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Development of a Two-Part Biomaterial Adhesive Strategy for Annulus Fibrosus Repair and *Ex Vivo* Evaluation of Implant Herniation Risk

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Abstract

Intervertebral disc (IVD) herniation causes pain and disability, but current discectomy procedures alleviate pain without repairing annulus fibrosus (AF) defects. Tissue engineering strategies seal AF defects by utilizing hydrogel systems to prevent recurrent herniation, however current biomaterials are limited by poor adhesion to wetted tissue surfaces or low failure strength resulting in considerable risk of implant herniation upon spinal loading. Here, we developed a two-part repair strategy comprising a dual-modified (oxidized and methacrylated) glycosaminoglycan that can chemically adsorb an injectable interpenetrating network hydrogel composed of fibronectin-conjugated fibrin and poly(ethylene glycol) diacrylate (PEGDA) to covalently bond the hydrogel to AF tissue. We show that dual-modified hyaluronic acid imparts greater adhesion to AF tissue than dual-modified chondroitin sulfate, where the degree of oxidation is more strongly correlated with adhesion strength than methacrylation. We apply this strategy to an *ex vivo* bovine model of discectomy and demonstrate that PEGDA molecular weight tunes hydrogel mechanical properties and affects herniation risk, where IVDs repaired with low-modulus hydrogels composed of 20kDa PEGDA failed at levels at or exceeding discectomy, the clinical standard of care. This strategy

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Declaration of interests

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Declaration of competing interests

None.

bonds injectable hydrogels to IVD extracellular matrix proteins, is optimized to seal AF defects, and shows promise for IVD repair.

Keywords

Intervertebral Disc; Annulus Fibrosus; Biomaterial Integration; Hydrogels; Tissue Engineering

Introduction

Intervertebral disc (IVD) herniation is one of the most frequent spinal pathologies, with an incidence rate of up to 20 cases per 1000 adults annually.[1] Symptomatic IVD herniation, and IVD defects more generally, results in back and neck pain as well as disability. The tremendous socioeconomic costs and diminished quality of life make the burden of chronic low back pain a leading cause of global disability.[2] Defects in the annulus fibrosus (AF) play a critical role in the pathophysiology of symptomatic IVD herniation, where nucleus pulposus (NP) tissue protrudes through AF defects and compresses upon spinal nerve roots leading to neuropathy.[3] Discectomy is the surgical standard-of-care to treat symptomatic IVD herniation, in which a surgeon removes prolapsed NP tissue with a rongeur and relieves mechanical compression of the nerves.[4] Although effective in relieving pain compared to non-operative controls, this procedure does not repair AF defects after NP removal, rendering this treatment option as a palliative response to symptomatic herniation and does not aim to seal IVDs or promote healing to prevent recurrent herniations that may occur after surgery.[5,6]

Recurrent herniation is the leading cause of reoperation following discectomy with a surgical revision rate up to 25%, and is associated with worse clinical outcomes and a greater socioeconomic burden than those who do not require reoperation.[7–11] Large unrepaired AF defects are a significant risk factor for symptomatic reherniation, and due to the poor healing capacity of the IVD, reflect a gap in current surgical practices for patients that bear a high risk of reoperation.[12–14] Next generation treatment strategies incorporate the repair of AF defects to address this critical challenge with primary goals to prevent recurrent herniation, biomechanical dysfunction, and progressive degeneration.[15,16] Mechanical devices such as the Barricaid® Annular Closure Device recently received FDA approval and aims to prevent reherniation following discectomy, however this device lends itself to an invasive approach since it requires anchorage to an adjacent vertebra and bears considerable risk of vertebral subsidence and endplate damage.[17] Additionally, purely mechanical devices do not have the ability to prevent degeneration after surgery. To that end, emerging approaches in regenerative medicine utilize hydrogels as minimally invasive AF repair biomaterials that serve as surgical sealants and void-filling tissue engineering constructs to promote functional restoration.[18–21] These water-swollen polymeric matrices are extraordinarily versatile by design and, depending on composition, have demonstrated ability to restore functionally important biomechanical properties or promote biological repair processes.[21]

Despite their considerable potential as a next generation treatment strategy, hydrogels for AF repair have yet to be translated into the clinic in part due to poor tissue integration with the complex IVD architecture.[22] Integration of polymeric materials is directly related to the adherence to tissue surfaces, which can be imparted by physical and/or chemical interactions at the tissue-hydrogel interface.[23] Strongly adherent surgical sealants employ both physical and chemical means of adsorption to achieve suitable tissue integration, however these materials are often highly cytotoxic and cause damage to treated tissue.[24] Given the high magnitudes of biomechanical loads exerted on the IVD, there is an unmet clinical need to develop an implantable hydrogel system with strong adherence to AF tissue such that it can durably seal AF defects without compromising cellular viability and either match or diminish the risk of herniation compared to current discectomy procedures.[14,25,26]

In addition to the high interfacial adhesion strength needed to successfully seal AF defects, the bulk mechanical properties of void-filling hydrogels are also critically important and determine whether the strategy is more amenable to a biomechanical or biological approach for repair.[19] For strategies that aim to restore biomechanical function, AF repair hydrogels should ideally match the native tissue properties so as to re-establish intact IVD behavior under physiological loading, which requires either a high macromer or crosslinking density.[18] However, a tissue engineering construct for the delivery of biologics (i.e. cells and/or bioactive factors) requires a significantly softer gel to maintain high cell viability or sufficient biologic release to elicit regenerative effects.[27,28] Synthetic polymer networks enable highly tunable construct properties; for example the bulk elastic moduli can be adjusted by the macromer molecular weight (MW) to generate either biomechanically-favorable or biologically-favorable strategies.[29–31] However, the effect of elasticity (or inversely, mechanical compliance) on implant herniation risk has yet to be assessed by synthetically tuning macromer MW.

In this study, we addressed adhesion and bulk material properties in IVD tissue engineering by developing a novel two-part strategy for AF repair composed of: (1) an interpenetrating network (IPN) hydrogel comprising synthetic (poly(ethylene glycol) diacrylate/PEGDA) and natural (fibronectin-conjugated fibrin/FN-Fibrin) polymer networks, and (2) a dual-modified (oxidized and methacrylated) glycosaminoglycan (GAG) that covalently bonds this injectable hydrogel to extracellular matrix proteins in the IVD. (Figure 1) The objectives of this study are three-fold: (1) to optimize adhesive properties of the tissue-hydrogel interface by screening across sulfated and unsulfated GAGs and enhancing degrees of GAG methacrylation and oxidation, (2) to assess the cytocompatibility of the optimal dual-modified GAG product used to bond the void-filling hydrogel to AF tissue, and (3) to scale this method of AF repair to a large animal model of simulated discectomy *ex vivo* and determine the effect of hydrogel elasticity on implant herniation risk. The global hypothesis of this study is that dual-modified GAG products with the highest biochemical degrees of modification will impart the greatest hydrogel adhesion strength with no demonstrable cytotoxicity. Furthermore, repairing AF defects with mechanically compliant hydrogels will exhibit a lower risk of implant herniation than stiffer, less compliant hydrogels and match the herniation risk of the clinical standard of care. Three major innovations are presented in this work. First, we oxidize and methacrylate both chondroitin sulfate (CS) and hyaluronic acid (HA), and systematically quantify their extents of conversion and adhesive strengths to

AF tissue, given the same reaction stoichiometries. Second, this two-part repair strategy has yet to be applied to a discectomy model and evaluated for AF repair. Third, this is the first study to examine the effect of hydrogel mesh size on IVD herniation risk.

2. Materials and Methods

2.1 Glycosaminoglycan Oxidation

In order to synthesize the dual-modified GAG products, unmodified GAGs first underwent an oxidation reaction to produce an oxidized intermediate. Chondroitin sulfate Type A (CS) (Alfa Aesar, Haverhill, MA) and hyaluronic acid (HA) (Acros Organics, Fair Lawn, NJ) were dissolved in ddH₂O at a concentration of 6% (w/v) and 0.25% (w/v), respectively. Once fully dissolved, GAGs were oxidized by adding sodium periodate (NaIO₄) (Sigma-Aldrich, St. Louis, MO) to solution at a 1:2.4 or 1:3.5 GAG:IO₄⁻¹ molar ratio for 16 hours devoid of light with vigorous stirring at room temperature.[32,33] The reaction was stopped by adding 10% (v/v) ethylene glycol (Sigma-Aldrich, St. Louis, MO) to the reaction mixture and subsequently purified by dialyzing against ddH₂O for 3 days using Spectra/Por® 1 dialysis membranes (MWCO = 6–8kDa) (Spectrum Laboratories, Rancho Dominguez, CA). During the dialysis period, solutions were transferred to a new dialysis membrane once a day and ddH₂O was changed twice a day. After dialysis, intermediate products were frozen down at –80°C for 24 hours and subsequently recovered by lyophilization for 7 days.

2.2 Glycosaminoglycan Methacrylation

Following oxidation, intermediate products then underwent a methacrylation reaction to produce the final dual-modified GAG products. Upon recovery, CS aldehyde and HA aldehyde intermediates were dissolved in ddH₂O at a concentration of 25% (w/v) and 0.5% (w/v), respectively. The pH of solution was first raised to 8.00 with the addition of 1M NaOH prior to the start of the methacrylation reaction. Oxidized GAGs then underwent methacryloyl substitution by adding methacrylic anhydride (MAH) (Sigma-Aldrich, St. Louis, MO) to the reaction mixture at a 1:10 or 1:20 GAG:MAH molar ratio.[34,35] The pH of the reaction mixture was maintained at 8.00 by the addition of 1M NaOH and proceeded for 24 hours at 4°C devoid of light with vigorous stirring. Final products were purified by dialyzing against ddH₂O for 3 days using Spectra/Por® 1 dialysis membranes (MWCO = 6–8kDa) (Spectrum Laboratories, Rancho Dominguez, CA). During the dialysis period, solutions were transferred to a new dialysis membrane once a day and ddH₂O was changed twice a day. After dialysis, final products were frozen down at –80°C for 24 hours and subsequently recovered by lyophilization for 7 days to obtain all dual-modified GAG products (CSMA Aldehyde and HAMA Aldehyde).

2.3 Quantification of Dual-modified GAG Degree of Oxidation

A 2,4,6-trinitrobenzenesulphonic acid (TNBS) assay was used to determine the degree of oxidation for dual-modified GAG products.[36] *tert*-Butyl carbazate (*t*-BC) (Sigma-Aldrich, St. Louis, MO) reacts with aldehyde moieties forming a stable carbazone in a similar manner to imine formation, enabling the quantification of aldehyde modification. A standard calibration curve from aqueous *t*-BC solutions (0–50 mM) was used to determine the amount of unreacted *t*-BC and in turn compute aldehyde content for each of the eight dual-

modified GAG formulations screened in this study. First, a 2% (w/v) solution of dual-modified CS, 0.25% (w/v) solution of dual-modified HA, and 1% (w/v) solution of trichloroacetic acid (TCA) (Fisher Chemical, Fair Lawn, NJ) was prepared in ddH₂O. Differences in weight by volume concentrations account for the difference between molecular weight and solubility between the two GAG types. Additionally, 0.1M sodium borate buffer was prepared by dissolving sodium tetraborate decahydrate (Fisher Chemical, Fair Lawn, NJ) in ddH₂O and the pH was adjusted down to 8.0 with 0.5N HCl (Fisher Chemical, Fair Lawn, NJ). A stock 50mM *t*-BC solution for standards and experimental samples was made with 1% TCA as the solvent. 25μL of the 2% (w/v) dual-modified CS solution (0.5mg of dual-modified CS product) was mixed with 25μL of the 50mM *t*-BC in TCA solution, and the reaction mixtures were vigorously agitated on an orbital shaker devoid of light for 16 hours. In order to keep stoichiometry consistent between the total molecular weight of CS and HA reacted with *t*-BC, 200μL of the 0.25% (w/v) dual-modified HA solution (0.5mg of dual-modified HA product) was mixed with 25μL of the 50mM *t*-BC in TCA solution, and the reaction mixtures were vigorously agitated on an orbital shaker devoid of light for 16 hours. Following incubation, 0.5mL of 6mM TNBS solution in 0.1M borate buffer was added to standard and experimental samples and the reaction mixtures were vigorously agitated on an orbital shaker devoid of light for 1 hour. After incubation, 20μL of each experimental and standard sample was added into wells of a 96 well plate in triplicate, and 180μL of 0.5N HCl was added to each well. Optical density measurements were taken at 340nm using a SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA).

2.4 Quantification of Dual-modified GAG Degree of Methacrylation

Proton nuclear magnetic resonance (¹H NMR) spectroscopy (500 MHz Varian Mercury 300, Agilent Technologies, Santa Clara, CA) was used to verify oxidation and methacrylation of dual-modified GAG products when compared to the unmodified polymer (Supplementary Figure 1).[37,38] Additionally, ¹H NMR was used to compute the degree of methacrylation for each formulation by determining the integration of the downfield vinyl peak at $\delta = 6.5\text{ppm}$ relative to the integration of the HA or CS backbone $\delta = 3.20\text{--}4.45\text{ppm}$ on iNMR software. [35,39–41]

2.5 Hydrogel Fabrication

Single Network (SN) and Interpenetrating Network (IPN) hydrogels were initially fabricated using 575Da PEGDA (Sigma Aldrich, St. Louis, MO) across a volumetric concentration range of 10–20% (v/v) for mechanical characterization using 11mM L-Ascorbic Acid (AA) (Fisher Scientific, Fair Lawn, NJ) and Oxone monopersulfate (Alfa Aesar, Haverhill, MA) as a redox initiation system to crosslink acrylate end groups. (Supplementary Figure 2) Due to the pH sensitivity of the Schiff base reaction, the redox initiation system was changed to ammonium persulfate (APS) (Acros Organics, Fair Lawn, NJ) and N,N,N',N'-tetramethylethylenediamine (TEMED) (Bio-Rad Laboratories, Hercules, CA) at either 20mM or 40mM for hydrogel mechanical testing, lap shear tests, and downstream *in situ* experiments.

SN hydrogels were fabricated using three MWs of PEGDA at 15% (v/v): 575Da (Sigma Aldrich, St. Louis, MO), 10kDa (Polysciences Inc., Warrington, PA), and 20kDa (Polysciences Inc., Warrington, PA) with 3.3% (v/v) (1.17U/mL) of Oxyrase-EC (Oxyrase®, Mansfield, OH) as an oxygen scavenger and either 20mM or 40mM APS/TEMED redox initiators. IPN hydrogels incorporated a fibronectin-conjugated fibrin network by the inclusion of 10µg/mL human fibronectin (Sigma Aldrich, St. Louis, MO), 0.5U/mL Factor XIII (EMD Millipore, Darmstadt, Germany), 10U/mL thrombin (Sigma Aldrich, St. Louis, MO), and 5mg/mL human fibrin (Sigma Aldrich, St. Louis, MO) into the prepolymer solution.

All constructs were fabricated by using a 1:1 dual-barrel syringe and 1:1 volumetric mixing tip (PacDent International, Walnut, CA) to cast prepolymer solution into cylindrical acrylic molds (8mm diameter by 3mm height) for rheological and compression testing or “dog-bone” ASTM D638–02a type V molds for tensile testing, and coverslipped for at least 5 minutes to ensure full gelation.

2.6 Hydrogel Mechanical Testing

After a 3-day swelling period in PBS to reach equilibrium, hydrogel specimens underwent unconfined compression (N = 10/group), parallel plate shear (N = 10/group), and uniaxial tensile (N = 5/group) testing to characterize the material properties across all SN and IPN hydrogel formulations according to ASTM F2150–19.[42] Unconfined compression tests were conducted on an Electroforce 3220 (TA Instruments, New Castle, DE), where specimens underwent a displacement-controlled ramp at 1% strain/sec to a total of 20% strain. Data was collected on WinTest 7 software (TA Instruments, New Castle, DE) and post-processed on Microsoft Excel, where the slope of the force-displacement curve at the top 10% of the linear region was used to obtain stiffness values for each specimen and normalized by cross-sectional area to convert stiffness to the unconfined compressive modulus. Parallel plate shear testing was conducted on a TA Instruments AR2000ex rheometer (TA Instruments, New Castle, DE), where specimens underwent a frequency sweep from 0.1–10Hz at 1% strain, and the complex modulus ($|G^*|$) and tangent phase angle ($\tan \delta$) values were obtained at 1Hz, which is a physiologically-relevant loading frequency. [43] Rheometry data was collected on Rheology Advantage software (TA Instruments, New Castle, DE). Uniaxial tensile testing was conducted on an Instron 8872 Fatigue Testing System (Instron, Norwood, MA), where specimens underwent displacement-controlled ramp at 0.2% strain/sec to either 50% strain for SN and IPN hydrogels composed of 10kDa PEGDA and 20kDa PEGDA, or failure for SN and IPN hydrogels composed of 575Da PEGDA since failure occurred below 50% strain for this MW. For SN and IPN hydrogels fabricated with 40mM APS/TEMED and 20kDa PEGDA, smaller rectangular casting molds of 9.5 mm × 27 mm were used to ensure complete gelation and minimize material usage. Data was collected and processed with Microsoft Excel software to compute stiffness values from the linear region of the force-displacement curves and converted to tensile modulus by normalizing to cross-sectional area.

2.7 Lap Shear Adhesion Testing

Hydrogel adhesion imparted by the dual-modified GAG products was quantitatively determined using a lap shear configuration according to the ASTM 2255–05 testing protocol (N = 10–15/group). Prior to testing, lap shear specimens were fabricated in 8mm diameter by 3mm height acrylic molds. First, 8mm diameter biopsy punches of AF tissue were obtained from skeletally mature and healthy bovine IVDs from coccygeal IVD levels cc1/2, cc2/3, cc3/4, and cc4/5 (Springfield Meat Co., Richlandtown, PA) using an axial orientation of the punch. AF tissue punches were then sliced into 1.5mm thick sections with Tissue Matrix (ASI Instruments, Warren, MI) and placed in the base of the acrylic mold. AF tissue punches were coated with either 25% (w/v) solution of the CSMA Aldehyde products or 10% (w/v) solution of the HAMA Aldehyde products for 5 minutes to enable Schiff base formation to occur. Differences in weight-by-volume concentrations between CSMA Aldehyde and HAMA Aldehyde were to control for the total weight of polymer applied to AF tissue samples. Negative control (-GAG) samples were treated with 1X PBS for 5 minutes instead of dual-modified GAG products to control for the effect of GAG treatment. After 5 minutes, the dual-modified GAG product or PBS was aspirated off AF tissue, and the IPN hydrogel prepolymer solution (20mM APS/TEMED) was casted directly over the specimens followed by coverslipping to create a 3mm thick AF tissue/dualmodified GAG/IPN Hydrogel specimens with parallel faces. PEGDA MW in the IPN hydrogel formulation was held constant ($M_n = 575\text{Da}$) in order to screen for differences in adhesion strength imparted by dual-modified GAG formulation. Specimens were then glued to custom aluminum platens with Loctite® 401 adhesive and fixed within Bose Electroforce 3220 equipped with a 5N load cell. Specimens underwent displacement-controlled ramp-to-failure at a shear strain rate of 0.2% strain/sec, and the maximum force at which interfacial failure occurred was used to determine the ultimate strength of the specimen. All data were collected on WinTest 7 software and post-processed on Microsoft Excel. The dual-modified GAG that imparted the highest average biomaterial adhesion strength (HAMA Aldehyde Formulation 4) was chosen for all downstream experimentation.

2.8 Dual-modified HA Visualization in AF Tissue

To visualize the depth of HAMA Aldehyde penetration into the AF, Alexa Fluor Cadaverine-594 (Thermo Fisher Scientific, Rochester, NY) was conjugated to the carboxylic moiety of the dual-modified HA via 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) chemistry. Prior to the reaction, sodium hydrogen phosphate heptahydrate (Alfa Aesar, Ward Hill, MA) and sodium phosphate monobasic monohydrate (Fisher Chemical, Fair Lawn, NJ) were dissolved in ddH₂O to prepare phosphate buffer with a pH of 8.2 and buffer strength of 100mM. A 10mg/mL solution of HAMA Aldehyde and 100mg/mL solution of EDAC was prepared in 0.1 M BupH™ MES buffered saline (Thermo Fisher Scientific, Rochester, NY) prior to the reaction as well. 4μL of 100mg/mL EDAC was added to 1mL of the 10mg/mL HAMA Aldehyde solution to generate the unstable *o* acylisourea ester intermediate. 6μL of 100mg/mL NHS solution was then added into the reaction mixture and incubated on an orbital shaker for 15 minutes at room temperature to produce the semi-stable amine-reactive ester. Following incubation, 3500μL of phosphate buffer was added to the reaction mixture to bring the pH of solution above 7.0. After the addition of buffer, 100μL of Alexa Fluor

Cadaverine-594 at a concentration of 0.002mg/mL was added to the reaction mixture and incubated on an orbital shaker for 2 hours at room temperature. After 2 hours, the final product was collected using Amicon® Ultra-0.5 centrifugal filter devices (Ultracel® –3,000 NMWL) (Merck Millipore Ltd., Darmstadt, Germany). Briefly, 500µL of the final product was aliquoted into Amicon® filter devices and spun down at 14,000g for 20 minutes using an Eppendorf centrifuge. The concentrated solute was recovered by inverting the filter device in a clean microcentrifuge tube and spinning the sample down at 1000g for 2 minutes.

A 10% w/v solution of HAMA Aldehyde with the conjugated Alexa Fluor Cadaverine-594 probe was cast over 8mm diameter punches of AF tissue for 5 minutes. Following incubation, the solution was aspirated off the AF and tissue specimens were either embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek USA, Torrance, CA) for cryosectioning to visualize cross-sectional depth-of-penetration or imaged on a Zeiss LSM 880 confocal microscope (Carl Zeiss Microscopy LLC, White Plains, NY) to assess spatial homogeneity of HAMA Aldehyde. Nine consecutive z-stacks from confocal imaging were imported into MATLAB (Release 2018b MathWorks, Natick, MA) and each pixel underwent thresholding according to fluorescent signal intensity. Pixel fluorescence data was smoothed across each z-stack using the MATLAB *smoothdata* function, and cumulatively added together at each spatial location to generate a surface topography map that visually represents HAMA Aldehyde depth across the entire area of the specimen. OCT-embedded tissues were sectioned on a cryotome to produce 12µm thick sections mounted on charged slides. Sections were then stained with a 1:1000 dilution of 1µg/mL stock solution of 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes to visualize AF cell nuclei. After coverslipping with Fluoro-Gel mounting medium (Electron Microscopy Sciences, Hatfield, PA), slides were imaged on a Zeiss AxioImager Z2 (Carl Zeiss Microscopy LLC, White Plains, NY).

2.9 Cell Viability Assay

Cytotoxicity of the optimal dual-modified GAG product (i.e. HAMA Aldehyde Formulation 4) was assessed using a cell viability assay across a large range of HAMA Aldehyde concentrations. Primary bovine AF cells were isolated from three healthy and skeletally mature biological donors (B21, B24, and B26) (Springfield Meat Co., Richlandtown, PA) from coccygeal levels cc1/2, cc2/3, cc3/4, and cc4/5 using a collagenous digestion protocol. Briefly, isolated IVDs were first dipped in 70% ethanol followed by a thorough rinse in washing solution containing 1.5% Amphotericin B (Fisher Scientific, Pittsburgh, PA), and 3% penicillin/streptomycin (Life Technologies Corporation, Grand Island, NY), and 1X PBS. After washing, the AF was dissected off of the NP and finely cut into ~3mm³ pieces. AF tissue was then sterilely transferred to a T75 Nunc™ EasYFlask™ (Thermo Fisher Scientific, Rochester, NY) with 25mL of 0.2% pronase (Fisher Scientific, Pittsburgh, PA) in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, Rochester, NY) and incubated at 37°C, 5% CO₂ for 90 minutes on a rocker plate. Partially digested AF tissue was washed twice with 1X PBS to remove pronase, and 25mL of DMEM with 200U/mL collagenase I (Fisher Scientific, Pittsburgh, PA) was added to the flasks for 13 hours. Digested AF tissue was then filtered through a 70µm filter (Fisher Scientific,

Pittsburgh, PA), centrifuged at 500g for 10 minutes, and the collected cells were analyzed for cell count and viability. AF cells (p0) were expanded to 90% confluence to obtain the appropriate yield for experimentation and used at p1 after TrypLE™ Express dissociation (Fisher Scientific, Pittsburgh, PA). AF cells (p1) were plated into Nunc™ MicroWell™ 96-Well Optical Bottom Plates with Polymer Base (Thermo Fisher Scientific, Rochester, NY) at a density of 4.4×10^3 cells/cm². Cells were cultured at 37°C, 5% CO₂ with 100μL of growth medium per well, where the growth medium was composed of high glucose (4.5g/L) DMEM, 10% FBS (Gemini Bio-Products, West Sacramento, CA), 1% penicillin/streptomycin (Life Technologies Corporation, Grand Island, NY), and 0.2% L-Ascorbic Acid (Fisher Scientific, Fair Lawn, NJ). When AF cells reached 80% confluency, cell viability upon exposure to HAMA Aldehyde was assessed using the CellTiter-Glo® 2.0 assay (Promega Corporation, Madison, WI). Growth medium was first aspirated and 100μL of medium supplemented with HAMA Aldehyde at concentration range of 10⁻⁹ to 10² (μM) was applied to AF cells for 1 hour. Live cell and dead cell controls were included by replacing medium with 0μM HAMA Aldehyde (Untreated) and 20% v/v EtOH, respectively. Blank wells were used to determine background luminescence readings. Prior to use, CellTiter-Glo® 2.0 reagent was thawed to room temperature and at the 1-hour timepoint, 100μL of CellTiter-Glo® 2.0 reagent was added to each well and incubated for 2 minutes on an orbital shaker devoid of light to induce cell lysis. After 2 minutes, well plates were taken off the orbital shaker and incubated at room temperature for 10 minutes to stabilize luminescent signal. After 10 minutes, luminescence in relative luminescence units (RLUs) was recorded on a SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA). All conditions were performed in triplicate for three biological donors.

2.10 Motion Segment Preparation

Bovine tails from healthy and skeletally mature animals were procured from a local abattoir (Springfield Meat Co., Richlandtown, PA) and coccygeal motion segments (vertebrae-disc-vertebrae) were isolated from levels cc2/3, cc3/4, and cc4/5 to histologically assess biomaterial integration and evaluate implant herniation risk. All facet and transverse processes were removed with a bone band saw (Mar-Med Inc., Strongsville, OH) in addition to the removal of extraneous musculature and ligaments with a scalpel, and motion segment samples were stored at -20°C until further use.

2.11 Histological Analysis

A subset of specimens prepared for hydrogel characterization, lap shear, and herniation risk tests were allotted for histological analysis. Specimens were first fixed in aqueous buffered zinc formalin fixative (Anatech Ltd., Battle Creek, MI) for 48 hours and subsequently infiltrated with a hydrophilic resin, 2-hydroxypropyl methacrylate (Sigma-Aldrich, St. Louis, MO), for 48 hours with two changes of monomer solution to avoid dehydration and clearing of specimens. The monomer solution was then polymerized by the slow addition of heat at 37°C to form blocks for sectioning. Histological sections were prepared by 5μm slices and mounted on silane slides (Matsunami Glass, Osaka, Japan). Sections from slides were first deplasticized by placing slides in toluene (Sigma-Aldrich, St. Louis, MO) for 30 minutes and changed with fresh toluene for another 30 minutes. Toluene was then replaced with a 50% volumetric mix of toluene and petroleum ether (Sigma-Aldrich, St. Louis, MO)

for 5 minutes. Slides were then dipped in ethylene glycol mono ethyl ether (EGME) (Sigma-Aldrich, St. Louis, MO) five times and rinsed with three changes of ddH₂O.

Sections from lap shear and motion segment specimens underwent tinctorial staining with picrosirius red and alcian blue dyes to visualize collagen and proteoglycan content, respectively, assess hydrogel integration, as well as observe overall specimen structure. After deplasticizing, sections were stained with Gomori's Hematoxylin (Fisher Healthcare, Houston, TX) for 15 minutes and rinsed with ddH₂O three times. Sections were then stained with alcian blue (pH = 2.5) (Poly Scientific R&D, Bay Shore, NY) for 30 minutes and rinsed with ddH₂O three times. Following alcian blue staining, sections were then stained with picrosirius red (Sigma-Aldrich, St. Louis, MO) for 1 hour and rinsed with 1% acid water for 2 minutes. Following staining, sections were dehydrated with EGME, cleared with Xylenes (Sigma-Aldrich, St. Louis, MO), and coverslipped with Eukitt mounting media (Electron Microscopy Sciences, Hatfield, PA).

Hydrogel-only sections (Figure 3) were stained with Gomori's Hematoxylin (Fisher Healthcare, Houston, TX) for 10 minutes and subsequently rinsed with ddH₂O three times. Slides were then stained with Protocol Eosin Y for 2 minutes (Fisher Healthcare, Houston, TX), followed by three rinses of ddH₂O. Following staining, sections were dehydrated with EGME, cleared with Xylenes (Sigma-Aldrich, St. Louis, MO), and coverslipped with Eukitt mounting media.

All slides were imaged on a Leica DM6B Upright Microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.12 Assessment of Herniation Risk

IVDs were isolated from motion segments by using an IsoMet® 1000 Precision Cutter (Buehler, Lake Bluff, IL) to make parallel cuts approximately 3mm from the superior and inferior vertebral end plates. Biomaterial implant herniation risk was characterized by a displacement-controlled (2mm/min) ramp-to-failure mechanical tests at a 5° incline to maximize stress at the repair site, as previously described. [44–48] 50 IVDs from bovine coccygeal levels cc2/3, cc3/4, and cc4/5 were systematically assigned to three cohorts to account for potential level effects: 'Intact', 'Discectomy', and 'Repair', where IVDs in the 'Repair' cohort were split into three separate groups in which AF defects were primed with HAMA Aldehyde Formulation 4 and subsequently sealed with IPN hydrogels of the three PEGDA molecular weights considered in this study (575Da, 10kDa, and 20kDa) (N=10/group). All motion segments assigned to 'Discectomy' or 'Repair' groups first underwent a clinically-relevant injury of a 4mm biopsy punch (Integra LifeSciences, Princeton, NJ) with 200mg (~25%) of NP removal. To simulate a clinically-relevant AF defect, a 4mm biopsy punch was inserted 7mm deep into the posterolateral face of the AF and the resulting plug of tissue was removed using a rongeur. Following initial tissue removal, the NP was then disrupted for 2 minutes with a curette and 200mg of fragmented NP tissue was removed from the IVD with a rongeur. For IVDs undergoing repair, ~150µL HAMA Aldehyde solution (10% w/v) was slowly injected into the AF defect using a 5mL syringe with a 20G × 1–1/2" BD PrecisionGlide™ Needle (Beckton, Dickinson and Company, Franklin Lakes, NJ) at a controlled rate to coat the tissue surface for 5 minutes to allow the Schiff base

formation to occur. After 5 minutes, HAMA Aldehyde solution was aspirated and IPN hydrogel prepolymer solution was injected into the AF defect with a 1:1 dual-barrel syringe and 1:1 volumetric mixing tip (PacDent International, Walnut, CA) and covered with parafilm. Specimens were set for 15 minutes prior to mechanical testing to allow full gelation (~2 minutes) and bonding with HAMA Aldehyde. The following mechanical output parameters that assess herniation risk were computed from the force-displacement curves using a custom MATLAB code and normalized to IVD cross-sectional area when necessary: failure strength, subsidence-to-IVD failure, failure strain, work-to-IVD failure, and ultimate strength to failure strength ratio. IVD failure occurred either by endplate fracture or NP herniation, where NP herniation was defined as 2mm of NP or implant protrusion from the outer radius of the AF. In the case of endplate fracture, IVD failure always coincided with the ultimate strength of the specimen (i.e. global maximum stress). IVD failure strength was defined as the stress at which endplate fracture or NP herniation occurred. Subsidence-to-IVD failure was defined as the displacement to which the point of failure for a given specimen occurred. Failure strain was defined as the percent deformation at the point of failure with respect to the original IVD height, where the IVD height prior to testing was measured in triplicate between parallel superior and inferior faces of the motion segment using a caliper. Work-to-IVD failure was defined as the area under the force-displacement trace until IVD failure occurred. The ultimate strength to failure strength ratio was computed by identifying the global maximum strength of a given specimen and normalizing that value to the failure strength of the same specimen.

2.13 Statistical Analyses

All quantitative data are presented as mean $\pm$ standard deviation. One-way ANOVA with Tukey's post-hoc test was used to assess significant differences for quantitative data pertaining to cell viability measurements. Two-way ANOVA was used to assess significant differences for quantitative data pertaining to the degree of GAG oxidation (TNBS assay), hydrogel mechanical properties, and lap shear adhesion strengths. Simple linear regression was used to assess the correlative relationship between GAG degree of oxidation and GAG degree of methacrylation with lap shear ultimate stress. Due to inhomogeneity of variance, Kruskal-Wallis nonparametric test with Dunn's post-hoc was used to analyze all quantitative output measures corresponding to the *in situ* herniation risk experiment. Statistical outliers were identified by the ROUT method ($Q = 1\%$) and consequentially excluded as necessary for output measures corresponding to the *in situ* herniation risk experiment. A non-linear semi-log fit was used to correlate failure strength and subsidence-to-failure to the tensile modulus of IPN hydrogels comprising different PEGDA MWs. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA) with threshold for significance across all experiments set to $\alpha = 0.05$.

Results

3.1 Synthesis and biochemical characterization of dual-modified GAGs

Dual-modified CS and HA were successfully synthesized according to a two-step reaction scheme, where unmodified GAGs are first oxidized by sodium periodate and the intermediate product is subsequently methacrylated by methacrylic anhydride. (Figure 2A)

Using this two-step reaction scheme, four products (Formulations 1–4) were synthesized per GAG type for stoichiometries that enhance the degree of oxidation as well as methacrylation. (Figure 2B) The degree of oxidation for dual-modified GAG products was computed by calculating the percent difference between OD₃₄₀ measurements between a given formulation and the unmodified GAG polymer. Significant differences were detected for the main effects of formulation number ($p_{\text{Formulation}} = 0.0005$) and GAG type ($p_{\text{GAG}} < 0.0001$), but no significant interaction between these effects ($p_{\text{interaction}} = 0.880$). The degree of oxidation for all dualmodified HA formulations were equal to or greater than the degree of oxidation for dualmodified CS formulations. (Figure 2C) ¹H NMR indicated that all dual-modified GAG products were methacrylated, indicated by the presence of vinylic peaks at 6.0ppm and 6.5ppm. The degree of methacrylation for dual-modified GAG products was computed by calculating the integral of the downfield vinyl peak, in which all dual-modified CS formulations had a degree of methacrylation greater than or equal to that of dual-modified HA. Peaks at 5.2ppm additionally indicate that all dual-modified GAG formulations were oxidized, as these chemical shifts correspond adjacent protons to the aldehyde moiety.[37] (Figure 2D) ¹H NMR of unmodified GAG polymers were used to ensure that all chemical shifts correspond to functionalization of the HA or CS backbone. (Supplementary Figure 1) All computed biochemical degrees of modification for a given formulation of dual-modified HA and CS are specified in Table 1.

3.2 Synthesis and mechanical characterization of SN and IPN hydrogels

All SN and IPN hydrogels underwent a sol-gel transition in the presence of APS/TEMED redox initiators, and the FN-Fibrin natural polymer network was homogeneously incorporated within the synthetic PEGDA network as histologically visualized by H&E staining. Gels were fabricated in geometric molds for mechanical testing and could also be injected directly into clinically-relevant AF defects. (Figure 3A) The compressive, tensile, and shear moduli for SN and IPN hydrogel formulations employed in this study were characterized to determine the effects of redox initiator concentration and hydrogel mesh size on construct stiffness. After initially screening across volumetric concentrations with 575Da PEGDA, it was found that a concentration of 15% v/v approximately matches AF properties in unconfined compression, uniaxial tension, and parallel plate shear. (Supplementary Figure 2) To determine the effect of hydrogel mesh size on construct stiffness and *in situ* herniation risk, hydrogel formulations of higher PEGDA MW (10kDa and 20kDa) were considered in the study while holding the 15% v/v PEGDA concentration constant. Across all mechanical parameters, there was a significant decrease in the modulus as the PEGDA MW increases ($P_{\text{PEGDA MW}} < 0.001$), where gels composed of 10kDa and 20kDa PEGDA were comparably less stiff than AF tissue in compression, tension, and shear. There was a significant effect of redox initiator concentration on the unconfined compressive modulus for SN ($p < 0.0001$) and IPN ($p < 0.0001$) hydrogels and IPN hydrogels in uniaxial tension ($p < 0.0001$) but had no significant effect on other mechanical properties ($p > 0.05$). (Figure 3B)

3.3 Lap shear adhesion testing

When treating AF tissue with either dual-modified CS or HA, hydrogel bonding with AF tissue was visually achieved when comparing gross specimens with negative controls after sample preparation. Histologically, this was observed through picrosirius red and alcian blue

staining, where dual-modified GAG treated samples had a contiguous boundary between the IPN hydrogel and AF tissue. Negative control samples (-GAG treatment) featured a considerable gap space between hydrogel and AF tissue, indicating no chemical adsorption amongst the gel and AF. (Figure 4A) Displacement-controlled lap shear tests indicated functional covalent bonding between the hydrogel and AF tissue, demonstrated by a continuous rise in force over time until failure occurred as shown in a representative loading curve. (Figure 4B) These force-displacement traces corroborate with visual observations during lap shear tests, where treated specimens slid together until failure occurred at the interface, whereas untreated controls slid over one another for the entire duration of the test. (Figure 4C) Quantitative assessments of specimen ultimate strength indicated that there were significant main effects of GAG type ($p < 0.0001$) and formulation number ($p < 0.0001$), as well as a significant interaction ($p < 0.0001$). (Figure 4D) When corresponding the ultimate strength to the biochemical degrees of GAG modification, there was a significant positive correlation between HA oxidation and ultimate strength ($R^2 = 0.88$, $p = 0.019$), but the positive correlation between CS oxidation and ultimate strength did not reach statistical significance ($R^2 = 0.63$, $p = 0.111$). Moreover, the positive correlation between HA methacrylation and ultimate strength trended towards significance ($R^2 = 0.76$, $p = 0.053$) and the positive correlation between CS methacrylation and ultimate strength did not reach statistical significance ($R^2 = 0.49$, $p = 0.183$). (Figure 4E)

3.4 HAMA Aldehyde tissue distribution

The spatial distribution of HAMA Aldehyde product in AF tissue after 5 minutes of treatment was histologically assessed and used to semi-quantitatively determine the maximum depth-of-penetration. A top-down view of HAMA Aldehyde-treated AF tissue shows that the biomaterial product homogeneously covered the surface of the specimen. (Figure 5A) When mapping the fluorescent signal intensity along the depth of an AF specimen, it was observed that HAMA Aldehyde product penetrated into the tissue evenly, with highest signal intensities localizing at the interfaces between lamellae. (Figure 5B) Cross-sections of HAMA Aldehyde-treated AF tissue demonstrated that the biomaterial was retained within 850 μ m from the tissue surface and that a considerable number of cells in the AF are exposed to this biomaterial at the working concentration (60 μ M) and lower, which is proportional to fluorescent signal intensity. (Figure 5C)

3.5 Assessment of cytocompatibility

Cell viability was screened after exposing AF cells to HAMA Aldehyde product for 1 hour across a concentration range of 10^{-9} to 10^2 μ M. At a concentration of 1 μ M and below, AF cell viability was not significantly different than the untreated controls ($p > 0.05$). Most notably, cell viability at the working concentration applied to AF defects (60 μ M, equivalently 10% w/v HAMA Aldehyde) was not different than untreated controls ($p > 0.05$). When cells were exposed to 10 μ M and 20 μ M of HAMA Aldehyde product, there was a significant increase in RLU output compared to the untreated controls ($p_{10\mu M} = 0.0291$; $p_{20\mu M} = 0.0042$). Lastly, when cells were exposed to a concentration of HAMA Aldehyde product above the working concentration applied to AF defects (100 μ M), there was a significant decrease in cell viability compared to the untreated controls ($p < 0.0001$) and no significant difference when compared to the 20% v/v ethanol treated control ($p > 0.05$).

(Figure 6A) Cell viability measurements were cross-validated with morphological observations through phase contrast microscopy. At 60 μ M HAMA Aldehyde (and lower), AF cells exhibited a healthy spindle-like fibroblast morphology comparable to the live cells in the untreated controls. In contrast, the 20% v/v ethanol treated control group exhibited an aberrant morphology that is representative of poor cell health and distinctly different than the HAMA Aldehyde treated AF cells at 60 μ M. (Figure 6B)

3.6 In situ histological assessments

Picrosirius red and alcian blue staining indicated collagen and proteoglycan content, respectively, as well as overall tissue architecture of bovine IVDs that were either intact, injured with a clinically-relevant AF defect (4mm biopsy punch with 200mg NP removal) that simulates discectomy, or repaired with the two-part strategy developed in this study. In the intact condition, the lamellar structure of collagenous AF fibers is present and contains a proteoglycan-rich NP. (Figure 7A) In the simulated discectomy model, mid-sagittal sections indicate the removal of NP along with complete disruption of AF integrity on the IVD's posterolateral side. (Figure 7B) Repair of clinically-relevant AF defects with the two-part repair strategy developed in this study corroborates with the histological findings indicated by lap shear specimens when scaling this approach up to a large animal model of simulated discectomy. The injectable hydrogel implant was able to volumetrically fill the void space of AF defects and featured a contiguous boundary between AF tissue and hydrogel implant at the interface, suggesting that the tissue and biomaterial are adsorbed to one another via GAG-mediated covalent bonds. (Figure 7C)

3.7 Assessment of IVD herniation risk

The herniation risk of this two-part repair strategy was characterized against the IVD model of simulated discectomy and intact IVD controls *ex vivo* in a displacement-controlled ramp-to-failure test. Within the repair cohort, AF defects were sealed with IPN hydrogels composed of 575Da, 10kDa, or 20kDa PEGDA, while holding all concentrations of the prepolymer solution constant. (Figure 8A) Representative force-displacement traces indicate two modes of motion segment failure: endplate fracture or NP herniation. Notably, all intact motion segments failed by endplate fracture, whereas all motion segments in the discectomy and repair groups failed by NP protrusion, exhibited by a discontinuous drop in force prior to reaching the ultimate strength of the motion segment specimen. (Figure 8B) IVD failure strength was significantly decreased compared to the intact condition for all IVDs that were repaired ($p_{575\text{Da}} < 0.0001$; $p_{10\text{kDa}} = 0.0013$; $p_{20\text{kDa}} = 0.0089$), however only a trend towards a decrease in failure strength was observed for the discectomy group ($p = 0.0631$). Notably, 70% of specimens in both the discectomy group and 20kDa PEGDA repair group endured supraphysiological stresses (2.3 MPa) before failure. (Figure 8C) Subsidence-to-IVD failure was significantly lower than intact levels for the discectomy condition ($p = 0.0087$) as well as the 575Da ($p = 0.0002$) and 10kDa PEGDA ($p = 0.0009$) repair conditions, however there was no difference between the intact group and 20kDa PEGDA repair group ($p = 0.1603$). (Figure 8D) IVD failure strain was significantly lower than intact levels for the 575Da ($p = 0.0002$) and 10kDa PEGDA ($p = 0.0323$) repair conditions, however there was no difference between the intact group and 20kDa PEGDA repair group ($p = 0.1089$) or discectomy group ($p = 0.0912$). (Figure 8E) Work-to-IVD failure was significantly

decreased compared to the intact condition for all IVDs that were repaired ($p_{575\text{Da}} < 0.0001$; $p_{10\text{kDa}} = 0.0009$; $p_{20\text{kDa}} = 0.0148$) as well as unrepaired in the discectomy group ($p = 0.0241$). (Figure 8F) The ultimate strength to failure strength ratio was significantly higher for all IVDs that were repaired ($p_{575\text{Da}} < 0.0001$; $p_{10\text{kDa}} = 0.0155$; $p_{20\text{kDa}} = 0.0266$) or unrepaired in the discectomy group ($p = 0.0295$). (Figure 8G) Nonlinear semi-log correlations were used to assess empirical exponential relationships between herniation risk parameters and indicated a strong negative correlation between IVD failure strength and hydrogel tensile modulus ($R^2 = 0.82$), as well as subsidence-to-IVD failure and hydrogel tensile modulus ($R^2 = 0.88$). (Figure 8H)

Discussion

This study developed a two-part repair strategy to seal annular defects composed of an interfacial priming agent (dual-modified GAG) that coats tissue surfaces and a void-filling IPN hydrogel system composed of both natural and synthetic networks that can chemically adsorb to IVD tissue through the dual-modified GAG compound. A similar strategy to bond injectable hydrogels to soft musculoskeletal tissues via dual-modified CS was first developed for articular cartilage repair, but this approach has neither been translated nor optimized with an analogous use of dual-modified HA for AF repair, which requires engineered constructs to endure distinct biomechanical loads and higher deformations for successful translation.[32,49–51] Since outcomes following articular cartilage repair do not inform the likelihood of success in AF repair due to differences in biomechanical behavior, a biomechanical evaluation of construct herniation risk using an *ex vivo* bovine IVD model of simulated discectomy was necessary in order to determine the integrative strength of this repair strategy after its application to AF defects.[18,52] Here, we show for the first time: (1) unsulfated GAGs (i.e. HA) undergo higher oxidation than sulfated counterparts (i.e. CS) given the same reaction stoichiometry, and impart greater biomaterial adhesion when applied to AF tissue surfaces; (2) the optimized dual-modified GAG, HAMA Aldehyde, was not cytotoxic; and (3) softer/more compliant hydrogel constructs bear a lower herniation risk than stiffer/less compliant constructs of the same material composition.

When developing this strategy for AF repair, we prioritized tissue integration of injectable hydrogels as the prominent clinical design requirement so as to minimize the risk of reherniation and thereby decrease the probability of reoperation. That motivated us to first optimize the adhesion strength between the IPN hydrogel and AF tissue by means of biochemical modifications incorporated on the dual-modified GAG. Different reaction stoichiometries produced dual-modified GAG formulations with varying degrees of methacrylation and oxidation, where it was observed that these parameters did not linearly correspond to the GAG: IO_4^- and GAG:MAH molar ratios. When comparing lap shear adhesion strength across GAG type, we found that treatment of AF tissue with dual-modified HA yielded significantly greater hydrogel adhesion than treating AF tissue with dual-modified CS across all formulations screened in this study. Relating this outcome to the biochemical degrees of modification, dualmodified HA had an equal or greater degree of oxidation compared to dual-modified CS whereas dual-modified CS had an equal or greater degree of methacrylation compared to dual-modified HA across all formulations screened in this study. Additionally, when comparing the coefficients of determination (R^2) between

ultimate stress and degrees of modification, the ultimate stress was more strongly correlated with the degree of oxidation than the degree of methacrylation ($R^2_{\text{HA, Oxidation}} = 0.88$ versus $R^2_{\text{HA, Methacrylation}} = 0.76$, and $R^2_{\text{CS, Oxidation}} = 0.63$ versus $R^2_{\text{CS, Methacrylation}} = 0.49$), irrespective of GAG type. This outcome suggests that GAG oxidation has a greater influence on biomaterial adsorption to tissue surfaces than GAG methacrylation. Mechanistically, this may be explained by the formation of two covalent double bonds via Schiff base formation between the two aldehyde moieties per GAG repeat unit of the dual-modified GAG and primary amines on extracellular matrix proteins in the IVD, versus the single covalent bond formed between the methacrylate group per GAG repeat unit and acrylate end group on the PEGDA macromer in the hydrogel when exposed to APS/TEMED redox initiators. A potential biochemical factor that may explain why HA exhibited greater degrees of oxidation than CS following the two-step reaction scheme, given the same stoichiometries, is the presence of sulfate groups at the C4 position on the GalNAc subunit of CS; sulfate groups may limit the extent of conversion in the first reaction step, where other studies demonstrated greater oxidation for HA compared to a similar carbohydrate, dextran sulfate. [33] Notably, this is the first study to simultaneously impart these two biochemical modifications to the HA backbone and implement this system for bioadhesive tissue engineering applications. We build upon prior studies that employ simultaneous oxidation and methacrylation with analogous polysaccharides by quantifying and systematically comparing the biochemical degrees of oxidation and methacrylation as well as bioadhesivity between dual-modified HA and dual-modified CS.[32,49,50,53]

When contextualizing these results to other studies that use bioadhesive hydrogels for AF repair, this approach yields comparatively higher lap shear adhesion strengths than riboflavin-crosslinked collagen and genipin-crosslinked fibrin.[54,55] When compared to studies that use oxidized and/or methacrylated materials, we demonstrate that this method matches or exceeds lap shear adhesion strengths with respect to single-crosslinked OMA-9/PEG (2kPa), dualcrosslinked OMA-20/PEG (15kPa), PNIPAAm-g-CS + CS aldehyde (2kPa), but is lower than that in the original Wang et al. study reporting the use of CSMA Aldehyde as a tissue adhesive (46kPa).[32,38,56] It should be noted that the stoichiometries used to synthesize dual-modified CS Formulation 1 in this study correspond to theoretical degrees of methacrylation higher than that reported in Wang et al., given the higher efficiency of methacrylic anhydride versus glycidyl methacrylate reported by Bryant et al., yet we observed that this product of CSMA Aldehyde only imparts an average adhesion strength of 9.51kPa with AF tissue.[35] These differences in observed outcomes might be attributed to dissimilarities in tissue composition and surface topography between cartilage and the AF, which are known to affect bonding at the tissue-biomaterial interface and thus impact adhesion strength values.[57]

Following the optimization of interfacial adhesion strength, we then assessed motion segment herniation risk with hydrogel implants of varying mechanical molecular weight to test the hypothesis that hydrogel elasticity is a critical factor impacting herniation strength *in situ*. We found that compliance of the hydrogel system, while keeping material composition constant, plays a considerable role in failure mechanics of the motion segment as well as the mechanism by which the motion segment fails. Surprisingly, we show that mechanically compliant hydrogels (low Young's modulus and high PEGDA MW) bear a lower herniation

Functionally important biological assessments, such as annulocyte cytocompatibility with the biomaterials comprising this repair strategy, were completed following biomechanical tests. Since fibronectin and fibrin are natural biopolymers, they are inherently cytocompatible and used in FDA-approved sealants that are commercially available, including TISSEEL.[58,59] In addition to synthetic tunability and functional moieties that enable hydrogel bonding, PEGDA was employed in this strategy because PEG-based materials have extensively been shown to be biocompatible and are FDA-approved for use in humans as well.[60] Although the constitutive polymer networks of the hydrogel are biocompatible, it has yet to be determined if the oxidized and methacrylated HA priming

agent in this repair strategy is non-cytotoxic to AF cells. Results from the cell viability assay suggest that there is no demonstrable cytotoxicity to AF cells when exposed to HAMA Aldehyde product at the working concentrations (and lower) used in this repair. 1 hour of exposure was chosen as the timepoint for AF cell viability measurements, since it is substantially longer than the 5-minute application period in our workflow and would account for both acute and relatively long-term exposure to the dual-modified HA product. It was observed that there was a significant increase in viability measured at 10 μ M and 20 μ M, indicating some benefit of HAMA Aldehyde for cell survival. These findings may be attributed to an increase in cell proliferation when exposed to HAMA Aldehyde at these concentrations, which can be elicited downstream through CD44 (i.e. homing cell adhesion molecule) intracellular signaling.[61] CD44 is a non-kinase transmembrane glycoprotein that binds to unconverted regions of HAMA Aldehyde and can promote a proliferative response through the PI3K/AKT pathway.[62] Moreover, it was observed that there was a significant decrease in viability at a concentration of 100 μ M HAMA Aldehyde, which may suggest that the culture conditions were too acidic for AF cell survival, as indicated by a change in the phenol red indicator of culture media from red to yellow.

With respect to clinical utility, the ease-of-application and required time for repair were prominent factors of consideration when developing this strategy. The injectability of the prepolymer solutions lends itself to a minimally invasive approach for AF repair, in which the biomaterials can be easily applied to defect spaces of human or large animal model IVDs. When considering clinical utility in the context of the simulated discectomy model used in this study, 25% of NP tissue was removed to enhance biomaterial delivery to the repair site as well as mitigate the risk of recurrent herniation following repair, since NP removal is known to increase IVD failure strength.[44] This outcome is validated by Carragee et al., in which patients that undergo a more aggressive discectomy procedure with NP removal demonstrate a lower reherniation rate (9%) than those without any NP removal (18%).[63] Moreover, the APS/TEMED redox initiator system used for macromer crosslinking can overcome issues regarding cure depth for UV-catalyzed photopolymerization *in situ*. [64] The current repair workflow takes approximately 7 minutes to apply this two-part biomaterial adhesive given the timescales of HAMA Aldehyde bonding and hydrogel gelation (Figure 1), and considering the average length of discectomy procedures is approximately 78 minutes, this procedure would only extend surgical time by approximately 9%. [65] Taken together, this strategy is expected to be an effective, cytocompatible method of repairing AF defects within a reasonable timeframe.

There are few limitations of this study and avenues of future investigation with respect to the outcomes reported here. A key limitation is the *ex vivo* focus of these studies, where systemic immune responses, changes in pain behavior, *in vivo* degradation kinetics, and endogenous cellular repair processes have not been evaluated. Although higher degrees of HA oxidation lead to greater hydrogel adhesion to AF tissue, it is known that higher degrees of polysaccharide oxidation can lead to faster biodegradation rates.[38,66] Since the dual-modified HA is an interfacial primer applied to tissue surfaces in liquid form, the only way to assess biodegradation is to histologically examine the presence/retention of these biomaterials over time in the AF repair site using a large *in vivo* animal model. Future large animal *in vivo* investigations are necessary to assess biomaterial degradation kinetics and

examine implant durability. Additionally, the FN-Fibrin natural polymer network within this hydrogel system incorporates integrin recognition sequences to enable cellular migration into the repair site, however this secondary hypothesis regarding endogenous cell recruitment and remodeling was considered beyond the scope of the current studies which focused on integration and herniation risk as the prime surgical design constraint.[67,68] Moreover, despite the presence of biochemical cues that permit cell attachment and motility, the current hydrogel system does not incorporate chemoattractants to stimulate resident cell migration or contain other bioactive factors to promote IVD healing, which may pose a limitation to long-term repair using this strategy in its current state. One potential avenue of advanced development is incorporating a bioactive component that directs biological repair responses after *in situ* application. Future work to address the aforementioned limitations and further demonstrate advanced preclinical validation, durability, safety, and effectiveness in an *in vivo* model are warranted following the successful integration and biomechanical optimizations achieved in this study.

Conclusions

This study is the first to: (1) optimize biomaterial adsorption through the use of oxidized and methacrylated GAGs to seal IVD defects by covalently bonding injectable space-filling hydrogels to native IVD tissue, and (2) determine the effect of construct elasticity on IVD failure mechanics when translating this strategy to a large animal model. Taken together, these results underscore the need to consider interactions at the tissue-hydrogel interface as well as the material properties of void-filling biomaterials in order to mitigate herniation risk. This two-part strategy is amenable to clinical use for AF repair since it is minimally invasive, easily applied to AF defects in a short time span, and demonstrates non-inferiority to the current standard of care. Future studies warrant investigation with an *in vivo* model of simulated discectomy to further examine endogenous repair processes as well as long-term durability of repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Schematic illustration of AF repair workflow and conceptual model of the two-part repair strategy.

(A) Workflow to repair annular defects with this two-part biomaterial system compared to the surgical standard of care. (B) Schematic of hydrogel composition and molecular working principle of dual-modified GAGs that enable covalent bonding of injectable hydrogels to native IVD collagen.

Figure 2: Synthesis and biochemical characterization of dual-modified GAGs.

(A) Two-step reaction scheme used to synthesize dual-modified CS and HA. (B) Dual-modified GAG formulations screened in this study categorized by oxidation and methacrylation reaction stoichiometries. (C) TNBS assay optical density measurements, which are used to determine the degree of oxidation for all dual-modified GAG formulations synthesized in this study. (D) ¹H NMR spectra for dual-modified CS and HA, which are used to determine the degree of methacrylation for all formulations synthesized in this study.

Figure 3: Fabrication and mechanical characterization of injectable SN and IPN hydrogels. (A) Hydrogels were fabricated in cylindrical molds for mechanical testing and bovine AF defects to demonstrate proof-of-concept *in situ* gelation. H&E staining of SN and IPN hydrogels demonstrates homogenous incorporation of the FN-Fibrin network up to 5mg/mL. (B) Bulk mechanical characterization of compressive modulus, tensile modulus, complex shear modulus, and tangent phase angle for SN and IPN hydrogel formulations across PEGDA MW and redox initiator concentrations.

Figure 4: Dual-modified HA imparts greater hydrogel adhesion to AF tissue than dualmodified CS.

(A) Gross specimen visualization and picrosirius red/alcan blue staining of samples fabricated for lap shear adhesion testing. (B) Representative load-displacement curve of a lap shear specimen that underwent displacement-controlled ramp-to-failure until the maximum force (F_{max}) was reached. (C) Lap shear specimens pre- and mid-test to visualize hydrogel adhesion to AF tissue with and without treatment of dual-modified GAGs. Circled area indicates slippage between the hydrogel and AF tissue during the lap shear test. (D) Lap shear ultimate stress factored by GAG type and formulation number. (E) Lap shear ultimate stress as a function of biochemical modifications and linear correlations between ultimate stress and degrees of oxidation and methacrylation.

Figure 5: Dual-modified HA homogenously covers the surface of AF tissue and penetrates below the tissue surface.

(A) Top-down view of lap shear specimens to visualize HAMA Aldehyde coverage on AF tissue. (B) Depth of HAMA Aldehyde fluorescent signal intensity as a function of spatial position. (C) Cross-sectional view of HAMA Aldehyde treated AF to determine depth of biomaterial penetration.

Figure 6: Dual-modified HA does not exhibit cytotoxicity at or below the working concentration used to repair AF defects.

(A) CellTiter-Glo® 2.0 cell viability assay to assess AF cytocompatibility to HAMA Aldehyde. (B) Phase-contrast images of HAMA Aldehyde treated AF cells compared to untreated and ethanol treated controls.

Figure 7: Application of this two-part biomaterial repair strategy leads to successful integration with AF tissue in an *ex vivo* bovine model of simulated discectomy.

Picrosirius red/alcan blue staining of an (A) intact IVD, (B) IVD that underwent simulated discectomy, and (C) IVD that was repaired with the two-part strategy. Arrow points to adhesive interface between AF tissue and IPN hydrogel.

Figure 8: Repairing AF defects with low-modulus hydrogels matches the herniation risk of the current surgical standard of care and leads to partial restoration to intact levels.

(A) Experimental design to assess implant herniation risk. (B) Representative loading curves for IVD motion segments that failed by vertebral endplate fracture and NP herniation. 1 = IVD failure strength, 2 = subsidence-to-IVD failure and IVD failure strain, 3 = work-to-IVD failure, 4 = ultimate strength, and 5 = ultimate strength/failure strength ratio. Quantification of (C) IVD failure strength, (D) subsidence-to-IVD failure, (E) failure strain, (F) work-to-IVD failure, and the (G) ultimate strength/failure strength ratio to mechanically characterize *in situ* implant herniation risk. (H) Nonlinear semi-log correlations between IVD failure strength and hydrogel tensile modulus and subsidence-to-IVD failure and hydrogel tensile

modulus. Dashed line in (B) represents physiological upper bound (2.3 MPa) of intradiscal pressure. (NS = Not Significant)

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Table 1:

Biochemical Degrees of Modification for Dual-modified GAG

	Formulation	GAG:MAH	GAG: IO4 ⁻	Degree of Oxidation (x)	Degree of Methacrylation (y)
CSMA Aldehyde	1	1:10	1:2.4	31%	57%
	2	1:20	1:2.4	21%	76%
	3	1:10	1:3.5	26%	37%
	4	1:20	1:3.5	38%	56%
HAMA Aldehyde	1	1:10	1:2.4	31%	47%
	2	1:20	1:2.4	31%	34%
	3	1:10	1:3.5	34%	47%
	4	1:20	1:3.5	42%	63%