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Screening of Hyaluronic Acid-Poly(ethylene glycol) Composite Hydrogels to Support Intervertebral Disc Cell Biosynthesis using Artificial Neural Network Analysis

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Abstract

Hyaluronic acid (HA) poly(ethylene glycol) (PEG) composite hydrogels have been widely studied for both cell delivery and soft tissue regeneration applications. A very broad range of physical and biological properties have been engineered into HA-PEG hydrogels that may differentially affect cellular “outcomes” of survival, synthesis and metabolism. The objective of this study was to rapidly screen multiple HA-PEG composite hydrogel formulations for an effect on matrix synthesis and behaviors of nucleus pulposus (NP) and annulus fibrosus (AF) cells of the intervertebral disc (IVD). A secondary objective was to apply artificial neural network (ANN) analysis to identify relationships between HA-PEG composite hydrogel formulation parameters and biological outcome measures for each cell type of the IVD. Eight different hydrogels were developed from preparations of thiolated HA (HA-SH) and PEG vinylsulfone (PEG-VS) macromers, and used as substrates for NP and AF cell culture *in vitro*. Hydrogel mechanical properties ranged from 70–489 kPa depending on HA molecular weight, and measures of matrix synthesis, metabolite consumption and production, and cell morphology were obtained to study relationships to hydrogel parameters. Results showed that NP and AF cell numbers were highest upon the HA-PEG hydrogels formed from the lower molecular weight HA, with evidence of higher sGAG production also upon lower HA molecular weight composite gels. All cells formed more multi-cell clusters upon any HA-PEG composite hydrogel as compared to gelatin substrates. Formulations were clustered into neurons based largely on their HA molecular weight, with few effects of PEG molecular weight observed on any measured parameters.

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Keywords

Nucleus pulposus; annulus fibrosus; cell culture; metabolites; intervertebral disc; tissue engineering; artificial neural networks; self-organizing map; modeling; polymers

1. Introduction

Intervertebral disc (IVD) disorders such as disc stenosis, spondylolysis, and herniation, contribute to pain and disability in millions of affected individuals annually [1]. In 2008 alone, IVD disorders resulted in more than 663,000 inpatient stays in US hospitals and cost more than \$9.5 billion, making spinal problems one of the most burdensome health conditions in the US [1]. Currently available non-surgical therapies can only treat pain and symptoms of IVD disorders, while surgical options, including discectomy, spinal fusion and total disc replacement, do not restore the structure and function of the native disc. A majority of tissue engineering therapies have focused on restoration of the nucleus pulposus (NP) region, as NP cell death, loss of cell phenotype, and loss of matrix hydration are believed to mediate disease related to IVD disorders. Numerous cells have been studied for the potential to repopulate and thus regenerate the NP region, including mesenchymal stem cells, nucleus pulposus cells, annulus fibrosus cells, chondrocytes and fibroblasts [2–6]. Different cell populations will respond to cell carrier materials with very specific and distinct biosynthetic profiles, such that it is not clear if delivery of annulus fibrosus cells or chondrocytes is appropriate to provide for restoration of nucleus pulposus matrix. Therefore, there remains a need to better understand the benefits and features of varying cell sources, and their interactions with varying cell carrier materials, in advancing the use of cell therapy for the NP region of the IVD [7, 8].

Selection of biomaterials is one of the key factors for the success of tissue regeneration strategies, both to ensure successful cell delivery and retention and to promote the appropriate matrix cues that regulate cell-matrix interactions aimed at functional tissue regeneration [5, 9–15]. A large number of studies have explored the potential for biomaterial scaffolds to support cell delivery and promote new tissue formation for tissues of the IVD, with a focus on injectable materials that can sustain cell viability [8, 16–21]. The rules of materials success for supplementation to the IVD remain unclear, however, as features supporting mechanical properties may compete with features supporting cell viability, biosynthesis or preservation of an appropriate cell phenotype. Hyaluronic acid (HA) has been a popular choice as an injectable biomaterial for delivery to the NP region of the intervertebral disc, as HA is a key component of the native extracellular matrix. HA biomaterials have several advantages in that they are biocompatible with a long history of clinical use, non-immunogenic, and their side chains can be readily manipulated to present various functional groups [8, 22–25]. Studies have demonstrated proof-of-concept for HA-based biomaterials, alone or in combination with a second constituent such as collagen, gelatin or poly(ethylene glycol), as cell carriers to the disc [14, 19, 26–31]. The advantages of the HA-composite-hydrogels include an ability to “tune” physical properties for the HA hydrogel as scaffolds or cell carriers [32–34]. Several studies [18, 35–40] have shown that HA-based scaffolds can maintain human NP cell phenotype, viability and proliferation, and

can promote proteoglycan and type II collagen synthesis by human NP cells *in vitro*. Few studies have focused on the diversity of cellular responses to these tunable HA-composite hydrogels, however, and those responses may vary amongst cell types.

For HA-composite hydrogels, a very broad range of physical and biological properties may be engineered into the biomaterial design that may differentially affect cellular “outcomes” of survival, synthesis and metabolism, complicating selection of an individual formulation. Statistical approaches such as principal components analysis or cluster analyses have been used to group biomaterial features based on multiple “outcomes” for material selection, particularly when large datasets are generated. Artificial neural network (ANN) analysis is another, probability-based approach to identify relationships between material formulations parameters and biological outcomes [41–43]. ANN analyses to generate a “self-organizing map” will identify connectivity amongst formulations based on similarities in a diverse array of measured outcomes [44–46]. An ANN network first consists of input, hidden and output layers of “nodes” or “neurons,” with connections across neurons in successive layers that are reinforced based on similarities in measured inputs. By feeding an array of measurement variables to the input layer, numerical differences across all neurons are evaluated that reinforce the connectivity or weighting between neurons. This iterative process is repeated for all measured arrays (samples) until a trained network of connected neurons is generated, leading to a “map” showing similarities across sets of measured variables that has parallels to outcomes from cluster analyses. In our prior work that used ANN to identify a suitable elastin-like polypeptide scaffolds for cartilage tissue engineering [41], polypeptide scaffolds formed from moderately crosslinked polymers and low protein density were mapped together based on the observation that they provide for the greatest matrix production and cell viability; nevertheless, the physical properties in these formulations were considered “weaker” than those of the native cartilage tissue but factored less into the material selection than the weighting assigned by ANN to the biological outcomes. A specific advantage to using this ANN in material optimization for biomedical applications is that ANN analysis does not require prior input parameters or defined assumptions of relationships among data components [45–50]. Additionally, large, incomplete, noisy, or complex data sets can be easily analyzed for distinct groupings based on similarities in measured parameters.

Among synthetic polymeric hydrogels, poly(ethylene) glycol (PEG) based hydrogels have been widely used in the field of tissue engineering due to their non-fouling nature, non-immunogenicity, tunable mechanical properties, resistance to protein adsorption, and the ability to incorporate functional groups for coupling to peptides and proteins [51–53]. The majority of studies involving PEG-based hydrogels use photopolymerization of PEG acrylates in the presence of UV light, and photoinitiator to promote crosslinking for cell encapsulation. Alternate chemistries that provide for crosslinking of PEG macromers have been widely demonstrated, including the Michael-type addition reaction of thiols to vinylsulfones [54–56]. Recently, our group utilized this reaction in the design of an injectable PEG-laminin composite hydrogel for cell delivery to the IVD [57, 58]. Since this chemistry allows gelation to occur without the need for an initiator or UV light, it was considered to be more suitable for cell delivery to the IVD space.

In this study, HA-PEG composite hydrogels were prepared via the Michael-type addition of thiolated HA (HA-SH) and 4-arm PEG-vinylsulfone (PEG-4VS) of various molecular weights and total polymer concentrations. In order to rapidly screen multiple formulations for an effect on cellular synthesis and phenotype, these HA-PEG composite hydrogels were studied for their ability to interact with and support IVD cells (NP and AF cells) in two-dimensional, *in vitro* culture. Under these conditions, important features of cell morphology, proliferation, matrix synthesis, and metabolite consumption and production can be assessed that will reveal and reflect cell-matrix interactions. A secondary objective was to apply ANN analysis in order to identify relationships between HA-PEG composite hydrogel formulation parameters and biological outcome measures for each cell type of the IVD. Overall, this work has revealed how different cell populations interact with these HA-PEG composite hydrogels, and also identified a set of HA-PEG hydrogels that can be supportive of IVD cells in culture.

2. Materials and Methods

2.1 Materials

Hyaluronic acid (HA) was purchased from Beta Pharma (MW = 637 kDa determined by viscosity measurement [59], Branford, CT). N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI), N-hydroxysuccinimide (NHS), dithiothreitol (DTT), calcium hydride and divinylsulfone (DVS) were purchased from Sigma-Aldrich (St. Louis, MO). Cystamine dichloride, phosphorus pentoxide and water (ultrapure, HPLC Grade) were purchased from VWR International, LLC (Radnor, PA). All 4-arm-polyethylene glycols (4-arm-PEGs, MW = 20 kDa, PDI = 1.02 and MW = 40 kDa, PDI = 1.02) were purchased from Jenkem Technology USA (Allen, TX).

2.2 Preparation of thiolated hyaluronic acid

Pulsed-ultrasonication [60, 61] was used to produce low molecular weight HAs from commercially-available HA, as described here. HA solutions (6.25 mg/mL) in ultrapure water were degassed with bubbling N₂ (30 minutes) and aliquots were exposed to pulsed ultrasound for 120, 20 or 5 minutes (13 ml, 8.7 W/cm², 1s on/1s off, 6–9 °C, Vibracell Model VCX500, 12.8 mm tip probe, Sonics and Materials, Inc. Newton, CT). After sonication, the solution was passed through a nylon syringe filter (pore size=0.45 µm) to yield low molecular weight HAs (~27 kDa, 59 kDa or 98 kDa, as determined by viscometry [49]), and the filtrate was freeze-dried as white foam (Figure 1 (a)).

Thiolated HAs were prepared by a two-step procedure [54, 62]. First, to a stirring solution of low molecular weight HA (27 kDa, 742 mg in 70 mL ultra-pure water) was added EDCI (1.45 g), and the stirring was continued at room temperature for 10 minutes under N₂. NHS (0.87 g) was then added and the pH value of the solution was adjusted to 4.5 by addition of 4 M HCl. After 2 hours, cystamine dichloride (1.70 g) was added. The reaction mixture was kept under N₂ and stirred at room temperature for 2 days, then dialyzed exhaustively against NaCl solution (4 g/L) and water for 2 days. The resulting dialysate was freeze-dried to yield white foam, and then dissolved in H₂O (70 mL) at the start of the modification. DTT (11.5 g) was added and the pH value of the solution was adjusted to 8.2. The reaction mixture was

stirred (2 days, room temperature) under N₂ and the pH value adjusted to 5 by adding 4 M HCl. Following the reaction, the solution was first dialyzed against NaCl/HCl (NaCl: 4 g/L, pH = 5), followed by exhaustive dialysis against diluted HCl solution (pH = 5). After dialysis, the solution pH was adjusted to 7 by adding 1 M NaOH, and then freeze-dried to yield a thiolated hyaluronic acid product (HA-SH-1, yield = 81%). ¹H-NMR (D₂O; 400MHz Varian spectrophotometer): δ 4.3–4.5 (HA anomeric proton), 3.0–4.1 (HA ring protons), 2.6 (NH-CH₂-CH₂-SH), 2.4 (NH-CH₂-CH₂-SH), 1.9 (NHCOCH₃). The degree of substitution (DS) of free thiol groups was determined to be 4.0% via Ellman method [63] and the molecular weight of HA-SH obtained was determined to be 27 kDa. Similar synthetic methods were used to synthesize HA-SH with different molecular weights and degrees of substitution (DS) of free thiol groups (HA-SH-2: MW = 59 kDa, DS = 4.0%; HA-SH-3: MW = 98 kDa, DS = 3.9%).

2.3 Preparation of poly(ethylene glycol) vinylsulfone

PEG vinylsulfones (4-arm-PEG-VS) were synthesized from 4-arm-PEGs and DVS as described (Figure 1(b)) [64]. Before use, all 4-arm-PEGs were dissolved in dichloromethane (DCM), precipitated into cold methanol three times, and dried over phosphorus pentoxide under high vacuum over 2 days to yield a fine powder. The powder 4-arm-PEG (*M_n* = 20,000, 6.0 g, 2.40 mmol-OH) was dissolved in anhydrous dichloromethane (90 mL), and the mixture was stirred under argon for 30 minutes. NaH (303 mg) was suspended in anhydrous DCM (30 mL), and the suspension was added into the flask dropwise and stirred for one hour. DVS (14.1 g, 120 mmol) was then dissolved in anhydrous dichloromethane (60 mL), and the suspension of PEG and NaH was added to the DVS solution dropwise over 1 hour. The reaction mixture was then stirred for 3 days under argon in the dark, neutralized with acetic acid (pH = 7), filtered, and concentrated. The final product was purified by precipitation in cold Et₂O twice, yielding a white powder of vinylsulfone functionalized 4-arm-poly(ethylene glycol) (4-arm-PEG-VS-20kDa: MW=20.5 kDa, 82% yield). The end group conversion percentage was determined to 96% based on ¹H-NMR, with a MW for the 4-arm-PEG-VS-1 calculated to be 20.5 kDa. ¹H-NMR (CD₃Cl): δ 6.8 (CH₂=CH-), 6.4 (CH₂=CH-), 6.1 (CH₂=CH-), 3.2–3.9 (–CH₂-CH₂-O- and -O-CH₂-CH₂-SO₂-). By employing the same method, other 4-arm-PEG-VS with different molecular weight and similar end group conversion percentages were prepared (4-arm-PEG-VS-40kDa: MW = 40.5 kDa, EGCP = 97%).

2.4 Crosslinked HA-PEG hydrogel formation and mechanical characterization

The physical properties of hydrogels formed from crosslinking the modified HA-SH with PEG-VS were studied in rheological testing for multiple formulations. 400 µl samples of synthesized HA-SH (MW=27, 59, or 98 kDa) and PEG-VS (4-arm, MW=20 kDa or 40 kDa) (molar ratio of thiol to vinylsulfone = 1.2 for all gels) in PBS were mixed in microtubes by gentle vortexing until complete dissolution. Thoroughly well dissolved and mixed solutions were transferred to individual wells of a 96-well tissue culture plate (70 µl/well, n=4/gel type) and kept at 37°C for complete gelation (1.5–2 hour) as determined previously by a vial tilting method (Figure 1(c)) [54]. The concentration (% w/v) is defined as the total dry weight of both PEG-VS and HA-SH per volume of PBS, and the dimensions of hydrogel samples prepared with this method were approximately ~6.4 mm diameter by 2.5 mm height

(approximate gel surface area: $\sim 0.32 \text{ cm}^2$). Hydrogel samples were then washed by application of pre-warmed PBS (37°C , $200 \mu\text{l}$, 20 min, 3x) to remove any unreacted residues.

Hydrogel samples were tested in torsional dynamic shear on a parallel plate geometry (8 mm dia.) using an AR-G2 Rheometer (TA Instruments, New Castle, DE). Plates were modified with a porous platen to provide friction for sample gripping during testing. Hydrogel samples prepared as described above were stored in physiological conditions (high humidity, 37°C , pH 7.4) for 24 hours prior to testing. After 24 hours, the gels were individually removed from the wells of the 96-well plate and placed in the center of the modified porous plate. To prevent sample evaporation, DI water was placed in the area surrounding the plate and the temperature of the plate was raised to 37°C . A 10% compressive pre-strain was applied to the sample for a 10 minute equilibration period prior to torsional shear testing to insure gripping. The storage modulus (G') and complex modulus (G^*) were then recorded for samples subjected to dynamic torsional shear at a frequency of 1 Hz and a strain of 0.01.

2.5 Cell isolation and culture upon HA-PEG hydrogels

Skeletally immature pig lumbar spines (3–6 months old, Nahunta Pork Center, Raleigh, NC) were obtained within 8 hours post-sacrifice. Cells were isolated from the nucleus pulposus (NP) or annulus fibrosus (AF) regions of IVD tissues via pronase-collagenase enzymatic digestion [65]; isolated NP and AF cells were cultured in monolayer 5–7 days in culture media (Ham's F-12 media (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 10mM HEPES (Gibco), 100U/ml penicillin (Gibco) and 100mg/mL streptomycin (Gibco)) with media change every 2 or 3 days prior to cell seeding. HA-PEG hydrogels were prepared as described above (Section 2.4) for multiple formulations (eight formulations: HA27k-PEG20k, HA59k-PEG20k, HA98k-PEG20k (2%), HA98k-PEG20k; HA27k-PEG40k, HA59k-PEG40k, HA98k-PEG40k (2%), HA98k-PEG40k). All crosslinked hydrogels were formed from 4% PEG solutions unless otherwise noted. For cell culture, the surface of each hydrogel sample was sterilized by UV exposure (30 min), followed by washing with culture media (1h, 37°C), followed by washing with sterile PBS (3x). Primary NP or AF cells (10,000 cells/gel) were seeded on the top of each gel ($n=5-6$ /hydrogel formulation/cell type) and cultured with a 180 μl culture media overlay for 7 days (5% CO_2 37°C) without any media change.

2.6 Measurement of biochemical content (DNA & sulfated glycosaminoglycan)

The sulfated glycosaminoglycan (sGAG) and DNA contents for papain digests of cell-hydrogel samples cultured for 7 days ($n=5-6$ per hydrogel formulation/cell type) were determined [41, 66]. In brief, papain digests of each cell seeded HA-PEG hydrogel (10 units/mg papain: Sigma Aldrich #P4762, St. Louis, MO; 1X PBS, 5mM cysteine HCL, 5mM EDTA, pH=6.0; mixed for 2h at 37°C then filtered; digested for less than 24 hours at 60°C) were analyzed by a dimethylmethylene blue (DMMB) assay for sGAG content using commercially available chondroitin-4-sulfate as a standard (Sigma). The DNA content was measured in papain digests (10 μl) by mixing with solutions of Hoechst 33258 dye (200 μl ,

Sigma, #DNA-QF) followed by reading absorbance at 460 nm (355nm excitation, PerkinElmer, Waltham, MA) with calf thymus DNA as a standard (Sigma, #DNA-QF) [67].

2.7 Measurement of secreted metabolites

Conditioned media was collected at day 7 of cell culture for each cell-seeded hydrogel sample and each cell type, for determination of lactate and pyruvate concentration ($n=5$ /hydrogel formulation /cell type); media collected from cultures incubated without cells for the same 7 days were used as a control and values were normalized to the values of the control media. Lactate and pyruvate were measured using a colorimetric reaction kit (#MAK064 & #MAK071 respectively, Sigma-Aldrich) following manufacturer's instructions.

2.8 Cell morphology

The morphology of AF or NP cells seeded on the surface of gelatin (control) versus HA-PEG hydrogel samples (HA58k-PEG20k and HA98k-PEG20k) was visualized by light microscopy (Zeiss Axiovert S100, X-Cite series 120Q Expo, Nikon Digital Sight) on day 3. Results were reported for cell clustering and cell spreading behaviors on varying HA-PEG hydrogel formulations as compared to gelatin.

2.9 Unsupervised ANN simulation

Unsupervised ANN simulation was used to distinguish HA-PEG hydrogels for their effects on AF and NP cells (outcomes) as has been described previously for cell-laden hydrogels [41]. Briefly, seven experimental outcomes were measured for AF or NP cell behavior on HA-PEG hydrogels following 7 days of culture and were gathered as an input vector, or array of values for each sample as follows: $INPUTS = [Lactate, Pyruvate, DNA, G', |G^*|, sGAG, sGAG/DNA]_i$, where i is the sample number. Separation amongst the input vectors representing 7 experimental outcomes for each cell type were identified with use of a self-organizing map (SOM) containing three "neurons" ("newsom," Matlab Neural Network Toolbox, MATLAB; The MathWorks, Natick, MA). To increase repeatability in the training of the SOM, pseudo-experimental outcomes ($n=10$ data points for each measurement outcome) following methods established in application of ANN modeling for biomaterials [41, 43, 68] were generated from measures for each cell-hydrogel sample using Monte Carlo methods and sample mean and standard deviation. Input vectors were fed to the SOM until convergence was achieved for AF and NP cell cultures separately. Values assigned to each of the three neurons were examined for the range and mean of HA molecular weight, PEG-VS molecular weight, and PEG concentration to identify hydrogel formulation parameters that regulate cell behaviors.

2.10 Statistical analyses

Data were expressed as mean $\pm$ standard deviation. Analysis of variance (ANOVA) with Tukey's *post hoc* tests or student t-tests was used to test for differences amongst hydrogel formulations for experimental outcomes (JMP 10, SAS, Cary, NC). Data were taken to be significant when a p-value of 0.05 or less was obtained.

3. Results

3.1 Mechanical testing

Mechanical testing results revealed that the HA98k-PEG40k composite hydrogel had the highest mean storage modulus (G') and complex modulus (G^*), with values of 489 ± 120 Pa and 490 ± 117 Pa, respectively (Table 1). Note that all findings pertain to the hydrogels formed from 4% w/v polymer concentration unless otherwise noted. Hydrogels formed from the HA98k-PEG20k composite formed at 2% w/v had the lowest mean storage modulus and complex modulus, with measured values of 111 ± 31 Pa and 109 ± 24 Pa, respectively (Table 1). In general, HA-PEG hydrogel stiffness increased with increasing HA molecular weight for hydrogels containing the same size PEG and equal polymer concentrations. In addition, hydrogels containing higher molecular weight PEG (40 kDa) were stiffer than gels containing 20 kDa PEG, when HA molecular weights and total polymer concentration were held constant. Hydrogels formed from the low molecular weight HA, HA27k-PEG20k and HA27k-PEG40k, were too viscous to obtain a consistent reading for G'' or the loss angle, δ , exhibiting behaviors that were very fluid-like during testing. Storage moduli of these hydrogel formulations were found to be in the range of 70–180 Pa (Table 1) and were lower than those of other HA-PEG hydrogel formulations of higher HA MW.

3.2 Biochemical content

DNA content was highest for NP and AF cells cultured on top of HA-PEG hydrogels containing the lowest HA molecular weight (27 kDa), regardless of PEG molecular weight (Figure 2). Fewer cells were retained on the surface of HA-PEG gels at the culture endpoints as HA molecular weight increased (Figure 2A). While HA-PEG gels containing 27 kDa HA were associated with significantly higher cell numbers as compared to tissue culture plastic (TCP, Figure 2A), these hydrogel substrates promoted very little sGAG accumulation (Figure 2B). HA-PEG hydrogels containing 59 kDa HA promoted significantly more sGAG accumulation by NP cells as compared to NP cells seeded upon all other hydrogel formulations. While trends were similar for NP and AF cells, there was no significant effect of hydrogel formulation on sGAG production by cultured AF cells (Figure 2B). For both DNA and sGAG contents, there were no significant differences observed between NP and AF cell types when grouped together (ANOVA, $p < 0.05$).

3.3 Media metabolites

For both NP and AF cells cultured upon HA-PEG substrates, there were no significant differences in measured pyruvate and lactate levels amongst hydrogel formulations (Figure 3). Average measured levels of pyruvate for AF cells plated on the surface of HA-PEG composite hydrogels were significantly lower than that of NP cells; however, there was no significant difference between cell types on tissue culture plastic (TCP, Figure 3A). In addition, pyruvate levels for AF cells on TCP were significantly higher than that of AF cells upon any other hydrogel formulation. As for pyruvate levels, lactate levels were not sufficiently different amongst formulations or between cell types to deduce any obvious trends (Figure 3B).

3.4 Cell morphology

To observe spreading or clustering behavior of cells seeded upon selected gel formulations, NP and AF cell morphology on a subset of HA-PEG hydrogels were visualized for comparison to that of cells on control gelatin surfaces on day 3 after seeding. NP cells on HA-PEG formulations formed multi-cell clusters while maintaining their rounded cell morphology (Figure 4). In comparison, NP cells on control gelatin surfaces remained attached as single cells (Figure 4). Similar to NP cells, AF cells formed multi-cell clusters on HA-PEG hydrogels (arrows), but not on control gelatin surfaces. However, a population of AF cells attached and spread on both HA-PEG hydrogel formulations, as well as control gelatin surfaces.

3.5 ANN Simulation

ANN analysis was used here to identify relationships between HA-PEG hydrogel parameters and a diverse set of mechanical and biological outcome measures. ANN simulation revealed that HA molecular weight is the main factor determining separation of the neurons followed by % polymer concentration (Figure 5) for both AF and NP cells. When considering the outcomes associated with each distinct neuron, it is clear that values for mechanical stiffness (Figure 6A) and DNA and sGAG contents (Figure 6B) are well separated by the neuron grouping; this is in contrast to the values for metabolites (Figure 6C) as they demonstrated almost no separation amongst the neurons for both cell types. Formulations mapping to Neuron 1 for AF and Neuron 3 for NP cells were associated with highest HA molecular weight, highest magnitudes of storage and complex moduli, and lowest DNA contents (Figure 5). For NP cells, a good contrast was observed for formulations in Neuron 2 that were of lower HA molecular weight and lower % w/v, and that gave rise to higher DNA contents and lower hydrogel stiffness. A similar contrast was observed between Neuron 1 for AF cells and Neuron 3, for which lower HA molecular weight and lower %w/v were associated with higher DNA content and lower hydrogel stiffness. The only notable difference between NP and AF cells in this contrast was that formulations contained in Neuron 1 for AF showed the highest sGAG levels amongst all Neuron groupings, whereas formulations contained in Neuron 3 for NP produced the lowest amount of sGAG. There were no significantly noted separations between neurons or trends in metabolite concentrations for any cell types.

4. Discussion

This study was designed to use ANN analysis to identify parameters of an HA-PEG composite hydrogel that could be used to support IVD cell metabolism and matrix synthesis *in vitro*. The application of ANN simulation was intended to identify global patterns in formulation parameter effects on biological outcomes such as cell number, matrix synthesis, and metabolism. Here, ANN simulation was successful in revealing relationships between HA molecular weight and polymer concentration that affected several and competing biological outcomes. Overall, the molecular weight of HA was found to be the most influential parameter of both mechanical properties and biological outcomes for IVD cells cultured on top of HA-PEG hydrogels. Biochemical results indicate that total cell content is influenced by HA molecular weights and in a similar manner for AF and NP cells. The

presentation of the HA ligand was anticipated to promote IVD cell attachment against the background of the non-fouling PEG, although the terminal DNA content measured here cannot be used to distinguish between DNA values attributed to initial cell attachment or cell proliferation over time. That DNA content was higher on the lower molecular weight HA-PEG hydrogels, while sGAG content was so low, suggests that both NP and AF cells on these substrates may have been undergoing a de-differentiation towards a more proliferative and less synthetically active phenotype. While this illustrates that HA molecular weight mediates cell number, it does not reveal if the mechanism is through reduced hydrogel stiffness or reduced ligand density, both associated with the lower molecular weight HA. A secondary observation was the effect of HA-PEG hydrogels upon AF or NP cell morphology. Studies have suggested that cell clustering may promote NP or AF cell survival [19, 69, 70] with consequences for increased sGAG production by immature NP cells [71]. The HA-PEG hydrogels all acted to promote multi-cell clusters as compared to gelatin-coated substrates, an effect observed more for NP than for AF cells. Additional studies would be needed to determine if specific hydrogel formulations of HA-PEG could be generated that would promote increased cell clusters, and to study effects on cell proliferation and matrix synthesis in this system.

ANN groupings of biochemical content and metabolite data demonstrated that neurons were separated on both sGAG and DNA contents, but not on metabolite measurements. Metabolite measures of lactate and pyruvate, as well as glucose, for cells in culture had previously been shown to relate to sGAG accumulation for chondrocytes in three-dimensional culture as a way to rapidly screen or predict biological outcomes and matrix synthesis [41]. Here, these values contributed to no separation in predicted performance for the NP or AF cells in culture upon different HA-PEG hydrogel substrates, which may relate to the use of two-dimensional culture conditions. Indeed, gas and nutrient exchange may be the most critical factors determining cell matrix synthesis for IVD cells, or chondrocytes, in culture with an expectation that these values could differ amongst hydrogel formulations in three-dimensions but not two-dimensions, as studied here. This is an important lesson from the use of ANN and suggests exclusion of metabolite measures in future studies of two-dimensional cultures. Another interesting observation was the separation in neurons obtained by using DNA and sGAG content as reported here, as opposed to the sGAG/DNA ratio, which is commonly accepted as a representative measure of matrix synthesis. Values for sGAG/DNA ratio in the NP cells show that while hydrogels assigned to Neuron 3 promote the lowest sGAG synthesis and lowest DNA content, they are associated with the values very similar to that for Neuron 1 (Figure 5). This is perhaps counter to the interpretation that formulations in Neuron 1 promote a de-differentiated, proliferative NP cell phenotype, while those in Neuron 3 are in a biosynthetically active, yet non-proliferative state. This observation does not hold for AF cells, where the sGAG/DNA ratio remained separated amongst Neurons 1 and 3, consistent with the concept that hydrogels in Neuron 1 promote a less proliferative and more synthetically active phenotype for AF cells. Why the finding that mapping with two biochemical outcomes (DNA and sGAG) as compared to a single outcome measure (sGAG/DNA) would differ for AF and NP cells suggests that there is an underlying behavioral difference that is not captured with measures in the current study.

Following HA molecular weight, the % polymer concentration was found to be an important variable driving separation of the formulations into neurons for both AF and NP cells, likely due to the the importance of this variable on determining physical properties. Here, the range of dynamic shear moduli for all HA-PEG formulations fell between 0.1–0.6 kPa, which fall at the very low end of values reported for the native NP tissue (0.1–70 kPa, [72–74]) and AF tissue (1–400 kPa, [75–77]). Hydrogels formed from the lower polymer concentrations (2% w/v) resulted in most soft gels with dynamic shear moduli of only 0.1–0.2 kPa, not really approximating values for the native tissues. These lower stiffness formulations were largely mapped to Neuron 2 for NP cells and Neuron 3 for AF cells. In general, stiffer polymeric hydrogels formed from higher %w/v polymer (e.g. HA) or a higher degree of crosslinking may approximate the native tissue properties, but will fail in their ability to support cell survival. Indeed, a prior study by our group has suggested that NP cells prefer attachment to and matrix synthesis upon very soft substrates of ~ 200 Pa stiffness, although those substrates contained laminin-PEG composite hydrogels, rather than HA-PEG as used here [57]. Also, our other prior studies [71, 78] have also demonstrated that soft (<700 Pa), laminin-containing extracellular matrix substrates promote a biosynthetically active and juvenile NP cell phenotype, as measured by NP cell morphology, cell-cell interactions, and proteoglycan production *in vitro*. Surprisingly, the results in the current study suggest that softer hydrogels formed from HA-PEG were found to be the least favorable substrates for promoting high matrix synthesis as measured by sGAG/DNA and for both cell types. Together, these findings suggest that while HA may serve as an important ligand for promoting cell-matrix interactions and/or disc cell survival, at least NP cell organization and phenotype may be highly sensitive to proteins native to their extracellular matrix environment such as laminin. Clearly, this suggests that multiple other hydrogel formulation parameters must be considered in interpreting hydrogel effects on biological outcomes. Overall, PEG molecular weight was found to be the least influential parameter in the current study of HA-PEG composite hydrogels, which may be useful to limit studies of composite hydrogel formulations in the future.

While it is possible to gain important understanding of the effects of composite HA-PEG based hydrogel formulations on IVD cell-material interaction from independent experimental outcome measures, this study confirms that the use of ANN simulation enriches the overall interpretation of data based on dissimilar input material parameters and multiple output measures, offering improved predictive ability for identifying biomaterial formulation parameters appropriate for a targeted application.

5. Conclusions

The work presented here demonstrated how different formulations of composite HA-PEG hydrogels can influence IVD cell behavior and demonstrates that injectable, *in situ* forming HA-PEG hydrogels have the potential to support NP and AF cells for cell culture *in vitro*, with potential to serve as a 3D carrier for cell delivery in the future. Findings suggest that careful tuning of formulations to incorporate lower molecular weight HA is helpful to insure maximum cell survival and sGAG production. This study also reveals the ability of unsupervised ANN simulations to recognize non-obvious patterns in outcomes measures based on composite HA-PEG hydrogel formulation. The ANN was able to successfully

identify HA molecular weight and polymer concentration as the more influential formulation parameters, and provided further insight on how to prioritize material parameters when forming composite HA-PEG based hydrogels.

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Figure 1. Schematics for synthesis of HA-SH from HA (a), 4-arm-PEG-VS based on 4-arm-PEG (b), and formation of the HA-PEG composite hydrogel via the Michael addition reaction between HA-SH and 4-arm-PEG-VS (c).

Figure 2.

DNA (A) and sGAG contents (B) on eight differently formulated composite HA-PEG hydrogels compared to regular tissue culture plate (denoted as ‘TCP’). In general, HA molecular weight appeared to regulate DNA content, but not sGAG content across formulations. Note all hydrogels are formed from 4% w/v polymer concentration unless denoted with ‘2%.’ Groups for DNA content denoted by a common letter were not found to be statistically different (Figure 2A, $p < 0.05$, $n = 5-6$, ANOVA or post hoc tests). Asterisks illustrate that sGAG content for NP cells and AF cells were similar amongst all groups

within each PEG molecular weight grouping, except for NP cells cultured upon formulations containing 59 kDa HA (Figure 2B, $p < 0.05$, $n = 5-6$, ANOVA or post hoc tests).

Figure 3. Metabolite concentrations (normalized to cell-free control media) in media aliquots collected from NP or AF cells grown on the surface of composite HA-PEG hydrogels for 7 days. Trends for differences amongst pyruvate (A) or lactate (B) contents across formulations were not apparent. An asterisk illustrates the observation that values upon tissue culture plastic (TCP), AF cells consumed more pyruvate and NP and AF cells produced more lactate than on any HA-PEG composite hydrogel (n=5, p<0.05).

Figure 4.

Cell morphology on the surface of composite HA-PEG hydrogels. Representative images of cell morphology attached on select HA-PEG hydrogel surfaces and gelatin at day 3. Arrows denote the center of clustering. There was no significant difference or trends observed in spreading or clustering and cell morphology between the two different HA-PEG formulations shown here; however, the composite hydrogels for both cells clearly showed more center points (arrows) of clustering compared to gelatin.

Figure 5. Results of artificial neural network analysis showing separation of composite HA-PEG formulation parameters by neuron (mean±standard deviation).

Figure 6. Normalized values for (A) mechanical properties, (B) biochemical data, and (C) metabolite data from artificial neural network modeling by neuron (mean $\pm$ standard deviation).

TABLE 1

Stiffness measured for HA-PEG hydrogels.

	G' (Pa)		 G* (Pa)	
	PEG 20K	PEG 40K	PEG 20K	PEG 40K
HA 27K	70 – 180	70 – 180	-	-
HA 59K	214±40	308±60	217±47	301±67
HA 98K	111±31*	157±21*	109±24*	162±22*
HA 98K	310±92	489±120	307±91	490±117

Values are given for hydrogels formed from 4% w/v polymer concentration unless denoted by an asterisk (2% w/v polymer concentration).