM system revealed that MSC expression of immunomodulatory markers is differentially impacted by the viscoelasticity and stiffness of the matrix. Together, these results describe and validate a novel material system for investigating how viscoelastic mechanical properties of ECM regulate cellular behavior.

Introduction

The extracellular matrix (ECM) regulates cells in development, regeneration, and disease [1–3] through presentation of biochemical cues, and mechanical cues that alter the intrinsic and extrinsic forces generated by and imposed on cells, respectively [1]. Adherent cells respond to the resistance of the ECM to cellular traction forces, in a manner dependent on both the static mechanical properties of ECM, such as stiffness or modulus [4], and the time-dependent viscoelastic [5, 6] or plastic properties [7]. However, in vivo and traditional cell culture model systems often allow for limited control of ECM mechanics or do not present native ECM molecules for cell binding, so there is a significant need for material systems

*Corresponding author - mooneyd@seas.harvard.edu.

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Data availability

The raw/processed data required to reproduce these findings will be made available on request.

that allow one to specifically tune viscoelasticity without altering other properties of the matrix, such as ligand presentation and density, matrix architecture, and diffusional transport. Furthermore, stiffness and viscoelasticity have not yet been examined in terms of cells' immunomodulatory phenotype in three-dimensional matrices.

A variety of material systems are currently being used to mimic various biochemical cues of native ECM while tuning the mechanical properties, and to explore how these impact stem cells. Polyacrylamide or poly(dimethylsulfoxide) hydrogels have been widely used in two-dimensional cell culture studies to tune the stiffness of cell-adherent substrates coated and/or patterned with fibronectin or collagen ECM proteins [4, 8]. In addition, the effects of time-dependent mechanical properties, such as stress relaxation, on cell behavior have also been investigated on two dimensional substrates by using materials formed via non-covalent crosslinking [9, 10]. A variety of naturally-derived and synthetic polymers are used as three-dimensional cell culture substrates, typically following covalent crosslinking by photoinitiated- [11, 12], free-radical- [13], click- [14–16], or dynamic [17, 18] mechanisms to form substrates with tunable stiffness and viscoelasticity. However, changes in the weight percent (% w/v) of the polymers or degree of crosslinking may also affect the resulting polymer mesh size and transport properties of macromolecules. One can instead utilize physical crosslinking mechanisms, as in the ionic crosslinking of alginate, to form viscoelastic hydrogels with tunable mechanical properties [6]. However, neither of these systems typically feature the hierarchical architecture of native ECM, such as the fibrillar structure of collagen type I. In native ECM, collagen type I is secreted by cells as a procollagen triple helix non-fibrillar protein, which physically and chemically crosslinks into collagen fibers [19]. Fibrillar collagen can be challenging to incorporate and maintain in three-dimensional matrices, because its fibers can be inhibited or altered by changes in collagen content and matrix crosslinking mode and density. Collagen type I can form interpenetrating networks with hydrogels formed with non-covalently [20] or dynamic covalently [17] crosslinked polymers, as well as methacrylated-gelatin [21]. However, none of these studies have reported that collagen fiber architecture can be maintained independent of matrix viscoelasticity. Previous alginate-collagen IPN studies used diluted alginate (0.5% w/v) and collagen (1.5 mg/mL) concentrations over a narrow range of storage moduli (0.03 – 0.3 Pa), and also did not investigate or tune the viscoelastic properties of the matrix [20]. It is important to be able retain fibrillar ECM structures to mimic native ECM, while also tuning both stiffness and viscoelasticity.

We hypothesize that sequential ionic and covalent crosslinking of interpenetrating networks of type I fibrillar collagen and click-functionalized alginate will enable independently tunable shear moduli (or stiffness) and viscoelasticity, without significantly altering the diffusional transport or architectural properties of the matrix. To create this artificial ECM (aECM), we generated an interpenetrating network of collagen type I and alginate, in which the alginate could be crosslinked via covalent and/or ionic bonds (Figure 1a–c). Collagen is used in this system to mimic the architectural and biochemical cues of fibrillar collagen in native ECM of various tissues. Specifically, we model how the matrix mechanics of tumors or sites of tissue injury regulate the immunomodulatory properties of mesenchymal stem cells (MSCs). Alginate was chosen to tune mechanics, because it can be ionically crosslinked by divalent ions (e.g., calcium) cooperatively bound to specific sugars,

glucuronic acid residues, that are arranged in blocks on the alginate chains (G-blocks); as one increases the availability of divalent ions, the cooperative binding of adjacent sugars in the G-blocks is reinforced rather than adding additional crosslinks.[22] Thus, ionic crosslinking of alginate can be used to tune the crosslinking density and modulus of the matrix, without changing the total amount of alginate, ligand density, or resulting mesh size of the network.[23] Covalent crosslinking is introduced into the system by incorporating low molecular weight alginate (Alg) that has been functionalized to contain either norbornene (Nb) or tetrazine (Tz), which are bio-orthogonal chemical groups that “click” together via an inverse electron demand Diels-Alder click reaction with a release of nitrogen gas [16]. Previous studies with this system did not contain fibrillar collagen matrix and were not yet developed for independently tuning stiffness and viscoelasticity [16]. Ionic and covalent crosslinking are tuned to be sequential in order to permit self-assembly of collagen type I into a fibrillar ECM structure during the reversible ionic crosslinking (Figure 1d–e). Calcium carbonate nanoparticles are homogeneously mixed with the hydrogel polymer solution prior to crosslinking (Figures 1a–b and S1a). Calcium carbonate is used instead of other calcium salts, e.g., calcium sulfate [22], because it remains insoluble at neutral pH and allows for uniform distribution of calcium prior to crosslinking. Ionic crosslinking is initiated by the pH-dependent release of soluble calcium ions by the addition of a weak acid, glucono-delta-lactone (GDL). In the presence of calcium ions, a combination of Alg-Nb and Alg-Tz polymers will rapidly undergo ionic crosslinking at their constituent G-blocks to form a hydrogel network, because the majority of carboxyl groups on each alginate chain are unmodified (>90%) and are available for ionic crosslinking. Following initial gelation and collagen self-assembly, it was hypothesized that Nb and Tz groups, now spatially confined in the crosslinked alginate G-blocks, could provide additional covalent crosslinks in the network and impart elasticity without altering gel architecture.

Materials and Methods

Materials synthesis and preparation

Low molecular weight (M_w) ultra-pure sodium alginate (Provona UP VLVG, NovaMatrix) was used for these studies, with an approximate M_w of ~32 kDa, ratio of glucuronic acid to mannuronic acid of 1.5, and 100 endotoxin units per gram, according to the manufacturer. (4-(1,2,4,5-Tetrazin-3-yl)phenyl)methanamine - Trifluoroacetic acid (Tz) was synthesized as previously described [16]. Briefly, 50 mmol of 4-(aminomethyl)benzonitrile hydrochloride and 150 mmol formamidine acetate were reacted with 1 mol of anhydrous hydrazine and quenched with 0.5 mol of sodium nitrite. Next, the product was isolated sequentially in 10% HCl and NaHCO_3 , extracted with DCM, and purified via reverse phase HPLC. The final product was recovered by rotary evaporation and purified by HPLC. After synthesis of Tz, click-modified VLVG was obtained by covalent coupling of either Tz or 5-(aminomethyl)bicyclo[2.2.1]hept-2-ene (Nb, Norbornene Methanamine, TCI America) as previously described [16]. The following protocol results in 5% degree of substitution, which refers to 5% of available carboxylic acid groups modified with Nb or Tz per mol of high molecular weight alginate ($M_w = 32$ kDa). First, VLVG alginate was dissolved at 1% w/v in pH 6.5 buffer (0.1 M MES, 0.3 M NaCl). Second, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were added in 5x

molar excess based on the number of carboxylic acid groups of the molecular weight alginate ($M_W = 32$ kDa). Third, Nb or Tz was added at 1 mmol per gram of alginate, stirred at room temperature for 16 h. Finally, the product was centrifuged and purified via tangential flow filtration utilizing a 1 kDa molecular weight cutoff column (Spectrum Labs) using a decreasing salt gradient from 150 mM to 0 mM NaCl in de-ionized water, followed by treatment with activated charcoal, sterile filtration (0.22 μ m), and freeze drying for long-term storage. All chemicals were purchased from Sigma-Aldrich.

Fabrication of interpenetrating network (IPN) hydrogels

First, collagen and alginate biopolymer solutions were prepared prior to hydrogel fabrication. Bovine atelo-collagen, Type I (9–10 mg/mL, FibriCol, Advanced BioMatrix) or rat tail telo-collagen, Type I (8–11 mg/mL, Corning) was neutralized on ice to $5 < \text{pH} < 6.5$ to prepare a stock solution of collagen with 10x HBSS (without calcium and magnesium, with phenol red, Sigma-Aldrich), 1 M N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (20 mM final concentration, HEPES, Gibco), and 1 M sodium hydroxide (~1% final concentration, NaOH). Unmodified (Alg) or modified VLVG alginates (Alg-Nb or Alg-Tz) were dissolved in a buffered salt solution (HBSS, 20 mM HEPES) at desired concentration (5% to 8% w/v). The pH was adjusted with 1 M NaOH to pH 7, as necessary. Second, a calcium carbonate (CaCO_3) slurry was obtained by ultrasonication (70% amplitude, 15 seconds) nanoparticles of precipitated calcium carbonate (nano-PCC, Multifex-MM, Specialty Minerals) in sterile water (Water-For-Injection, WFI, Gibco) at 100 mg/mL. Third, immediately prior to casting the hydrogels, an appropriate amount of collagen was adjusted to pH 7.5 and mixed with appropriate amount of CaCO_3 slurry on ice for final concentrations of 0.10% to 0.45% w/v calcium and 4 mg/mL collagen. Fourth, alginate was mixed with the collagen-calcium solution on ice prior to gelation. For unmodified alginates (Alg), VLVG, collagen-calcium, and HEPES-buffered HBSS (HBSS/HEPES) were stirred thoroughly with micro-stir bars in appropriate amounts for desired final concentration (1.5% w/v alginate). For click-modified alginates (NbTz), the ratio of Alg-Nb to Alg-Tz was adjusted depending on calcium condition (Table 1). For these gels, Alg-Nb, collagen-calcium, and HBSS/HEPES were stirred thoroughly, and Alg-Tz was reserved to add later. Immediately prior to gelation an appropriate amount (4x molar excess of the calcium concentration) of freshly dissolved glucono-delta-lactone (0.4 g/mL in HBSS/HEPES, GDL, EMD Millipore) was added during rapid stirring. Then, the reserved amount of Alg-Tz was added. The resulting hydrogel solution was quickly transferred by micro-pipets to a mold or plate for characterization. Initial gelation for 1 h was performed at 37° C in tissue culture incubator, followed by equilibration in HBSS/HEPES buffer at 37° C for 1 h, and then replacement of buffer with cell culture media. The initial pH of the polymer solution tunes the ramping of crosslinking from 30 seconds after adding GDL at pH 7 to 200 seconds at pH 8 (Figure S1b), which provides working time for handling the materials.

Rheological characterizations

Rheological characterization of the resulting IPN hydrogels was performed on a stress-controlled rheometer (AR-G2, TA Instrument) using a 20mm cone plate. 75 μ L of hydrogel solution was loaded immediately onto the peltier plate after mixing. A humidity chamber was used to prevent dehydration. The oscillatory rheology was used to measure the storage

modulus (G'), loss modulus (G''), and loss angle (δ) during the onset of crosslinking and after equilibrium was achieved. Rheological tests included an oscillatory time-sweep for 1 h at 25° C (1 Hz, 1% strain) during calcium carbonate-crosslinking, a second oscillatory time-sweep for 0.5 – 1 h at 37° C (1 Hz, 1% strain) for gel equilibration and collagen crosslinking, followed by oscillatory frequency sweep (1% strain, 0.1 to 10 Hz). A shear stress relaxation test was performed by applying 15% shear strain in 1 s and then maintaining 15% strain while recording shear stress for 2 – 10 h. The initial time-sweep at 25° C was used to avoid carbon dioxide and nitrogen gas bubbles, which cause rheological artifacts. Unless otherwise noted, frequency sweep and stress relaxation tests were conducted at 25° C.

Macromolecular transport analysis

Model macromolecules, fluorescently-labeled bovine serum albumin (BSA, 66 kDa, Invitrogen) and FITC-IgG (mouse isotype Ab controls), were used to assess biologically-relevant transport within the IPN hydrogels, as previously described [20, 24]. Briefly, unmodified and Nb-Tz IPNs at low and high calcium crosslinking were used to encapsulate 1 mg/mL AlexaFluor 488-BSA or 33.3 µg/mL IgG in microwells (10 mm diameter) in a 24-well plate (MatTek). Gels were allowed to equilibrate for 1 hour at 37° C in a solution of 1 mg/mL labeled BSA or 33.3 µg/mL IgG in HBSS/HEPES. This media was selected to maintain neutral pH during gel equilibration with physiological salt concentrations, while avoiding calcium, magnesium, or inorganic phosphate ions in the media that may alter the gel properties. Altering the media composition may affect the molecular weight cutoff (MWCO) of the gels, but we expect the changes to be uniform across the conditions tested. Then, this solution was replaced by release media (0.2 mL, HBSS/HEPES, 2.5 mM calcium chloride), which was subsequently collected at 0, 0.25, 0.5, 1.0, 1.5, and 2 hours while samples were continuously agitated on an orbital shaker. Fresh release media was replaced at each time point. Fluorescence of the collected release media was measured by a plate reader (Biotek, 492 nm excitation, 518 nm emission). The diffusion coefficient D was determined by normalizing the cumulative fluorescence intensity to the initial equilibration solution and fitting with a semi-infinite slab approximation (Equation 1, A = matrix area, V = media volume).[24]

$$\frac{C_{media}}{C_{matrix}} = \frac{A}{V} 2\sqrt{\frac{D_{matrix}t}{\pi}} \quad (1)$$

Immunostaining and confocal imaging of collagen and alginate networks

VLVG alginate was modified with TAMRA (rhodamine, Sigma) to fluorescently label the alginate network, as previously described [6]. VLVG-TAMRA was added to IPN formulations (Table 1) at 10% of the final alginate % w/v. IPN hydrogels of unmodified (Alg) and Nb-Tz alginates at low and high crosslinking were fabricated and then equilibrated overnight in staining media of HBSS/HEPES, 1% BSA, and 2 mM calcium chloride at 37° C. Immunostaining for collagen was performed in staining media for 2 h at room temperature (1/500, rabbit polyclonal anti-collagen I, ab34710), followed by three

washes and secondary staining for 1 h at room temperature (1/1000, goat anti-rabbit IgG Alexa Fluor 488, ab150077). For Fast Green staining, IPNs were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes, followed by washing with PBS and staining for 2 hours with 0.1% Fast Green FCF (Sigma-Aldrich) in 30% (v/v) ethanol according to manufacturer recommendations. Confocal fluorescence and second-harmonic imaging (SHG) of stained IPNs was performed with a 20x or 40x objective on a Leica SP5 X multi-photon inverted confocal microscope. At least three images were acquired per condition. Histograms from each image were analyzed by Matlab to solve for Gaussian fits. The mean fit parameters were used to plot histograms of the mean frequency distribution of labeled alginate, collagen, and protein signal intensity per pixel. Collagen SHG signal was quantified by the mean intensity per pixel of a z-stack of 15 μm depth. Intensity line scans were obtained from ImageJ.

Cell culture and encapsulation in IPNs

Fresh human bone marrow from donors less than 45 years old was obtained from Lonza. Human MSCs were isolated by a density gradient with Lymphoprep (StemCell Technologies), followed by adherent culture to tissue culture plastic in alpha-minimal essential media (aMEM, no nucleosides, +GlutaMax, Gibco) supplemented with 20% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (Thermo). Media was changed every 3–7 days and cells were passaged at 70–90% confluency. Isolated MSCs were cryopreserved after 2 passages in complete media and 7.5% dimethylsulfoxide (DMSO, Thermo) and thawed for subsequent experiments. Cells for experiments were used at passage 3–6. For encapsulation, cells were retrieved from culture and suspended at 40×10^6 cells/mL in HBSS/HEPES on ice. An appropriate volume of stock cell solution for the desired final concentration (2×10^6 cells/mL) was added during hydrogel fabrication to the collagen-calcium solution prior to adding alginate, to ensure homogeneous distribution of cells. After mixing all components on ice, hydrogel solutions were transferred to microwells (10 mm diameter) of a 24-well plate (MatTek) and incubated at 37 °C for 1 h. Then, 1.0 mL of HBSS/HEPES buffer was added to each well and the hydrogels were incubated at 37° C for 1 – 2 h until complete calcium carbonate dissolution and hydrogel equilibration. The buffer pH was monitored during crosslinking and the buffer was replaced when it dropped below pH 7. Finally, media was replaced with aMEM complete media with 10% FBS and cultured at 37° C, 5% CO₂. Blank Alg IPN hydrogels (no cells) ionically crosslinked with calcium carbonate were cultured in transwell inserts in the same wells as adherent MSCs to determine the effects of any differences in soluble calcium on MSCs.

Phase contrast imaging and retrieval of conditioned media and cells of IPNs

Phase contrast images were taken of cells encapsulated in IPNs with an EVOS microscope. The relative cross-sectional area of cells was measured in ImageJ. Conditioned media (CM) from each well was collected for ELISA (PGE2 Parameter Assay, R&D). CM was centrifuged at 5000 x g for 10 minutes and the supernatant was diluted 1/10 in calibrator diluent. ELISA measurements of CM samples were performed as according to manufacturer's instructions and reported in pg/mL. Following collection of CM, hydrogels were transferred with spatulas to 96-well U-bottom plates and digested with 150 μL of 300 U/mL collagenase IV (Worthington) and 34 U/mL of alginate lyase (Sigma-Aldrich) at 37°

C for 15 minutes. Next, 0.1 mL wash buffer (DPBS without Ca/Mg, 2 mM EDTA, 0.5% BSA) was added, the plate was centrifuged at 400g for 5 minutes at 4° C, and the supernatant discarded. Then, 0.2 mL of 50 mM EDTA/DPBS (without Ca/Mg, Thermo) was added and triturated with a multi-well pipet until the gels fragmented. The plate was centrifuged at 400g for 5 minutes at 4° C and the supernatant was discarded. A final set of washes was performed with cold 0.2 mL wash buffer on ice, followed by centrifugation at 400g for 5 minutes at 4° C, and then repeated to obtain a cell pellet. Cell count and viability were measured by viability staining and flow cytometry (MUSE) to confirm successful isolation and to quantify cell yields. RNA lysis buffer was added, transferred to microcentrifuge tubes, vortexed, and then flash frozen in dry ice and stored at -80° C or immediately used for RNA isolation.

Relative gene expression measurement

Relative gene expression was measured as previously described [25]. Briefly, RNA was isolated and with PureLink RNA Micro Kit (Invitrogen), quantified by NanoDrop spectrophotometer, and cDNA was reverse-transcribed by iScript Advanced Reverse Transcription Supermix for Real-time quantitative PCR (RT-qPCR) (Bio-Rad). qPCR was performed using CFX96 (Bio-Rad) in duplicate with 10 ng of cDNA and 2x AdvancedSSO SYBR Green Supermix (Bio-Rad) in each reaction. qPCR measurements were repeated on at least three replicate samples using Bio-Rad PrimePCR primers. Relative gene expression was computed by the delta-delta Ct method, which compared Ct values to a control sample and reference gene (*GAPDH*). See Table S1 for list of individual gene primers.

Statistical analysis

Statistical analysis was performed on Prism GraphPad software. Statistical tests used one-way or two-way ANOVA and post-hoc tests for multiple comparisons, or Student's t-tests for comparison between two groups. P-values less than 0.05 was assumed to be significant in all analyses. Results from qPCR are reported as fold change relative to a control, but statistics were performed on the log-2 transform of the delta-Ct values, which are the relative expression normalized to housekeeping gene *GAPDH*. Error bars represent standard error of mean, unless otherwise noted.

Results

First, the gelation kinetics, shear moduli (or stiffness), and viscoelastic properties of calcium crosslinked, unmodified alginate (Alg) hydrogels and NbTz modified alginate hydrogels (NbTz) without calcium crosslinking were characterized by oscillatory rheology. At low crosslinking density (0.15% w/v calcium carbonate), Alg transitions from a more fluid phase to a solid-like gel phase at about 375 seconds, as defined by the time when the loss angle falls below 45 degrees (Figure 2a). The loss angle measures the phase difference between the oscillatory applied strain and measured shear stress, and values below 45 degrees indicate the fluidity of the polymer mixture is diminished, and larger-scale re-arrangements of the networks are reduced. At a higher crosslinking density (0.30% w/v calcium), Alg gels more rapidly (~70 s) (Figure 2b). The rate of Nb-Tz crosslinking was explored by adjusting the ratio of Nb-to-Tz groups (N:T) and weight percent (% w/v) of NbTz; a constant total

quantity of alginate was maintained in gels by adding unmodified Alg as needed. The fluid-to-solid transition time for Nb-Tz increased 10-fold from ~90 s to ~900 s as the crosslinking density of Nb-Tz was reduced from high (1.5% w/v NbTz, 1.0 N:T ratio) to low (1.0% NbTz, 0.25 N:T ratio) (Figure 2c–d). Thus, at both high and low crosslinking densities, onset of gelation (loss angle < 45 degrees) was faster for ionic crosslinking (Alg) than for covalent Nb-Tz crosslinking.

Several gel formulations (selected from Table 1) were subsequently used to fabricate the collagen-alginate aECM hydrogels with either solely ionic crosslinking (Figure 2e–f, Alg), or a combination of ionic and covalent crosslinking (Figure 2e–f, NbTz), and were mechanically characterized. The storage moduli G' of Alg and NbTz hydrogels were similar to one another at both low or high crosslinking density. Conversely, the loss angle was lower for NbTz hydrogels across a range of frequencies (Figure 2e–f), which indicates more elastic and solid-like behavior. These data indicate one can achieve tunable and matched shear storage moduli with Alg and NbTz aECM hydrogels at these crosslinking conditions, while maintaining constant at each stiffness the % w/v of calcium carbonate, collagen content, and overall alginate content. Furthermore, Nb-Tz click chemistry can be used to tune viscoelasticity of aECM hydrogels, as measured by the loss angle, at low and high crosslinking densities, without significantly altering the storage moduli compared to ionic-only Alg hydrogels.

It was hypothesized that the sequential ionic and then covalent crosslinking of alginate would not alter nutrient transport and the architecture of the interpenetrating network at the cell-scale. First, the diffusion of a model macromolecule, BSA, was analyzed through the aECMs. Fluorescently-labeled BSA was encapsulated in Alg and NbTz aECM hydrogels, and its cumulative release was uniform across conditions (Figure 3a). The release data were fit to a semi-infinite slab diffusion approximation (Eq. 1). The curve is not linear, but was only evaluated at early time points (< 2h), during which the release appears linear, to better meet assumptions for the semi-infinite slab approximation. The diffusion coefficients (D) calculated from this data (Eq. 1) revealed no significant differences in BSA diffusion between Alg and NbTz aECM hydrogels, as well as between calcium crosslinking conditions (Figure 3b). The transport of larger macromolecules (>150 kDa) was examined by diffusion studies using IgG isotype control antibodies (Figure S2). Diffusion was not significantly different between low and high crosslinking for each system. Although, differences in diffusion of IgG were observed between NbTz and Alg materials.

The uniformity of interpenetration of the polymers at the micron-scale and the consistency in collagen self-assembly in the aECM was also determined. Analysis of the fluorescence signal of rhodamine-labeled alginate (TAMRA) and anti-collagen type I immunostaining demonstrated that alginate and collagen were uniformly distributed in Alg hydrogels across low and high crosslinking densities (Figure 3c–d). The collagen immunostaining was similar in low NbTz hydrogels, but a very low signal was observed in high NbTz hydrogels (data not shown). FastGreen staining was then performed on NbTz hydrogels as an alternate approach to visualize non-fibrillar collagen protein distribution, and collagen protein was found to be uniformly distributed (Figure 3e–f). Fluorescence intensity line scans clearly indicate interpenetration, as there is no spatial separation of intensities as a function of

distance at a cellular size scale (Figure S3). Visualization of the collagen fibrillar network using second harmonic generation (SHG) confocal imaging revealed that the SHG signal intensity was higher in low Alg compared to high Alg and low NbTz, but the overall architecture appeared similar (Figure S4). In addition, encapsulation of cells did not inhibit the assembly of collagen during crosslinking (Figure S5). However, a significant loss of collagen architecture was observed in the NbTz hydrogels at high crosslinking density (0.35% Ca, 1.5% NbTz, 1 N:T ratio). These findings indicate that while collagen is evenly distributed throughout the various hydrogels, collagen's assembly into fibrils is compromised in the high NbTz gels.

Next, we investigated if manipulating the rates of collagen physical crosslinking and Nb-Tz covalent crosslinking could rescue collagen self-assembly in the high calcium NbTz gels. First, the gel phase transition time of atelo-collagen, which was used in the IPNs in all studies to this point, and telo-collagen type I were determined by oscillatory rheology. The loss angle of atelo-collagen type I reached less than 45 degrees in about 200 seconds, while telo-collagen type I reached less than 45 degrees after less than 10 seconds (Figure 4a), which is consistent with the manufacturer's information. The slow transition of atelo-collagen type I likely underlies the lack of collagen fiber formation in the high NbTz aECM hydrogels. NbTz crosslinking was subsequently slowed by reducing the composition of NbTz from 1.5% to 1.0% w/v, while adding 0.5% Alg to maintain a constant alginate concentration, and maintaining the N:T ratio at 1.0. The 1.0% NbTz hydrogels loss angle reached below 45 degrees at 200 s, instead of 90 s (Figure 4b). Complete NbTz aECM hydrogels were fabricated at 0.30% w/v calcium for 1.0% and 1.5% NbTz, and both reached a similar plateau modulus G' (Figure S6a). The frequency sweep for G' was also similar, and the loss angle was 2.3 degrees for 1% NbTz compared to 1 degree for 1.5% NbTz (Figure 2f and Figure 4c). The phase transition times for all NbTz crosslinking conditions are summarized in Figure S6b. SHG confocal imaging of collagen fibrillar networks in Alg, 1.0% NbTz, and 1.5% NbTz showed that collagen self-assembly was significantly reduced in 1.5% NbTz aECM hydrogels, even with use of telo-collagen instead of atelo-collagen (Figure 4d). However, 1.0% NbTz rescued collagen self-assembly, with similar collagen fiber morphology and intensity as Alg hydrogels. No statistically significant differences were observed in collagen fiber mean fluorescence intensity between Alg and 1.0% NbTz aECM hydrogels (Figure S7).

Finally, the impact of the physical properties of these artificial ECM hydrogels on the paracrine function of mesenchymal stem cells (MSCs) was analyzed, as this is central to MSC immunomodulatory behavior in cancer and stem cell therapies [26–30]. Human bone marrow-derived MSCs were isolated from fresh donor bone marrow aspirates and expanded to passage 3–6. Cells were then encapsulated in the different aECM hydrogels with various levels of crosslinking with a range of storage moduli and viscoelasticity (Figure 5a–b). Oscillatory rheology was performed to characterize the properties of all hydrogels utilized in these studies (Figure S8). Rheology confirmed the gels' storage moduli were not statistically significantly different between Alg and NbTz at each crosslinking density (Figure 5a), while a significant reduction in the loss angle was achieved with the addition of NbTz crosslinks (Figure 5b). Shear stress relaxation tests were also performed to further characterize the materials' viscoelastic properties (Figure S9). Neither the addition of cells nor the absence

of collagen significantly altered the resulting properties of the materials (Figure S10 and Figure S11). Although it is possible that cells could interact with collagen fibers prior to covalent crosslinking, this is not expected to have a significant effect because the crosslinking occurs over a time-scale of minutes, which is relatively short compared to time-scale of biological effects analyzed in these studies. Upon routine observation, we also noted an increase in cross-sectional area of the cells in the high NbTz hydrogels (Figures S12), which did not appear to correlate with results of following studies on immunomodulatory gene expression of MSCs. Next, the MSCs' gene expression of a panel of immunomodulatory paracrine markers were characterized in aECMs with these matching rheological properties. Gene expression was measured in MSCs on tissue culture plastic (TCP) for 72 hours after an initial pulse stimulation with TNF α and IFN γ inflammatory cytokines, and in unstimulated MSCs isolated from aECMs after 72 hours. Cells in all aECM hydrogels demonstrated significant upregulation of immunomodulatory paracrine markers cyclooxygenase-2 (*PTGS2*), interleukin 1 receptor antagonist (*IL1RN*), and TNF α -stimulated gene 6 (*TNFAIP6*), compared to cells adhered to tissue culture plastic (TCP) (Figure 5c–e). MSCs on TCP upregulated monocyte chemoattractant protein-1 (*CCL2*), galectin-9 (*GAL9*), and TNF α -stimulated gene 6 (*TNFAIP6*) after an initial pulse stimulation (Figure 5d and Figure S13), which was consistent with previous studies on MSC polarization to an immunomodulatory phenotype.[31] The gene expression levels of *PTGS2* and *TNFAIP6* were further upregulated as the stiffness (G') was increased in the Alg hydrogels (Figure 5c–d). The levels were also plotted versus loss angle at each G' , which showed a viscoelastic-dependent increase in expression at 0.5 and 2.5 kPa G' . Expression of the immunomodulatory markers *CCL2* and *GAL9* also showed an increasing trend as a function of stiffness and viscoelasticity in the Alg hydrogels, although the increase in expression was much less than upregulation by cytokine stimulation alone (Figure S7). Conversely, increasing the stiffness in the more elastic NbTz hydrogels did not induce significant upregulation of *PTGS2* and *TNFAIP6*. The gene expression of interleukin 1 receptor antagonist (*IL1RN*) was increased in cells encapsulated in both Alg and NbTz hydrogels, as compared to tissue culture plastic, and no effects of aECM stiffness were noted. Although, a trend of increasing expression as a function of loss angle was observed, but not statistically significant. Notably, expression of *PTGS2* and *IL1RN* were significantly upregulated by aECM, but not by pulse cytokine stimulation alone. To confirm the differences in the calcium used to crosslink the various gels were not responsible for the changes in gene expression, empty gels were formed with calcium crosslinking, and added to cells on tissue culture plastic. This did not significantly impact expression of any of the analyzed genes. To confirm the findings of stiffness and viscoelasticity of aECMs on gene expression, secreted levels of prostaglandin E2 (PGE2), the product of cyclooxygenase-2, were quantified from these same rheological conditions. PGE2 was chosen to be evaluated, because *PTGS2* was expressed at the highest level (Ct ~ 20) of all the markers evaluated. PGE2 levels were increased in conditioned media from 2.5 kPa Alg hydrogels gels after 72 hours, compared to 0.25 kPa Alg (Figure 5f). PGE2 levels were also significantly upregulated by an increase in loss angle at 0.5 and 2.5 kPa moduli, which indicates that PGE2 production is jointly regulated by both stiffness and viscoelasticity.

Discussion

A novel aECM material system consisting of an interpenetrating network of collagen type I and click-functionalized alginate was fabricated that provides tunable covalent and ionic crosslinking to vary the rate of stress relaxation of the matrix, or viscoelasticity, independently from the storage moduli, or stiffness. Hydrogels with only ionic crosslinking (Alg) and click-alginate hydrogels with both ionic and covalent crosslinking (NbTz) were generated that reached the same plateau storage moduli at 0.25 kPa, 0.5 kPa and 2.5 kPa. Across this range of moduli, ionic crosslinking yielded hydrogels with a rate of stress relaxation ($\sim 10^2$ seconds) similar to native tissues.[1] The rate of stress relaxation was significantly increased to greater than 3000 seconds by the addition of covalent crosslinks with click-functionalized alginate, without changing the modulus, calcium concentration, or total content of alginate and collagen. Existing material systems for tuning matrix mechanics typically yield either viscoelastic or purely elastic mechanics, but native ECM is modified in development, regeneration, and disease to exhibit varying types of both viscous and elastic properties [1]. The resulting range of elastic moduli of 1.5 to 7.5 kPa (assuming $E = 3 \times G'$) from these studies is physiologically relevant, as it is similar to the range of stiffening previously reported in mouse models of pancreatic ductal adenocarcinoma [32]. Solid tumors also show an increase in elastic energy, independent of stiffness [33]. This mechanically-tunable aECM provides a facile approach for investigating the roles of both stiffness and viscoelasticity on cellular behavior.

Crosslinking conditions of the aECM were varied without significantly impacting the diffusional transport properties of BSA and interpenetration of collagen and alginate at the cell-scale. The diffusion constant for BSA was relatively constant with different mode and magnitude of crosslinking, which suggests the nutrient diffusion is not significantly altered in the various hydrogels used in these studies. The reduction in diffusional transport of IgG of the NbTz gels compared to Alg gels suggests IgG is on the order of the characteristic size of the mesh. However, macromolecules on the scale of BSA or smaller (e.g., growth factors, cytokines, amino acids, sugars, etc.) are expected to uniformly transport through the material. Moreover, in each material system IgG transport was not altered between low and high crosslinking, so larger macromolecular transport can still be compared between moduli. In addition, the fluorescence signal of alginate and collagen was uniform across the conditions, indicating that the interpenetrating network was maintained without phase separation at the cell-scale. These results for the ionically crosslinked hydrogels are consistent with previous studies using other ionically crosslinked alginate hydrogels [20, 23]. However, the addition of covalent crosslinking typically would be expected to change the network architecture. Here, the use of small crosslinking agents which can only bridge polymer chains in close proximity, and temporal sequencing of the various gelation processes in the gels likely resulted in click-crosslinking occurring only in the G-blocks of adjacent alginate chains instead of randomly along the polymer chains. The relatively large mesh size of alginate gels (~ 5 nm)[34] results in different alginate chains generally being too separated for the click crosslinking agents (size ~ 0.2 – 0.3 nm)[35] to effectively bridge and form crosslinks. However, the ionic crosslinking will bring the G-blocks into close contact, allowing the click groups on the sugar residues in these G-blocks to react. Therefore, the

sequential kinetics of ionic and then covalent crosslinking allows for independently tunable mode and magnitude of crosslinking without significantly altering the alginate network architecture.

Further, sequencing the timescales for ionic and covalent crosslinking allowed for collagen self-assembly to mimic the fibrillar architecture of native ECM. Second generation harmonic (SHG) confocal imaging of the collagen fibers showed a significant reduction in fiber assembly in the faster gelling 2.5 kPa covalent and ionic (NbTz) aECM hydrogels. This result indicates that covalent crosslinking of Nb-Tz can disrupt the ability of collagen to self-assemble. However, the fiber assembly was largely rescued by decreasing the w/v % of NbTz, which delays the covalent crosslinking gelation time. In agreement with our results, static covalent crosslinking has previously been reported to be incompatible with collagen self-assembly into fibrillar structures [17]. The sequential ionic and covalent crosslinking of aECM here is analogous to how collagen is covalently modified in tissues. Following collagen self-assembly, enzymatic crosslinking by lysyl oxidase further crosslinks assembled collagen fibers [26, 36, 37] to alter their mechanical properties without altering the overall ECM content of the tissue [38]. Together, these studies demonstrate one approach to build complex hierarchical structures of extracellular matrix with tunable mechanical properties.

Finally, encapsulation of MSCs in aECM upregulated gene expression of immunomodulatory markers and was regulated to varying degrees by the stiffness and viscoelasticity of the aECM. Cells were encapsulated in the tunable aECM hydrogels in a robust and facile manner that is similar, in terms of complexity, to existing hydrogel systems [11, 14, 15, 20]. Expression of cyclo-oxygenase-2 (COX2) and TNF α -stimulated gene-6 were upregulated by stiffness and viscoelasticity in aECM hydrogels. Interestingly, the expression of COX2 was upregulated by changes in the mechanical properties of this aECM, but not by transient pulse stimulation with inflammatory cytokines IFN γ and TNF α in standard cell culture, whereas TNF α -stimulated gene-6 was upregulated by both cytokines and mechanics to similar extents. Further investigation is warranted to determine the differential and/or joint regulation of these markers by mechanical and cytokine cues. Conversely, increasing the stiffness of the more purely elastic hydrogels (NbTz) did not significantly regulate MSCs' gene expression. These results suggest the physical properties of the ECM can regulate immunomodulatory phenotypes of cells, which is relevant for cell therapies and cancer immunology. Altogether, these findings have significant implications for MSC therapies, as these cells are being widely investigated to treat inflammatory disease such as graft-versus-host-disease [39], and hydrogel materials are being developed to prolong their half-life and secretion of factors after systemic delivery [40–42].

Conclusions

Novel artificial ECM hydrogels with tunable stiffness and viscoelasticity were fabricated from interpenetrating networks of collagen type I and click-functionalized alginate, using a combination of ionic and covalent crosslinking. Viscoelasticity was controlled by covalent secondary crosslinking of click-functionalized alginate, without altering the diffusional nutrient transport or alginate architecture at the cell-scale. Temporal control of sequential

ionic and covalent alginate crosslinking permitted collagen self-assembly into a fibrillar structure. This artificial ECM system was used to investigate how stiffness and viscoelasticity regulated the gene expression of immunomodulatory markers by human MSCs. Increased elasticity of the matrix from the addition of covalent crosslinks altered the cells' response to mechanical cues. These results demonstrate how material systems can be used to generate hierarchical and tunable ECM and inform the development of physical models of tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Artificial extracellular matrix (aECM) hydrogel system features hierarchical and tunable covalent and ionic crosslinking. **a)** Collagen type I protein (green) is mixed with calcium carbonate nanoparticles (red) to obtain a homogenous suspension at 4° C. **b)** Next, an alginate polymer solution and cells are added to the mixture while maintaining a neutral pH and 4° C. **c)** After the addition of weak acid glucono-delta-lactone, the gel mixtures are rapidly cast and form hydrogels at 37° C. Alginate (blue) and self-assembled fibers of collagen type I form an interpenetrating network (IPN) hydrogel. Crosslinking domains of alginate (dotted circle) contain both covalent and ionic bonds (inset). **d–e)** Sequential ionic and covalent crosslinking of alginate permits collagen type I self-assembly into a network. **d)** Collagen self-assembles with a representative time scale ($t_{Collagen}$). **e)** In parallel, alginate reversibly crosslinks within blocks of glucuronic acid residues (inset) following addition of divalent cations, such as calcium, (red circles), with a representative reaction time (t_{Ionic}). Following initial alginate gelation and collagen self-assembly, click chemistry groups norbornene (Nb, purple) and tetrazine (Tz, light blue) provide secondary covalent crosslinks within the G-blocks over an addition time (t_{NbTz}) and release N_2 gas.

Figure 2.

Sequential ionic and covalent crosslinking tune the viscoelasticity of aECM. **a–d)**

Oscillatory shear rheology (1 Hz, 1% strain, 25° C) with a cone -plate geometry was used to determine the timescales of transition from a more fluid phase (loss angle > 45°) to solid-like gel phase (loss angle < 45°) of alginate hydrogels with either ionic or covalent crosslinking. Storage modulus, G' (solid line), loss modulus, G'' (dotted line), loss angle (open circle). **a–b)** Unmodified alginate with only ionic crosslinking, Alg (blue, 1.5% w/v total alginate, 4 mg/mL collagen type I) at low and high crosslinking densities (0.15% and 0.3% w/v calcium carbonate). **c–d)** A combination of alginate polymers functionalized with either norbornene (Nb) or tetrazine (Tz) form hydrogels upon mixing via Nb-Tz covalent crosslinking, NbTz (red, 1.5% w/v total alginate, 4 mg/mL collagen type I) at low and high crosslinking density (low - 1.0% w/v NbTz, 0.25 N:T ratio; high - 1.5% w/v NbTz, 1 N:T ratio). **e–f)** Frequency sweeps after equilibrium of aECM hydrogels formed with either only

ionic (Alg, blue) or sequential ionic and covalent crosslinking (NbTz, red) at low and high crosslinking densities (low – 0.15% Ca, 1.0% NbTz, 0.25 N:T ratio; high – 0.3% Ca, 1.5% NbTz, 1 N:T ratio). Storage modulus, G' (solid line), loss modulus, G'' (dotted line), loss angle (open circle). Graphs show representative data from rheological measurements.

Figure 3. Macromolecular diffusion of bovine serum albumin (BSA) and interpenetration of aECM hydrogels are uniform across Alg and NbTz hydrogels. **a)** Cumulative release of fluorescently-labeled BSA from Alg and NbTz hydrogels at low and high crosslinking densities. Low – 0.15% Ca, 1% NbTz 0.25 N:T ratio; High – 0.35% Ca, 1.5% NbTz 1 N:T ratio. **b)** The diffusion coefficient (D) of BSA, calculated from release curves, for various hydrogels. Error bars represent the 95% confidence interval (CI) of D for each condition. **c)** Confocal fluorescent imaging of rhodamine (TAMRA)-modified Alg (left) and anti-collagen I antibody staining of Alg (right). **d)** Mean distributions of fluorescence intensities for TAMRA-Alg hydrogels, $n = 3$. **e)** Confocal fluorescent imaging and **f)** mean distribution of FastGreen staining of collagen protein in NbTz hydrogels. Images shown are representative of across all conditions. Scale bar 100 μm .

Figure 4.

Self-assembly of collagen fibrillar architecture in click-crosslinked aECM depends on rate of collagen and norbornene (Nb) and tetrazine (Tz) crosslinking. **a)** Gelation kinetics of collagen type I (4 mg/mL) crosslinking at 37° C with telopeptides (Tel o-Col I, black) or without telopeptides (Atelo-Col I, gray) with 1.5% w/v alginate and 0% w/v calcium carbonate. Storage modulus, G' (solid line), loss modulus, G'' (dotted line), loss angle (open circle). **b)** Gelation kinetics of NbTz hydrogels can be controlled by their composition. Shear moduli (left) and loss angle (right) with only click alginate crosslinking (1.5% w/v

total alginate, 4 mg/mL collagen type I, 0% w/v calcium carbonate) of 1.5% w/v NbTz (red, 1 N:T) and 1.0% w/v NbTz (purple, 1 N:T). Storage modulus, G' (solid line), loss modulus, G'' (dotted line), loss angle (open circle). **c)** Frequency sweeps after equilibrium of 1.0% NbTz aECM hydrogels with high calcium (0.3%) and high covalent crosslinking (1 N:T ratio) at 1% strain (1.5% total alginate, 4 mg/mL telo-collagen type I). Storage modulus, G' (solid line), loss modulus, G'' (dotted line), loss angle (open circle). Graphs show representative data from rheological measurements. **d)** Second harmonic generation (SHG) confocal imaging of fibrillar collagen in Alg (blue), 1.5% NbTz (red), and 1.0% NbTz (purple) with high crosslinking (0.3% Ca, 1 N:T ratio), 4 mg/mL telo-collagen type I, and 1.5% w/v total alginate. 40x objective, maximum intensity projection of 15 μm z-stack, scale bar 25 μm .

Figure 5.

Viscoelastic properties of aECM regulate the immunomodulatory phenotype of bone marrow-derived MSCs. **a–b**) Shear moduli (a) and loss angle (b) of aECM with various compositions. **a**) G' storage modulus (black), G'' loss modulus (gray), no statistically significant difference between Alg and NbTz groups, two-way ANOVA with Sidak's multiple comparison test, $p > 0.05$. **b**) * statistically significant difference between Alg and NbTz, student's T test, unpaired, $p < 0.05$, $n > 3$. **c–e**) Relative gene expression of cyclooxygenase-2 (*PTGS2*), TNF α stimulated gene 6 (*TNFAIP6*), and interleukin-1 receptor

antagonist (*IL1RN*) by human primary MSCs after 72 hours, normalized to housekeeping gene *GAPDH* and cells adhered on tissue culture plastic (TCP) control. aECM – MSCs were encapsulated in Alg (blue) or NbTz (red) of various moduli and loss angles. * statistically significant difference, two-way ANOVA, Sidak's post hoc test, $p < 0.05$, $n = 3$. Controls – MSCs adhered to TCP were cultured with empty ionically crosslinked Alg (Ca) to determine the effects of freely soluble calcium in these gels on gene expression. 8h pulse – MSCs adhered to TCP were pulse stimulated with TNF α and IFN γ (+, 10 ng/mL) or vehicle control (–) for 8 hours. ND, not detected. * statistically significant difference, unpaired T-test, two-tailed, $p < 0.05$, $n = 4$. f) Prostaglandin E2 (PGE2) in conditioned media measured by ELISA after 72 hours in Alg (blue) and NbTz (red) hydrogels of various moduli and loss angles. * statistically significant difference, two-way ANOVA, Sidak's post hoc test, $p < 0.05$, $n = 3$.

Table 1.

Formulations of collagen-alginate IPN hydrogels

IPN Hydrogel	Collagen (mg/mL)	Total alginate (% w/v)	VLVG alginate (% w/v)	Nb- alginate (% w/v)	Tz- alginate (% w/v)	CaCO ₃ (% w/v)	GDL (mM)	Nb:Tz ratio
Alg	4	1.5	1.5	0	0	0.10 – 0.45	40 – 180	n/a
1.0% NbTz, 0.25 NT	4	1.5	0.5	0.2	0.8	0.10 – 0.15	40 – 60	0.25
1.5% NbTz, 1 NT	4	1.5	0	0.75	0.75	0.30 – 0.35	120 – 180	1.0
1.0% NbTz, 1 NT	4	1.5	0.5	0.5	0.5	0.30 – 0.35	120 – 180	1.0