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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 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 to 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 sulfated glycosaminoglycan 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.

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 USA [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, NP cells, annulus fibrosus (AF) 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 AF cells or chondrocytes is appropriate to provide for the restoration of NP 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].

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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 IVD, 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) (PEG), 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 cross-linked 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 ultraviolet (UV) light, and photoinitiator to promote cross-linking for cell encapsulation. Alternate chemistries that provide for cross-linking 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 four-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 (2-D), 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

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 purchase from VWR International, LLC (Radnor, PA). All four-arm PEGS (MW = 20 kDa, polydispersity index (PDI) = 1.02 and MW = 40 kDa, PDI = 1.02) were purchased from Jenkem Technology USA (Allen, TX).

2.2. Preparation of thiolated HA

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^{-1}) in ultrapure water were degassed with bubbling N_2 (30 min) and aliquots were exposed to pulsed ultrasound for 120, 20 or 5 min (13 ml, 8.7 W cm^{-2}, 1 s on/1 s 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

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