3D PRINTING OF HYALURONIC ACID SCAFFOLDS FOR TISSUE ENGINEERING APPLICATIONS

BY

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THESIS

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ABSTRACT

Direct-write assembly is used to fabricate 3D microperiodic scaffolds composed of hyaluronic acid (HA), a natural, biocompatible, biodegradeable polymer, for cell culture and tissue engineering applications. HA is functionalized with UV-curable glycidyl methacrylate (GM) and printed to create 3D scaffolds with filament diameters ranging from 10-250 µm. The potential for 3D HAGM scaffolds in injectable applications is demonstrated by flowing 1.5 mm x 1.5 mm scaffolds through a channel tapered to 0.5 mm x 0.5 mm x 0.5 mm without damage. The compressive moduli of HAGM scaffolds can be tuned by varying the spacing between pattern filaments, and optimal designs exhibit values akin to the compressive modulus of articular cartilage.

Porcine adipose derived stem cells (ASCs) are cultured on HAGM scaffolds with the aim of inducing chondrogenic differentiation. Cartilage formation on 3D printed scaffolds is better distributed within the scaffolds relative to 2D controls, and new tissue is best able to infiltrate their microperiodic structure when a cell-adhesive RGD peptide is incorporated. To achieve the most effective distribution of ASCs within the 3D scaffolds, seeding of cells within dilute mixtures of HAGM is explored. Such mixtures may be combined with 3D printed scaffolds to produce a cell-laden scaffold that provides both a substrate for cartilage formation and pervasive structural elements that bear compressive loads when used in in vivo tissue repair applications. ASCs within the 2% HAGM bulk gels proliferate, transport, and undergo chondrogenic differentiation. Finally, HAGM scaffolds are implanted in porcine mandibular bone to confirm their biocompatibility and potential to support tissue growth in in vivo models.
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CHAPTER 1
INTRODUCTION

1.1 Introduction

Recent emphasis on biomimetic designs has advanced the field of tissue engineering beyond two dimensions into the domain of three-dimensional (3D) platforms for both in vitro and in vivo replication, regeneration, and replacement of biological tissues. Tissue damage repair, organ transplant needs, and alternatives for disease and drug therapy models have driven the demand for new and innovative technologies for replicating 3D microenvironments within the body.

As tissue engineering evolves, a variety of fabrication methods have made significant contributions towards producing 3D, biologically relevant structures for culture and growth of cells and tissues. While random, porous constructs have been popular due to their ease of fabrication, relatively low costs, and large sample volumes, recent methods such as stereolithography[1,2,3], colloidal templating[4], and 3D additive manufacturing [5,6,7,8] have been used to produce architectures with high a degree of control over porosity, orientation, and other features of the microenvironment that may affect cell and tissue behavior.

Materials selection is critical in the design of 3D constructs for tissue engineering applications. Key parameters that must be considered include biocompatibility, biodegradability, and cell-substrate interactions. Many naturally derived biopolymers meet these criteria, and are therefore ideal candidates. Hyaluronic acid (HA) is a large, unbranched, negatively charged polysaccharide found in extracellular matrix (ECM) and synovial fluids that naturally degrades via hyaluronidase and has proven to be well suited to tissue engineering with a variety of cell types and tissues. Functional groups along the backbone of the polymer allow for chemical
modification to tailor HA towards specific tissue engineering applications[9,10,11,12]. Functionalization with UV-crosslinkable methacrylate groups is especially useful for patterning 3D gels. In addition, co-polymerization with other biocompatible polymers such as (polyethylene) glycol diacrylate (PEGDA), which further allows grafting of integrin-binding peptides derived from ECM like RGD to form hydrogels that promote higher levels of cell attachment, a significant advantage for tissue engineering platforms[13,14,15,16].

When considering clinical applications, injectable 3D scaffolds are attractive as minimally invasive alternatives for regenerative therapies that would otherwise require traumatic surgical implantation. HA has been used in its natural form for basic injection therapies[17], and functionalized HA has been used for injecting crosslinked hydrogels [18,19,20]. Each of these strategies, however, relies on space-filling, soft gels without defined geometry; hence, they do not incorporate the design advantages of previously discussed 3D fabrication methods. Structural support and controlled architecture are not presently available in injectable HA systems, though significant steps forward have been made with other material systems[21]. While 3D printing of HA has been demonstrated [22,23], the ability to create spanning, periodic architectures have not yet been accomplished using those methods.

1.2 Thesis Scope

The objectives of this thesis are to synthesize, pattern, and characterize 3D scaffolds composed of UV-curable hyaluronic acid and to examine their applications in cell culture and tissue engineering.
1.3 Thesis Organization

In chapter 2, pertinent literature on HA and its functionalization methods is presented as well as a brief overview of 3D tissue engineering. In chapter 3, the design and synthesis of a UV-curable HA ink suitable for direct-write assembly are discussed. In addition, the fabrication of 3D HA scaffolds is described. In chapter 4, the application of these scaffolds for cell culture and tissue engineering is described. Finally, the thesis conclusions are provided in chapter 5.
2.1 Introduction

The chapter begins by providing an overview of hyaluronic acid (HA) and its functionalization and role in biological systems. Next, tissue engineering and regenerative medicine will be reviewed, with an emphasis on tissue mechanics, stem cells, and three-dimensional (3D) tissue engineering and fabrication. The use of HA in 3D tissue engineering and related injectable approaches is specifically highlighted. Finally, a novel 3D printing approach, known as direct-write assembly, is introduced with an emphasis on the ink design requirements and its potential applications in tissue engineering.

2.2 Hyaluronic Acid

Tissue engineering scaffolds should ideally promote natural wound healing and tissue growth in implant sites. Hence, biocompatibility, biodegradation, and functionality are key features of well-designed tissue engineering constructs. Naturally derived biopolymers are ideal candidates for scaffold materials where tissue regeneration is a primary goal. Hyaluronic acid (HA) is a large, unbranched, negatively charged polysaccharide composed of repeating units of glucuronic acid and N-acetylglucosamine (Figure 2.1).

![Chemical structure of hyaluronic acid](image.png)

Figure 2.1 Chemical structure of hyaluronic acid.[24]
It is naturally degraded in the presence of hyaluronidase, which is also present in the body. The prevalence of HA in the extracellular matrix (ECM) and its role in various cell functions such as motility, proliferation, and angiogenesis have made it an attractive candidate material for tissue engineering. Cells are able to interact with HA through cell-surface receptors CD44 and RHAMM to induce this cellular activity. [25] In addition, the functional groups along the polymer backbone present sites for chemical modification that permit the polymer to be tailored towards specific applications. As a result, HA functionalization, tissue engineering, and injectable motifs have been extensively investigated.

The primary site for functionalization of HA is in the exposed alcohol functional group on the N-acetylglucosamine unit. Modification of this group is often used to form photocrosslinkable moieties that allow chemical gelation of the polymer when exposed to UV light. The two primary methods used to chemically modify HA with photocrosslinkable functional groups are: (1) a free radical polymerization with methacrylic anhydride in the presence of acid [9,26-29] and (2) a ring opening reaction with glycidyl methacrylate. [10,30] These reactions, shown in Figure 2.2a, allow up to 90% of the available hydroxyl groups to be functionalized. The presence of methacrylate groups on the polymer backbone can be quantified by NMR following each reaction (Figure 2.2b).
Figure 2.2 Methacrylation of HA. (a) reaction mechanism for glycidyl methacrylate[10] and methacrylic anhydride[26] reactions. (b) $^1$H NMR of glycidyl methacrylated HA. Peaks at b and c are indicative of methacrylate functionalization.[10]

Burdick et al. demonstrated that UV-cured HA hydrogels could have controllable degradation rates and mechanical properties by varying the degrees of methacrylation through the
methacrylic anhydride reaction.[29] Bencherif et al. showed similar results for the glycidyl methacrylate reaction, demonstrating similar cell morphology and attachment across the degrees of methacrylation.[10] In my thesis, we build upon the synthesis of HA-glycidyl methacrylate (HAGM) to form a UV-curable hydrogel ink for printing of 3D HA scaffolds.

Thiol chemistry is another strategy that has been used for crosslinking of biocompatible HA materials, which takes advantage of the carboxyl group on the glucaronic acid unit (Figure 2.3a).[22,31,32] In one form of this method, HA is modified with a di-thiol containing dihydrazide in the presence of 3-(3-dimethylaminopropyl)carbodiimide(EDCI) activating agent. The gel forms by oxidation in air, and a simple reduction of the di-thiol bridge forms a reversible gel. No alternative energy source is needed for crosslinking, and gels form within minutes. Crosslinked gels are non-cytotoxic, making them suitable for tissue engineering.[31] Other crosslinking methods that utilize the carboxyl group on HA have been developed with aldehydes [33], azides [34], and tyramines [35]

HA can also be grafted with other polymer molecules to alter functionality and tailor HA to design strategies. Polyethylene glycol and poly(D,L-lactic acid-co-glycolic acid), well known biocompatible polymers, have been co-polymerized with hyaluronic acid to tailor degradation rates and the release of active compounds.[14,36,37,38] A Michael’s addition of RGD peptide sequences to HA or its derivative functional forms makes HA hydrogels more prone to cell attachment.[13,38] Macromolecules with unique functionality such as thermosensitive Pluronic F127 have been grafted on HA to combine the biofunctionality of HA with the unique temperature-sensitive characteristics of the Pluronic (Figure 2.3b).[39] Despite cytotoxic issues with pure Pluronic, the co-polymer demonstrated viable chondrocyte cultures over 4 weeks and
maintained the unique rheological properties characteristic of Pluronic F127 micelle formation.[39]

Figure 2.3 Reaction schemes for alternative HA functionalization (a) Di-thiol [31] and (b) Pluronic F127 co-polymerization. [39]

2.3 Tissue Engineering and Regenerative Medicine

Tissue engineering has developed over the past two decades to meet the demand for the replacement and regeneration of tissue and organs due to loss, catastrophic damage, and disease. Both in vitro and in vivo models have been utilized to mimic the complex microenvironment surrounding cell and tissue development. Bioactivity, degradation, soluble factors, mechanical cues, and fluid flow are all elements contributing to the complexity of the in vivo environment, which must be considered in tissue engineering.
2.3.1 Tissues of the Body and Associated Mechanics

The body is a composite composed of organic and inorganic materials, including fluids, tissues, and cells that display a wide range of characteristics that present challenges to tissue engineers. 3D scaffolds should reasonably mimic the target tissue for growth or regeneration. Not only do target tissues differ in structure and morphology, but their mechanical properties and responses throughout the human body vary greatly as well. As a result, a basic understanding of these differences is important for tissue engineering. There are four different types of tissue in the body: epithelial, muscle, nervous, and connective. This thesis focuses on connective tissues that support and connect other tissues throughout the body, particularly bone and cartilage. Bone and cartilage are extremely relevant to tissue engineering because of their critical relationship to mechanical support and movement. Annually, over 250,000 surgeries are directed at the repair of cartilage in tendons and ligaments in the United State alone.[40] The high demand for cartilage tissue provides the impetus for this thesis work.

Prior to engineering replacement tissue, it is important to understand the structure and mechanics of relevant connective tissues. Uncalcified articular cartilage has cells distributed throughout a collagen matrix that is anchored in the calcified layer, forming a three dimensional network of interlacing fibers that rise to the surface and turn parallel to the articular surface.[41] The Young’s modulus of cartilage is 1 MPa, while pure bone mineral is extremely stiff and strong with a Young’s modulus of 165 GPa. Human femur bone, a combination of these two tissues, is between these two values at ~18 GPa.[42] Research efforts in tissue engineering of each of these tissue types must carefully consider these mechanical characteristics of native tissue. The work in this thesis addresses how 3D HA scaffolds can be engineered to exhibit the material properties of cartilage and bone tissue.
2.3.2 Stem Cells and Effects of the Mechanical Microenvironment

Within tissue engineering, there is a fundamental requirement for cell sources that are capable of forming specific tissue types under properly controlled conditions. Stem cells are traditionally defined as cells that can proliferate while maintaining an undifferentiated phenotype, which are capable of differentiating into specialized cell types. There are a wide variety of stem cell types, but this work is focused on adult stem cells, which are primarily limited to specific cell lineages. Unlike embryonic stem cells, which can differentiate into cell types from all three germ layers, mesenchymal stem cells (MSCs) and adipose derived stem cells (ADSCs) are limited in their differentiation to particular lineages. They remain ideal candidates for tissue engineering because of their ability to differentiate into most tissues of the body, including cartilage, bone, fat, muscle and neurons. Prior to differentiation, they can be maintained in an undifferentiated state over the course of multiple in vitro passages. [34,43]

Discovery of MSCs was pioneered by Freidenstein et al. when they isolated polystyrene-adherent cells from bone marrow with tight fibroblast-like colony formations that were able to differentiate into both bone and cartilage. [44] These cells were soon found to expand to several other cell types, including adipocytes and myocytes [45]. The field grew rapidly and now yields over 10,000 publications annually. However, the procedure to obtain MSCs is highly invasive, available cell counts are low, and differentiation ability typically decreases with age. An alternative cell source that has potential to bypass these issues is ADSCs, taken from fat deposits removed during liposuction. Adipose tissue has been shown to contain stem cells with similar morphology and function to bone marrow-derived MSCs. [46] With nearly a half million liposuctions conducted per year in which the liposate is typically discarded, there is a large pool
of available cells. In addition, ADSCs can more easily be extracted from patients for potential autologous therapies.[47]

Cell differentiation of both MSCs and ADSCs has been proven to be highly dependent on extracellular cues. In addition to genetic and molecular in-cell markers, substrate stiffness has a major influence on stem cell fate. The extracellular matrix (ECM), which provides mechanical cues, has been carefully examined with respect to elasticity and geometry. Contractile forces arise as cells attach to a substrate, creating tensile stress on the cytoskeleton of the cell and cascading to the biochemical cues that determine differentiation.[48] Engler et al. showed that varying substrate stiffness, for instance, can affect stem cell fate. When seeded, MSCs initially exhibit similar morphology. After 96 hours, however, cells seeded on 0.1-1kPa substrates spread and showed the greatest expression of neurogenic transcripts. Substrates of stiffness 8-17 kPa led to spindle shaped, muscle-like cells with protein expression closest to muscle cells. Cells seeded on the stiffest 24-40 kPa substrate mimicked the morphology of osteoblasts and more strongly expressed osteogenic factors (Figure 2.1a).[49]

Geometric cues from the substrate can also influence stem cell fate. Independent of soluble factors, varying geometric features that altered contractile forces on the cell culture are able to induce differentiation along adipogenic and osteogenic pathways (Figures 2.4b and 2.4c).[50] Patterned PDMS micro-features are popular for determining mechanical cues on stem cell fate. MSCs cultured on longer posts (lower substrate stiffness) contained disorganized actin filaments and formed lipid droplets, indicating adipocyte differentiation (Figure 2.4d).[51]
Artificially engineered ECMs are a pathway through which researchers have proven the potential to promote specific tissues for regenerative medicine. Moving beyond the mechanical cues of planar surfaces demonstrated above, the fabrication of well-controlled 3D constructs is an important next step for properly mimicking the *in vivo* environment of stem cells in tissue engineering.

### 2.3.3 3D Tissue Engineering

A major goal of tissue engineering is to proliferate and manipulate cells in an environment that directly mimics the natural *in vivo* environment. Planar surfaces like those described above are useful for extracting information about specific extracellular cues, but they are inadequate for representing the 3D environment in which cells and tissue develop in the body. For example, in a seminal study in 1997, Weaver et al. used a cell line of tumorigenic and
non-malignant cells that were both derived from the same HMT-3522 mammalian cells, offering a unique tool for addressing the mechanisms behind malignant conversion in cancer cells. [52] The study revealed that the β₁ integrin was integral to tumorigenic cell fate. Since this molecule participates in cell-matrix adhesions, unique characteristics were found when the studies were done in 3D. By using an antigen to the β₁ integrin, tumorigenic cells were shown to revert to their original, non-malignant morphologies (Figure 2.5a), something never shown in 2D. Tissue engineering now emphasizes the 3D models for regenerative research to best simulate the environment and effects *in vivo*.

![Figure 2.5](image)

**Figure 2.5** Significance of third dimension in tissue engineering. (a) HMT-3522 cells (a) that become tumorigenic (a’) revert to benign morphology (a”).[52] (b) 3D culture of mouse embryonic stem cells in osteogenic media induces mineralization, indicated by black Von Kossa staining (white arrows).[53] (scale bars: a, 16 µm; b, 250µm).

Garreta et al. further demonstrated the necessity of the third dimension for stem cells in their study on osteogenic differentiation on self-assembling peptide scaffolds.[53] Loss of pluripotency was confirmed with fluorescently labeled Oct4 promoters in both 2D and 3D cultures of mouse embryonic stem cells, but mineralization associated with osteogenic differentiation was significantly higher in the 3D scaffolds compared to traditional 2D culture methods (Figure 2.5b). Morphology of cells derived from rat models were also matched only by the *in vitro* cultures utilizing 3D substrates.
2.3.4 3D Matrix and Scaffold Fabrication

3D tissue engineering initially relied on traditional materials, like Matrigel, to provide a research foundation.[52] However, there is a plethora of exciting materials and fabrication methods that have recently emerged for producing 3D constructs. Materials, such as collagen and gelatin, are widely utilized in the formation of random 3D porous structures due to their presence in the ECM. 3D fabrication techniques such as colloidal templating, stereolithography, and micro-extrusion broaden the scope of available materials to include chitosan, UV curable polymers, and polysaccharides.

Random 3D porous matrices are popular due to their ease of fabrication, low manufacturing costs, and large sample volumes produced by solution forming[54,55], particle leaching[56,57], gas foaming[58,59], or electrospinning[60,61] (Figure 2.6).

![Figure 2.6 Random 3D architectures for tissue engineering. (a) solution forming of collagen[55] (b) sucrose particle leaching in gelatin matrix[56] (c) poly (D,L –lactic acid) scaffold foamed with CO₂ gas [58] (d) electrospun collagen nanofibers.[60] (scale bars: a, b, c, 300µm; d, 5 µm)](image)

Solution forming, the most common method, relies on cooling dilute polymer suspensions to extremely low temperatures to induce ice crystal formation. Upon exposing this material to millitorr pressures, the ice crystals are sublimated, leaving behind a porous polymer network for tissue engineering. Collagen, one of the major proteins found in the ECM, is a popular matrix material. To date, researchers have also incorporated glycosaminoglycans, another primary
component of the ECM. The final result is a 3D matrix that is closely mimics the *in vivo* environments of cells.[62] The primary drawback is a lack of control over spatial architecture.

To address this limitation, researchers have used methods such as colloidal templating [63,64], stereolithography [2,3,65,66], and 3D filamentary printing [7,67,68] (Figure 2.7).

![Figure 2.7 3D architectures for tissue engineering. (a) Colloidal templating - chitosan inverse opal for mouse preosteoblastic cells.[63] (b) Stereolithography - porous poly (D,L-lactide) for fibroblast culture (c) 3D filamentary printing - multilayered carbohydrate glass lattice for sacrificial vascular networks.[7] (Scale bars: a,d, 1mm)](image)

These techniques result in highly controlled 3D architectures, allowing for increased control over porosity, orientation, and other features of the microenvironment that can affect cell and tissue behavior.

Colloidal templating of 3D scaffolds begins with the formation of uniform, degradable microspheres. Choi et al. used poly(caprolactone)(PCL) spheres, which were formed into a cubic close packed lattice and infilled with chitosan, a non-toxic, biocompatible, nanofibrous biomaterial.[63] Upon selective dissolution of the PCL template, a 3D inverse opal structure remained in which cells could be seeded and cultured. Stereolithography has been used to create matrices with controlled microporosity and stiffness from biodegradable. A typical flow diagram is depicted in Figure 2.8a. 3D scaffolds are designed in CAD and converted to STL format for fabrication. They are produced via UV curing of a photopolymerizable bath. The scaffolds are then cleaned or further processed prior to cell-seeding. Melchels et al.
demonstrated this method with poly (D,L-lactide) (PLDA), a common biodegradable polymer used in bone engineering. 3D scaffolds of arbitrary design were constructed with varying pore sizes at critical defect sizes of approximately 5 cm x 5 cm x 10 cm (Figure 2.8b). Mechanical properties of the scaffolds can be tuned via microstructure design for specific defect site applications.

![Figure 2.8](image)

**Figure 2.8** 3D stereolithography for tissue engineering. (a) Process flow for stereolithography.[65] (b) CAD designs and built PLDA 5x5x10 mm structures.[3]

Recent advances in tissue engineering have focused on scaffold architectures in which the biomaterial created by stereolithography[69], inkjet printing[70,71], or 3D printing[8,68,72] and pre-seeded with cells (Figure 2.9). Cell-seeded materials address several limitations of the more traditional biodegradable scaffold approach; that finite substrate attachment surfaces are not natural mechanisms for tissue growth, that different cell types may need to be placed in particular locations for complex tissues, and that vascularization is required in large areas of new tissue growth.
Figure 2.9. 3D cell printing. (A) Stereolithography - human ASCs in methacrylated gelatin after 22 days. Oil red O staining indicates intracellular lipid accumulation.[69] (B) Inkjet – 3D cell “pie.” Blue = human amniotic fluid-derived stem cells, Red = canine smooth muscle cells, Green = bovine aortic endothelial cells. [71](C) 3D printing – live(green)/dead(red) staining of rat heart endothelial cells in alginate scaffold after 14 days.[72] (scale bars: A, B, 1 mm: C 200 µm)

One novel approach towards this objective is the use of tissue spheroids.[8] In this method (Figure 2.10), cell-laden spheroids are arbitrarily patterned to form desired structures. Development from these spheroids produces whole tissue constructs. Narotte et al. utilized this method to form branching vasculature[8]. Chinese Hamster Ovarian cells were distributed in agarose molds to form vasculature with a diameter of 900 µm within a week. Heterogeneous tissue constructs were formed by placing spheroids of different cell types adjacently.

Figure 2.10 Bioprinting of vascular constructs with tissue spheroids. (a) Design template for tubular 3D construction. (b) Design template for multi-material tubule. (c) Printed human skin fibroblast (HSF) tubule in agarose. (d) Printed HSF branched tubes and pattern fusion after 7 days. (e) Cross-section of heterogeneous HSF and human umbilical vein smooth muscle cell tubule.[8]
2.3.5 HA in 3D Cell and Tissue Engineering

Because it is a natural, biocompatible, biodegradable polymer with potential for functionalization, HA has been used extensively for cell and tissue engineering. Seeded, crosslinkable gels have been used for growth and proliferation of human embryonic stem cells (hESCs) and MSCs. Gerecht et al. first introduced 3D hESC cultures with methacrylate HA that could sustain undifferentiated proliferation of hESCs for 30 days (Figure 2.11).[9] hESCs seeded in HA expressed markers for both undifferentiated proliferation and HA attachment. Upon enzymatic release from the hydrogels, hESCs formed colonies with undifferentiated morphologies within 24 hours. Thiolated HA gels previously described were co-polymerized with PEG to encapsulate MSCs for myocyte differentiation and repair. Live/dead staining confirmed MSC viability in 3D culture, and the reversible nature of the gel allowed for release of cells from the network.[37]

HA is a key material in cartilage engineering, which is extensively researched as a result of a quarter million knee and tendon surgeries every year, nearly all of which have a limited lifespan. HA is popular due to its role as major component of natural articular cartilage,
providing much of the compressive strength. The role of HA role in angiogenesis is also a consideration since cartilage is limited in healing capacity due to limited vasculature.[25] Both photocrosslinkable [27,28,30] and non-functionalized [73,74] HA have been used, and MSCs[73,75] and chondrocytes [28,76] have each been combined with HA to promote regeneration of cartilage tissue. Nettles et al. encapsulated chondrocytes in photocrosslinkable HA, and integrated the hydrogels with tissue in osteochondral defects in rabbit models.[28] Whole bone and histological images confirmed improved cartilage regeneration in HA trials (Figure 2.12), but implanted gels were two orders of magnitude below the compression modulus of native tissue. Hydrogels that evolve due to degradation and cellular uptake in cartilage defects with crosslinked HA also showed improved tissue distribution compared to static gels.[77]

![Gross (a,b) and histological (c,d) images of filled (a,c) and unfilled (b,d) rabbit osteochondral defects. Green Masson’s trichrome staining indicates deposition of collagenous matrix over filled defect.[28]](image)

**Figure 2.12** Gross (a,b) and histological (c,d) images of filled (a,c) and unfilled (b,d) rabbit osteochondral defects. Green Masson’s trichrome staining indicates deposition of collagenous matrix over filled defect.[28]

Patterning of fine-tuned spatial architectures of HA has been limited. Electrospinning[61] and layer-by-layer filamentary approaches[22,23] have been utilized, but spanning architectures have not been demonstrated like in other bioprinting efforts. Primitive vascular constructs of
cell-seeded HA were printed from a Fab@Home device with NIH 3T3 fibroblasts. Thiolated HA was crosslinked with a 4-armed tetracrylated PEG, and bioprinted cells and features retained shaped and viability over 28 days of culture (Figure 2.13 c,d).[23] A Fab@Home device was also used to pattern gold nanoparticle-doped HA and fibroblasts in large tube-like structures (Figure 2.13a,b). [22] While these methods are integral in their forward progress of cell-seeded HA gels, patterning finer features (<200µm) has not been demonstrated to date.

![Figure 2.13 3D patterning of HA hydrogels. (A) An acellular core I printed in a spiral pattern. Next, the cellularized AuNP-HA is added around the core. Then, and acellular, supportive “halo” is printed. (B) Printed tubular structure without acellular supports.[22] (C) Bioprinted thiolated-HA/tetracrylate PEG tubular structure printed from filamentary sections. Green fluorescence indicates calcein AM-stained live cells after 28 days of culture [23] (D) Surface view of electrospun diydrazide-HA/PEO nanofibrous scaffold.[61]]

### 2.3.6 Injectable HA and Injectable Scaffolds

Injectable approaches to 3D tissue engineering are attractive for their potential as minimally invasive strategies for regenerative therapy. Crosslinking of thiolated HA in ambient conditions has produced products such as HyStem and Extracel for commercially available, injectable HA hydrogels. Cell encapsulation and injectable HA gels have been investigated for repair of damaged cardiac[20], vocal[78], and spinal cord tissues[79].
Each of these strategies relies on space-filling, soft gels that lack defined geometry. Structural support upon implantation and controlled architecture are not presently available in injectable HA systems. Initial injections are liquid, which requires fine control of gelation, making specific placement of hydrogel without spreading difficult. Pre-formed, injectable polymer scaffolds offer a solution to such issues. Bencherif et al. recently addressed this need through shape-memory cryogels capable of passage through a standard syringe (Figure 2.14).

![Figure 2.14 Preformed, syringe injectable scaffolds. (A) Cryogel before and after syringe injection. (B) Arbitrary geometric shape of cryogels. (C) Confocal image of cells (6d incubation post injection) in RGD-modified methacrylate-alginate cryogel. (Scale bars: A, 2mm; B, 4mm; C, 20 µm).[21]](image)

Encapsulation and cell attachment via methacrylated alginate and an RGD peptide were used to load and inject MSCs into mice, where bioluminescent reporter cells were viable and locally retained. Connected macroporosity for injectable cell scaffolds has great potential in tissue engineering, and control of porosity and spatial architecture may greatly advance this novel approach.
2.4 Direct-Write Assembly

Direct-write assembly is an emerging printing route that enables patterning of 3D scaffolds with fine-scale features under ambient conditions. In this approach, a computer-controlled, 3-axis translation stage moves an ink deposition nozzle in a layer-by-layer manner to produce 3D architectures (Figure 2.15).[80] By designing ink formulations with appropriate rheological properties, a wide variety of materials can be patterned using this printing approach. Filamentary features with sizes ranging from 1µm to 1mm can be produced that are capable of spanning large gaps, a non-trivial task for other fabrication routes.

Figure 2.15 Direct-write assembly. (A) Printing set-up. (i) substrate (ii) print nozzle (iii) computer-controlled x-y-z translational air bearing gantry (iv) pressure input (v) imaging camera. (B) Schematic of layer-by-layer deposition under UV curing. (C) Camera image of printing first layer of hydrogel scaffold through a 30µm nozzle.

To date, a broad array of materials have been printed in arbitrary patterns, including hydrogel[5,6], fugitive organic[81-83], colloidal[84-88], polyelectrolyte[89], and nanoparticle [90,91,92,93] inks for applications in tissue engineering[5,6,87,88,94], biomimetic design [95,96], and self-healing [81-83] materials. These inks must flow through a fine deposition nozzle without clogging, yet quickly solidify to enable patterning of spanning filaments. Hence, the ink must be both shear thinning (Figure 2.16a) and viscoelastic, i.e., with a shear elastic
modulus ($G'$) that exceeds the loss modulus ($G''$) (Figure 2.16b). The inks typically have a viscosity of $\sim 10^1$-$10^2$ Pa·s at shear rates relevant for fine scale printing (~20-200 s$^{-1}$)[5].

Figure 2.16 Ideal rheology for direct-write inks. (A) Shear thinning and (B) viscoelastic behavior.

Figure 2.17 shows three recent examples of 3D scaffolds patterned by direct-write printing guiding fibroblast cell, neuronal cell, and bone growth. Barry et al. used a polyacrylamide hydrogel to fabricate small feature size periodic scaffolds.[5] 3T3 fibroblasts seeded onto the scaffolds aligned to parallel feature patterns on 2D surfaces and formed space filling morphologies within 3D compartments, while demonstrating interactions with neighboring cells. Direct-write assembly was also used to create 3D poly(2-hydroxyethyl methacrylate) (pHEMA) scaffolds for culture of rat hippocampal cells.[6] Confocal imaging was used to identify the optimal scaffold geometry for uniform incorporation of cells during seeding as well to characterize the growth and attachment of neuronal processes to these pHEMA scaffolds. 3D silk-hydroxyapatite scaffolds were fabricated to support co-cultures of human MSCs and human mammary microvascular endothelial cells (hMMECs).[8] Co-culture is required to produce vascular structures in constructed tissue, an important consideration when engineering any tissue. Over six weeks, MSCs and hMMECs remained viable, and osteogenesis and angiogenesis markers were both noted. The promising results to date with 3D scaffolds
produced by direct-write assembly necessitate further investigation of novel materials for 3D tissue engineering.

**Figure 2.17.** Direct-write printing of 3D scaffolds for tissue engineering applications. (A) Optical fluorescence microscopy image of 3T3 fibroblast seeded on polyacrylamide. Rhodamine-phalloidin stains actin red, DAPI stains DNA nucleus blue, and fluorescein-o-acrylate stains hydrogel scaffold green.[5] (B) Confocal image of primary rat hippocampal cells distributed in 60µm pitch pHEMA scaffold.[6] (C) Optical image of HA-silk gradient scaffold.[88] (D) hMMECs on silk-HA scaffold. Live cells are stained green.[88] (Scale bars: A, 3mm; C, 50µm; D, 20µm)
CHAPTER 3

DIRECT-WRITE ASSEMBLY OF INJECTABLE 3D HYALURONIC SCAFFOLDS

3.1 Introduction

In this chapter, we create 3D hyaluronic acid-glycidyl methacrylate (HAGM) scaffolds via direct-write assembly and demonstrate their potential as injectable platforms in tissue engineering. A novel hydrogel ink composed of HAGM, poly(ethylene glycol) diacrylate (PEGDA), solvent and photoinitiator is synthesized, formulated, and printed to produce 3D microperiodic scaffolds that are composed of fine features of <30µm that are retained when injected through small microchannels. HA molecular weight, degree of methacrylation, and PEGDA crosslinker as well as printing parameters and scaffold architecture add to the versatility and tunability of this system. Compared to other 3D scaffolds produced for cell culture [5,6], our HA ink printing approach produces scaffolds that are highly biocompatible and degradable.

3.2 Experimental Methods

3.2.1 Materials

Hyaluronic acid (~0.35 and 1.60 x 10^6 g/mol) is purchased from Lifecore Biomedical. Glycidyl methacrylate (GM), triethylamine (TEA), dimethylformamide (DMF), poly(ethylene glycol) diacrylate (PEGDA), poly(3-aminopropyl)triethoxysilane (APTES) and toluidine blue are purchased from Sigma-Aldrich and used as received. Nano-Strip® is purchased from Cyantek. All de-ionized water (DI-H₂O) is obtained from a Milli-Q® purification system (Millipore). All other reagents are from central facilities and are of the highest purity possible.

3.2.2 HA Methacrylation and Characterization

HA is modified with methacrylate functional groups following a reported method in the literature[10]. Specifically, HA is dissolved in a phosphate buffered saline (PBS):DMF co-
solvent (25:75, 50:50 or 75:25, v/v) at 0.5% (w/v) overnight at room temperature. 13.3 g of GM and 6.7 g of TEA are added drop-wise, and the reaction vessel is stirred for 5-10 days. The solution is precipitated in cold acetone (whose volume is 20 times greater than the volume of the reaction solution) and re-dissolved in DI-H$_2$O. The product is dialyzed against DI-H$_2$O for 3 days with 2 water changes per day, followed by 3 days of lyophilization. Hyaluronic acid-glycidyl methacrylate (HAGM) is stored at -20°C until further use.

The degree of methacrylation (DM) is determined by $^1$H NMR using a Varian Unity 500. 1% solutions of HAGM in D$_2$O (w/w) are measured at frequency of 500 MHz. Samples are analyzed with NUTS (Acorn NMR) software. DM is defined as the amount of methacrylate groups per HA disaccharide unit and is determined from peak integration ratios between methacrylate protons (δ ~6.1 and 5.6 ppm) and HA methyl protons (δ ~1.9).

3.2.3 Ink Design

HAGM inks are created by combining low (~0.35 x 10$^6$ g/mol) and high (~1.60 x 10$^6$ g/mol) MW HAGM of varying DM with PBS/glycerol co-solvent (50:50 w/w), Irgacure 2959, PEGDA (average 700 MW), and excess DI-H$_2$O. A typical ink consists of (w/w) 8.5% low-MW HAGM (19.8% DM), 8.5% high-MW HAGM (19.8% DM), 0.1% Irgacure 2959, 8% PEGDA, and 74.9% 50:50 (w/w) PBS/glycerol. Irgacure 2959 is first dissolved in the co-solvent overnight. The photoinitiator and co-solvent, HAGM, PEGDA, and excess DI-H$_2$O (3 mL for 5 g of ink) are combined and mixed for 180 s at 2000 rpm (ARE-310, Thinky USA) before being magnetically stirred for 12 h. Excess DI-H$_2$O is evaporated at 60°C while mixing with Thinky at 1 h intervals. As a qualitative measurement, a viable HAGM ink for printing spanning features forms a ball (Figure 3.2) immediately following centrifugal mixing. Final ink mixtures are stored at room temperature in the dark.
3.2.4 Rheological Characterization

Rheological properties of HAGM inks are measured using a controlled-stress rheometer (Bohlin CVOR-200, Malvern Instruments) fitted with cone and plate geometry (CP 4/40, cone diameter 40mm, 4° angle, 150 µm gap). Rheological properties of 5% HA solutions are measured with a controlled strain rheometer (AR-G2, TA Instruments) fitted with a concentric cylinder geometry. Viscosity measurements are carried out in controlled shear stress mode ascending from shear rates 0.01 to 100 s⁻¹. The elastic shear (\(G'\)) and viscous (\(G''\)) moduli are measured using an oscillatory logarithmic stress sweep at a frequency of 1 Hz. All measurements are acquired at room temperature with an aqueous solvent trap to mitigate evaporation.

3.2.5 3D Scaffold Printing

Prior to printing, glass coverslips are cleaned with stabilized sulfuric acid and hydrogen peroxide (Nano-Strip®) at 75°C for 40 min and rinsed with d-H₂O. Slides are dried by blowing with compressed nitrogen and further oven dried for 10 min at 125°C. Cleaned glass coverslips
are placed in a 5% (v/v) solution of APTES in toluene for 12 h at 60°C. Upon removal, coverslips are rinsed with IPA, dried with nitrogen, and stored in the dark at room temperature. APTES–coated coverslips promote filament adhesion of HAGM inks via electrostatic attraction between negatively charged HA polymer and positively charged amine groups.

Pre-pulled 10 µm and 30 µm silanized glass pipette tips (World Precision Instruments) are sputter-coated with a gold-palladium film (30 nm) (Denton Desk II TSC). 250 µm tapered dispensing tips are used as purchased (Nordson EFD).

HAGM inks are loaded into a 3 mL syringe barrel (Nordson EFD) with an attached tip of appropriate size and mounted onto a 3-axis micropositioning stage (ABL9000, Aerotech Inc.) controlled by customized software (NView). Extruded ink filaments are patterned in layer-by-layer fashion using applied pressures of 10-75 psi (Ultimus 5, Nordson EFD) at a speeds between 100-1000 µm s\(^{-1}\). During printing, the structures are exposed to UV light with \(\lambda = 320-400\)nm (Omnicure S200, Exfo) at ~10mW cm\(^{-2}\). Upon completion of printing, 3D scaffolds are further exposed to UV light at ~400 mW cm\(^{-2}\) for 10 min.

### 3.2.6 3D Scaffold Imaging

Printed HAGM scaffolds are imaged using a SEM (Hitachi S-4700) after the samples are coated with gold/palladium for 30s (Emitech K575).

To evaluate scaffold injection that mimics flow through a syringe into a larger cavity (or defect site), we created a model microfluidic test cell in a poly(methyl methacrylate) (PMMA) block mounted on a CNC-mill (Wabeco V6) and patterned with a 400µm square-end end mill (Performance Micro Tool). The acrylic blocks with milled microchannels are cut and polished on a polishing wheel (EcoMet, Buehler Inc.). Inputs are constructed with a hand drill and 2mm tapered tips attached to 3mL syringe barrel (Nordson EFD), and finished channels are mounted
onto glass slides with optical adhesive (NOA 78, Norland). We drill 2mm$^2$ and 0.5mm$^2$ channels in PMMA blocks connected by a tapered neck and depositing into a small reservoir (Figure 3.2a). After mounting on a glass slide and installing inputs and outputs, an 8-layer, 1.5mm x 1.5mm x 0.2mm HAGM-PEGDA scaffold with 30µm filaments is passed through the channel. Applied pressure of 30 psi (Ultimus 5) is used in the forward and reverse directions to manipulate scaffolds movements. Optical images are obtained with an inverted microscope (Leica DMI 6000b). Visual depictions of the microchannel design and imaging set-up are shown in Figure 3.2.

**Figure 3.2** Experimental design for scaffold injection simulation. (a) Schematic of milled channels. A 2mm$^2$ channel tapers into a 0.5 mm$^2$ channel that deposits into a 4x4x2mm reservoir. Glass slides were attached to the flat end for optical imaging on an inverted microscope (b). Pressure boxes are attached to inputs and outputs to drive fluid flow.
3.2.7 3D Scaffold Degradation

HA degradability is measured by cutting and obtaining the mass for 3D scaffolds (250µm features, 6x6x2 mm) and soaking overnight in PBS. Scaffolds for each treatment (n=3) are placed in a PBS solution with 0, 10, 100, and 1000 U ml⁻¹ hyaluronidase at 37°C. Aliquots of these solutions are removed and frozen for further testing and replaced at each time point. The concentration of HA degraded is determined using a carbazole assay[97].

3.2.8 Mechanical Characterization

A custom device consisting of a 100g +/- 0.1g load cell (RSP2 Single Point, Loadstar), a digital load cell interface (DI-100, Loadstar) and a motorized actuator with 0.2µm repeatable incremental accuracy (12mm Z8 Motorized DC Servo Actuator, ThorLabs) is used for collection of bulk and scaffold compression data (Figure 3.2a). The device is calibrated for load cell deflection prior to scaffold compression. 5% HAGM in PBS is poured into cylindrical PDMS molds (Figure 3.3b) and cured with UV, λ = 320-400nm (Omnicure S200, Exfo) at ~400mW cm⁻² for 10 min. Samples (Figure 3.3c) (n=3) are tested in a single pass, uniaxial, unconfined compression at 50µm s⁻¹, and data collected by LabVUE. Moduli are taken by fitting data from 5%-10% strain.

Printed HAGM-PEGDA scaffolds with 250µm filaments (n=3) are pre-soaked in PBS for 24 h and tested in a single pass, uniaxial, unconfined compression immediately after removal from media. Scaffolds are compressed at 50µm s⁻¹, and data is collected by LabVUE. Moduli are taken from 30g-60g loads in the linear regime, corresponding to strains of approximately 5%-10%.
Figure 3.3 Mechanical characterization of HAGM. (a) Custom device for mechanical compression testing, including the (i) load cell, (ii) digital load cell interface, (iii) motorized actuator, and (iv) sample location and alignment prism. (b) PDMS mold formed with 5mm biopsy punch. (c) Completed sample for mechanical characterization. (scale bars: b,c, 5mm)
3.3 Results and Discussion

Direct-write assembly is a process by which 3D architectures are patterned by extruding ink filaments in a layer-by-layer fashion using a 3-axis computer controlled stage (Figure 3.3).

![Image](image_url)

**Figure 3.4** Direct-writing of 12 mm HAGM scaffold with 250 µm filaments under UV light. (a) Optical zoom camera (b) Mounting block for syringe barrel connected to air pressure line (c) Attachment point to x-y-z positioning stage (d) 250µm nozzle. Inset: schematic of layer-by-layer filamentary deposition during the direct-write process.

Inks suitable for 3D printing must flow through fine nozzles, yet undergo rapid solidification upon deposition in order to retain their filamentary shape and span gap(s) across underlying. To promote the desired solidification, hyaluronic acid (HA) is modified with photo-reactive glycidyl methacrylate (GM) functional groups via a ring-opening mechanism. By varying reaction conditions (Table 3.1), we functionalized the polymer backbone with GM at 8.2%, 19.8%, and 29.8% (± 3.6%) degrees of methacrylation (DM), determined from NMR peak integration ratios between methacrylate protons (δ ~6.1 and 5.6ppm) and HA methyl protons (δ ~1.9) (Figure 3.5, peaks a,b). DM represents the amount of methacrylate groups per HA disaccharide unit (Figure...
3.5). Decreasing the ratio of PBS:DMF or increasing the molar ration of HA:GM leads to increases in the DM (Table 3.1). The respective compressive moduli of cured 5% HAGM hydrogels increase with DM (8.2%, 19.8%, and 29.8%). Values of 18.5 ± 1.3 kPa, 54.5 ± 1.4 kPa, and 80.8 ± 1.5 kPa are observed as shown in the inset of Figure 3.5. The scaffold stiffness is influenced by both the DM content as well as its 3D geometry.

<table>
<thead>
<tr>
<th>Mol. ratio</th>
<th>Solvent% (v/v)</th>
<th>Temp (°C)</th>
<th>Time (days)</th>
<th>DM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA:GM</td>
<td>PBS</td>
<td>DMF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:50</td>
<td>75</td>
<td>25</td>
<td>25</td>
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<tr>
<td>1:100</td>
<td>50</td>
<td>50</td>
<td>25</td>
<td>10</td>
</tr>
</tbody>
</table>

**Table 3.1** Reaction conditions for HA methacrylation.

**Figure 3.5** Methacrylation of hyaluronic acid. H\(^1\)-NMR spectroscopy of HAGM polymers. Proton peaks at (a) and (b) indicate methacrylate functionalization, calculated at 8.2%, 19.7%, and 29.8%. Inset depicts increasing load vs displacement fits for 5% cured hydrogels of the three DMs.
Direct-writing of HAGM inks requires a material that flows during nozzle extrusion, yet is solid-like to prevent feature sagging prior to UV curing. We must first design a DM HAGM ink that meets this requirement. The apparent viscosity as a function of shear rate for 5% solutions of each synthesized DM HAGM is shown in Figure 3.6.

![Figure 3.6](image.png)

**Figure 3.6** Ink viscosity as a function of shear rate of 5% HAGM solutions in PBS at 3 degrees of methacrylation.

At equivalent polymer concentrations and shear rates, the ink viscosity increases as DM decreases, varying by 2 orders of magnitude at 0.01 s\(^{-1}\) shear rate from 94.27 Pa·s for 29.8%DM to 0.24 Pa·s for 8.2% DM. To achieve homogenous inks at polymer concentrations high enough to support spanning features, excess water is added to the initial ink formulation to fully dissolve the hydrogel, which is subsequently evaporated. Because of low solution viscosity observed for higher DM concentrations, inks formulated from 29.8% DM HAGM require more water to be evaporated to enhance their concentrations above 5%. However, we found that more concentrated polymer solutions undergo premature curing. Thus, we opted not to design
printable inks composed of 29.8% DM HAGM inks. Fortunately, premature curing was not observed for the 8.2% and 19.8% DM HAGM solutions, so we used these materials to produce printable hydrogel inks. We determined an optimal total polymer concentration for 19.8% and 8.2% DM HAGM inks of 17.0% and 14.2% (w:w), respectively, with a 1:1 ratio of high:low MW HAGM. Both formulations exhibit shear thinning behavior (Figure 3.7a) that arises from physical entanglement of HA polymer chains, with shear thinning exponents $n \sim 0.10$ according to the power law $\eta \dot{\gamma}^{n-1}$, where $\eta$ is the solution viscosity and $\dot{\gamma}$ is the shear rate.

Shear rate experienced by the ink at the nozzle walls during printing can be estimated by:

$$\dot{\gamma}_{\text{wall}} = \left( \frac{3+b}{4} \right) \frac{4Q}{\pi R^3}$$

where $b$ is the inverse of the shear thinning exponent, $n$, $Q$ is the volumetric flow rate ($=v\pi R^2$), $v$ is the print speed, and $R$ is the nozzle radius.[6] It is determined using $b=0.9$ and an average print speed of 1mm·s$^{-1}$ that the shear rates during nozzle deposition are ~390s$^{-1}$ for 10µm nozzles, our smallest used, and ~15.6s$^{-1}$ for 250µm nozzles, our largest used. Even at the largest nozzle size and lowest associated shear rate, both inks exhibit viscosities of ~20 Pa·s, nearly two
orders of magnitude below low shear viscosities at 0.1 s\(^{-1}\) of 1618.90 (8.2% DM) and 1842.50 (19.8% DM) Pa·s. The dramatic reduction under relevant printing conditions ensures ink deposition without clogging.

The shear elastic modulus is another critical rheological parameter for the synthesis and design of these inks; it is a key parameter that determines the ability of the printed material to span gaps across underlying layers during 3D fabrication. The elastic moduli \((G')\) of the 8.2% DM HAGM (~8 kPa) and 19.8% DM HAGM (~7 kPa) are both higher than their respective loss moduli \((G'')\) of ~4kPa and ~5kPa (Figure 3.7b). This solid-like behavior is a hallmark of inks capable of patterning spanning filaments. To ensure that spanning features are maintained beyond printing times of 5 min, when feature sagging can occur, extruded filaments are exposed to UV light during printing. Cross-linking of the methacrylate groups functionalized on the polymer backbone form 3D features that are maintained for months beyond the printing window. 3D scaffolds are printed using this method with 10µm (Figure 3d), 30µm (Figures 3a-c) and 250µm (Fig 3.8a) filaments. Filament spacing can also be modified, with filament diameter-to-filament spacing ratios ranging from 2:1 (Figures 3.8a,b,d) to 8:1 (Figure 3.8c). Because the same materials system is used in all structures, the features are simply altered by changing nozzle size and/or computer code that controls the printhead.
Figure 3.8 SEM micrographs of printed HAGM scaffolds. (a) Low and (b) higher magnification images of 6-layer HAGM scaffolds printed with 30µm and 2:1 edge-to-edge spacing:filament diameter ratio. Printing parameters can be varied by adjusting the spacing (c) 30µm nozzle, 8:1 ratio or the nozzle diameter (d) 10µm nozzle, 2:1 ratio. (scale bars: a, 400µm; b-d, 100µm)

The effect of scaffold geometry on compressive modulus is shown in Figure 3.9. HAGM-PEGDA scaffolds are printed with a 250µm nozzle with center-to-center filament spacings of 750µm, 1250µm and 1750µm (Figures 3.9a i-iii). The compressive moduli of scaffolds (n=3) under unconstrained, uniaxial compression decreases from 584 ± 81 kPa to 155 ± 4 kPa as center-to-center filament spacing increases from 750 µm to 1750 µm (Figure 3.9b). For tissue engineering applications, HAGM-PEGDA scaffolds can be arbitrarily printed with feature sizes that produce compressive moduli matching the desired characteristics of surrounding tissue. Articular cartilage, for instance, is a commonly targeted load-bearing tissue for regenerative therapies that has a compressive modulus of approximately 600 kPa, which is akin to that observed for HAGM-PEGDA scaffold with a 750µm spacings. This feature size is therefore applicable to cartilage replacement implantations in load-bearing sites that require matching
compressive moduli to surrounding tissue. A library of feature sizes for 3D printed HAGM and HAGM-PEGDA scaffolds could be developed to complement other tissues. The maximum modulus achievable is 5.93 ± 1.13 MPa, which corresponds to the compressive modulus of the printed HAGM-PEGDA filament value. This is well below relevant values for bone repair which must withstand high compressive loads, but lies within range of articular cartilage, muscle, tendon, and ligament.

![Figure 3.9](image)

**Figure 3.9** Effect of 3D scaffold structure on compressive modulus. (a) Optical images of scaffolds printed with center-to-center spacings of i) 750µm, ii) 1250µm, and iii) 1750µm. (b) Compressive moduli decreases with increasing center-to-center spacing. (scale bar, 1mm)

Direct imaging of a 3D HAGM scaffold flowing through micro-machined tapered channel is carried out to simulate scaffold injection for minimally invasive implantation applications, such as arthroscopic surgery. An 8-layer, 1.5 mm x 1.5 mm x 0.2 mm HAGM-PEGDA scaffold composed of 30µm filaments is flowed through a tapered microchannel whose dimensions change from 2 mm x 2 mm to 0.5 mm x 0.5 mm, a 16-fold reduction, without
damage to the microperiodic structure (Figure 3.10b). The smallest dimension through which the 3D scaffold must pass through is one-third of its lateral size. By using glycerol as the flowing media, we slowed down scaffold movement through the channel so that we could directly capture evidence of the rolling mechanism of the scaffold through the taper and small channel region before its shape is restored to the original configuration as it enters the large cavity (4 mm x 4mm x 2mm) (Figure 3.10c). Their mechanical robustness also permits them to be stamped from larger printed sheets for potential commercial scale-up (Figure 3.11).

**Figure 3.10** Injectable HAGM scaffolds. (a) Channels tapering from 2mm$^2$ down to 0.5mm$^2$ formed out of PMMA with a CNC mill. (b) Optical image of 8-layer 1.5x1.5x0.2mm HAGM scaffold before (left) and after (right) passing through tapered channel. (c) Frame-by-frame shots of scaffold passing through tapered channel by fluid flow. (scale bars: b, 0.5mm; c, 4mm)
Figure 3.11  Demonstration of scaffold stamping for commercial scale-up applications. Multiple scaffolds printed in one session (a) can be stamped with a biopsy punch (b) to well defined scaffold geometries (c) with clean cut edges (d). Arbitrarily large scaffold sheets and stamping mechanism could be used for large-scale fabrication. (scale bars: a,b 5mm; c, 1.5mm; d, 500µm)

A desirable feature of implanted scaffolds in tissue engineering is biodegradability as cells and tissues of the body infiltrate and proliferate to fill a target defect. HA scaffolds benefit from natural degradation when exposed to hyaluronidase (HAase) in vivo by cleavage of the β-N-acetylhexosamine-[1→4] glycosidic bonds. To evaluate this feature, both HAGM and HAGM-PEGDA scaffolds are placed in PBS solutions of varying HAase concentrations ranging from 0-1000 U/mL over the course of 21 days, with media changes to supply fresh HAase at each time point (Figure 3.12). HAGM scaffolds degrade faster than HAGM-PEGDA scaffolds due to decreased crosslinking in the hydrogel. Depending on target applications, direct-write printed scaffolds may be tailored to degrade at specific rates by varying PEGDA concentration from 8% (Figure 3.12a) to 0% (Figure 3.12b).
Figure 3.12 Scaffold degradation of (a) HAGM-PEGDA and (b) HAGM scaffolds in HAase solutions over 21 days.

3.4 Conclusions

We demonstrated the ability to fabricate 3D hydrogel scaffolds by direct-writing of HAGM, a naturally derived, biocompatible, biodegradable polymer modified with UV-curable functional groups. Tunability of the HAGM scaffolds toward specific applications and material properties were established by variations in polymer DM and MW, scaffold feature size, and PEGDA concentration. Finally, the ability of the scaffolds to undergo compression through small channels without suffering structural damage showed potential for injection-based tissue engineering strategies.
4.1 Introduction

In this chapter, we utilize printed HAGM-PEGDA scaffolds in *in vitro* and *in vivo* 3D cell culture and tissue engineering applications. Adipose derived stem (ASCs) are cultured on these 3D scaffolds as well as 2D controls to investigate cartilage formation. By coupling an RGD peptide to the scaffolds, their cell binding is enhanced, promoting infiltration of developing cartilage. A strategy for infusing HAGM-PEGDA scaffolds with a cell-laden, dilute HAGM gel is explored to evenly distribute these cells throughout these 3D structures. Cell proliferation and mobility are observed, and cartilage formation is positively identified through a quantifiable assay and immunohistochemistry. Finally, an initial attempt at HAGM-PEGDA scaffold implantation in a live porcine model is discussed, and the biocompatibility and tissue growth in defect sites are demonstrated.

4.2 Experimental Methods

4.2.1 Materials

Primary antibodies for aggrecan and collagen II are obtained from Novus Biologicals (CN# NBP2-12447) and Millipore (CN# MAB1330), respectively. Texas Red secondary antibody is purchased from Vector Labs (TI-2000). Cyanoacrylate tissue adhesive is obtained from Vet-Bond. Pig analgesic is purchased from Banamine and antibiotic from Excede. Arg-Gly-Asp-Cys (RGDS) was obtained from Genscript. TGF-β1, 50 nM ascorbic acid-2-phosphate, hyaluronidase, toluidine blue, papain, ethylenediaminetetraacetic acid (EDTA), dithiotriol (DTT), chondroitin sulfate from shark cartilage, dimethylmethylen blue (DMMB), and Triton
X-100 are from Sigma-Aldrich. All other reagents are from central storeroom facilities and are of the highest purity possible.

4.2.2 RGD Scaffold Fabrication

To create inks containing cell-binding peptides, RGDC and PEGDA are dissolved in 0.1% Irgacure 2959 (w/w) in PBS for 4 h at room temperature with mechanical mixing at a 10:1 molar ratio of acrylate to thiol functional groups according to prior work[16]. HAGM, glycerol, and excess water are added and the ink synthesis (section 3.2.4) and printing (section 3.2.5) proceeds as previously described.

4.2.3 In Vitro Cell Culture

Details on porcine adipose-derived stem cell (ASC) primary culture are as previously reported.[98] All protocols are approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC). Frozen ASCs are thawed and plated (passage 2-3) at a density of 7.5 × 10^5 cells/mL. Cells are cultured to 80% confluency before trypsinization and plating onto scaffolds. Differentiation is induced by culturing cells in a micromass culture as previously described.[99] Ten-microliter drops containing 5 X 10^5 cells in media supplemented with 1% FBS containing transforming growth factor (TGF-β1, 10 ng/ml) are placed on scaffolds and incubated for 1 h before filling wells with chondrogenic media. Chondrogenic differentiation medium consisted of basic high-glucose DMEM supplemented with 10 ng/mL TGF-β1 (Sigma T7039), 50 nM ascorbic acid-2-phosphate (Sigma A4403), 1% FBS, 1% penicillin G-streptomycin, and 5.6 mg/L of amphotericin B. The cells are maintained in chondrogenic medium for 2 weeks with medium changed every 3 days.

Green Fluorescent Protein (GFP)-positive ASCs at a concentration of 3x10^6 cells/mL are encapsulated in 2% HAGM-PEGDA in DMEM gels in 125μl aliquots per 96 plate well. Cells
isolated from a pig transgenic for GFP[100] are cultured for 7 days and imaged with an inverted fluorescence microscope (IX71, Olympus). Micromass culture and high density seeded encapsulation are conducted according to previously published experimental procedures.[101,102] Culture expanded ADSCs are harvested and pelleted in DMEM. For HA encapsulation treatments (n=3), cells are re-suspended in 2% HAGM-PEGDA in chondrogenic media at 2x10⁷ cells/mL. Positive controls (n=3) are re-suspended in chondrogenic media at 2x10⁷ cells/mL. Negative controls (n=2) are re-suspended in DMEM with 1% FBS containing transforming growth factor (TGF-β1, 10 ng/ml). Droplets of 12.5 µl of each treatment are pipetted into 24 well plates and incubated for 2 hours at 37°C and 5% CO₂. Following incubation, 500µl of solution relevant to each treatment is added in each well, and cells are cultured at 37°C and 5% CO₂. Cells are maintained in media for 11 days with media changes every 2 days.

4.2.4 Mechanical Characterization

Compression moduli of 2% bulk HAGM in PBS and HAGM-PEGDA scaffolds are obtained with the custom device described previously. 3D scaffolds (10mm in diameter) of 8-layers with 250 µm filaments spaced 750µm center-to-center are printed and cured with UV light, λ = 320-400nm (Omnicure S200, Exfo) at ~400mW cm² for 10 minutes and injected with 2% HAGM in PBS before curing again. All samples (n=3) are soaked for 24 hours in PBS.

4.2.5 Cell Imaging

For immunohistochemistry (IHC), cell aggregates are collected, frozen in optimum cutting temperature (OCT) embedding compound, and stored at -80°C. Frozen blocks are cut into 10µm sections (Cm1900, Leica), mounted on glass slides, and stored at -80°C. Sections are fixed in cold acetone for 10 mins before bringing to room temperature. Slides are incubated in
5% horse serum in PBS containing 1% Triton X-100 (PBS-T) to block binding of nonspecific antibodies for 30 minutes, washed with 1% horse serum in PBS-T, and incubated with primary antibodies for aggrecan and collagen II (50:1 in 1% serum PBS-T) at 4°C overnight. Sections are washed with 1% serum PBS-T and incubated with 15µg/mL Texas Red secondary antibody in 1% serum PBS-T for 1 hour at room temperature. Sections are washed with 1% serum PBS-T, mounted, and imaged with an inverted fluorescence microscope (DMI 4000, Leica).

4.2.6 Bulk HA Degradation

Aliquots of HAGM containing 1333µg and 2667µg of polymer are put in 24 well plates and are cured with UV, $\lambda = 320-400$nm (Omnicure S200, Exfo) at ~400mW cm$^{-2}$ for 10 minutes. After soaking for 24 hours in PBS, 1mL of 1000 U/mL hyaluronidase is added, and samples are incubated for 48 hours at 37°C. HA concentrations are determined using a carbazole assay.[97]

4.2.7 s-GAG Quantification

Sulfonated glycosaminoglycan (s-GAG) content is determined by a DMMB assay.[75,103] After 4, 7, and 11 days, 3D chondrogenic cultures are removed and digested with papain for 48 hours at 60°C. Papain solution is prepared at 300µg/mL papain in 20mM sodium phosphate buffer (pH=6.8) containing 1 mM EDTA and 2 mM DTT. Following digestion, 2.5 mL DMMB solution is added to 100µl of the sample, and the absorbance at 525 is determined with a microplate reader (SpectraMax, Molecular Devices). DMMB solution is prepared by adding 16 mg DMMB, 3.04 g glycine, 2.37g NaCl, and 95 mL of 0.1M HCl to 1L distilled water. Chondroitan sulfate is used as a standard to provide a calibration fit.

4.2.8 In Vivo Scaffold Implantation

Prior to surgery, printed HA-PEGDA scaffolds (8 layer, 250µm nozzle, 750µm center-to-center spacing) are pre-swelled to 10mm diameter and 2 mm thickness. The pig is prepped
for surgery by shaving and sterilizing the lower jaw, followed by application of iodine and thyroid drapes. A scalpel and electrocautery pencil are used to penetrate fat and muscle, and periosteum is removed with a periosteal elevator (Figure 4.1a). Ten mm transcortial osteotomies (4 per side) are performed with a trephine on the posterior region of the mandible with adequate irrigation [104,105], and scaffolds are placed in 2 osteotomies on each side (Figures 4.1 b,c). In the left mandible, scaffolds were secured with a cell-laden, fibrin and blood mixture. Blood is collected from the ear vein and mixed with 1 mL of cell/DMEM solution to a total volume of 5mL. After quick mixing, the solution is immediately injected into the defect and combined with 0.1 mL of 0.3M calcium hydrogen phosphate to induce coagulation. Empty controls are also treated with the fibrin and cell mixture. In the right mandible, scaffolds are secured with tissue adhesive. The periostium, muscle, fat, and skin layers are sutured, and the wound is closed with tissue adhesive. Pigs are administered an analgesic and antibiotic and allowed to recover. Pigs are maintained on a soft diet for 1-3 days post-surgery and then resume standard feed.

Figure 4.1 Implantation of HAGM-PEGDA scaffolds in a pig mandible. (a) Exposure of the mandible. (b) 10mm defects formed with a trephine. (c) HAGM-PEGDA scaffolds implanted in defects
4.2.9 Computed Tomography

At 3 and 7 weeks post-surgery, the jaw region of the pig is scanned using computed tomography (CT) (Lightspeed 16 Slice, GE Medical Systems) at 120kV and 64 mA. 3D rendering of CT slices is accomplished using Carestream.

At 12 weeks post-surgery, the pig mandible is harvested and the defects are separated and trimmed with a band saw. After 2 weeks in pure ethanol, the remaining soft tissue is removed to expose defect sites. Micro-computed tomography (Micro-CT) conducted at 100 kV and 100 mA with a copper-aluminum filter is used to image bone defects every 0.7° for 360°. (Skyscan 1172, Bruker). 3D modeling of the 2D slices is accomplished via a reconstruction algorithm (NRecon 1.1.4) and rendering (Amira 5.0).

4.2.10 Bone Mineral Density

Bone mineral density (BMD) for bone growth within the defect area is measured using dual energy x-ray absorptiometry (DXA) (QDR 4500A, Hologic). BMD, given in g/cm³, is determined by absorption differential between two x-ray beams of different energy levels. Due to dependence on sample thickness, BMD is normalized against whole bone surrounding defect of similar thickness and is presented as defect BMD/non-defect BMD.

4.3 Results and Discussion

As an initial test for applications with cartilage tissue, HAGM-PEGDA scaffolds are seeded with ASCs and pushed towards chondrogenic differentiation. Toluidine blue stains purple for glycosaminoglycans, a primary component of cartilage, and is known as a positive indicator for cartilage.[106] After 14 days, cartilage from cells seeded on 6-layer HAGM-PEGDA scaffolds demonstrate a more distributed morphology compared to 2D controls (Figure 4.2a-b). Scanning electron microscopy is used to examine the 3D distribution of cartilage.
Cartilage formation on HAGM-PEGDA scaffolds was minimal, and the most significant development occurred in cartilage growths on the underside of the scaffold (Figure 4.2c). To resolve this issue, cell-binding RGD was incorporated with PEGDA via a Michael’s addition prior to mixing with the other ink components. The peptide promotes cartilage growth that was more evenly spread throughout and within the microperiodic architecture (Figure 4.2d).

![Figure 4.2 Scaffolds effects on cartilage morphology. Cartilage stained with toluidine blue after 14 days on (a) polystyrene culture plate and (b) polystyrene culture plate with HAGM scaffold. SEM micrographs of cartilage on scaffolds without (c) and with (d) an incorporated RGD peptide. (scale bars: a,b, 75µm; c,d, 50µm)](image)

Though cartilage was found to better infiltrate the 3D architecture with the incorporation of RGD, even distribution of ASCs is difficult during initial seeding, and cells tend to aggregate or fall to the bottom of the scaffold. These phenomena lead to pockets of cartilage rather than tissue growth over the entire scaffold.

A strategy for evenly distributed cell seeding is the use of hydrogels as a biocompatible, cell-seeded matrix for cell proliferation, tissue formation, and growth. We use 2% HAGM as our
cell-seeded hydrogel, the same polymer that serves as the primary material component for the hydrogel direct-write ink previously discussed. For tissue engineering applications, it is desirable to match the tissue implant to surrounding, native tissue. After UV curing, 2% HAGM has a weak compressive modulus of 9.1 +/- 0.8 kPa compared to native cartilage at approximately 600 kPa (Table 4.1). [28]

<table>
<thead>
<tr>
<th>Material</th>
<th>Compressive Modulus (kPa)</th>
<th>Std Dev (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk HAGM 2%</td>
<td>9.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Scaffold</td>
<td>584</td>
<td>82</td>
</tr>
<tr>
<td>Scaffold + Bulk 2% HAGM</td>
<td>516</td>
<td>26</td>
</tr>
</tbody>
</table>

Table 4.1  Compressive moduli of scaffolds and bulk HAGM

HA scaffolds have a compressive modulus that lies within the range of native cartilage (~600 kPa). Compressive moduli of scaffolds infused with 2% HAGM are similar to the pure scaffolds. By infusing 3D HAGM-PEGDA scaffolds with cell-seeded HAGM, it is possible to have cell distribution throughout the entire scaffold and robust compression mechanics to emulate surrounding native cartilage.

Cell viability and 3D distribution are necessary features of a cell-laden hydrogel system and are confirmed by imaging ASCs in a 2% HAGM gel and monitoring GFP fluorescence. Cells cultured on 2D well plate controls are only visible on plane of focus and tend to cluster (Figure 4.3a), while cells in 2% HAGM are more evenly dispersed and visible at all Z focal planes throughout the 2mm thick sample (Figure 4.3b-d).
Cells cultured in 2% are GFP-positive, fluorescent at 510nm, and used to positively confirm cell viability. Cells are viable over the course of 6 days of culture in the 2% HAGM gel, and increases in cell density demonstrate proliferation between days 3 and 6 (Figures 4.4 a,b). Cells clusters are also found within the samples after 6 days that are not present at day 0, confirming cell mobility within the hydrogel (Figure 4.4c).
This cell mobility is very important, as cell-cell interactions promote N-cadherin, a molecule involved in chondrogenesis.[107] Cluster formation increases the number of cell-cell interactions and enhances cartilage development.[108]

Encapsulating materials for tissue engineering applications must be biodegradable, breaking down as new tissue grows to fill defect sites. The ability of the 2% bulk HAGM to degrade is confirmed by measuring glycosaminoglycan (GAG) content before and after incubation of the cured hydrogel with hyaluronidase (HAase), a naturally occurring enzyme that cleaves the β-N-acetylhexosamine-[1→4] glycosidic bonds of HA (Table 4.2)

<table>
<thead>
<tr>
<th>Sample (µg/mL)</th>
<th>Experimental (µg/mL)</th>
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</thead>
<tbody>
<tr>
<td>2667 w/ HAase</td>
<td>2536 +/- 504</td>
</tr>
<tr>
<td>2667 w/out HAase</td>
<td>297 +/- 78</td>
</tr>
<tr>
<td>1333 w/ HAase</td>
<td>1300 +/- 200</td>
</tr>
<tr>
<td>1333 w/out HAase</td>
<td>294 +/- 42</td>
</tr>
</tbody>
</table>

Table 4.2 Comparison of initial sample GAG content and experimentally observed GAG content in solution after exposure to HAase
In aliquots of UV cured HAGM containing 2667 µg/mL and 1333 µg/mL of polymer (HA is a GAG), near equivalent amounts of GAGs are detected in solution after 48 hours incubation with HAase. All of the cured polymer degrades into solution, while much smaller concentrations are detected when HAase is not present. If implanted in vivo, HAGM encapsulating ASCs would be biodegradable due to the presence of HAase in body fluids.

To confirm chondrogenic differentiation in HA hydrogels and the formation of cartilage tissue, ASCs are cultured in densely seeded micromasses, which are optimal because they best mimic pre-chondrogenic cellular condensation in vivo.[101,109] Over the course of 11 days in culture, the sulfated glycosaminoglycan (s-GAG) content rises for ASCs cultured in HAGM gel as well as for ASCs cultured under standard micromass conditions (positive control) (Figure 4.5).

![Graph](image)

**Figure 4.5** s-GAG content in densely seeded ASC cultures over 11 days
s-GAGs are a primary matrix element of cartilage and indicate chondrogenic differentiation.[110] s-GAG content in ASCs in HAGM without chondrogenic media, the negative control, stay well below both the treatment and positive control over 11 days. There is no data point at Day 0 for the positive control, because there is no cell attachment immediately after plating. Error bars are high for early time points in Figure 4.5, which may be reduced in future experiments with increased protocol practice and sample size. After 11 days, immunohistochemistry (IHC) of the cultured aggregates is used to identify the presence of type II collagen, the predominant collagen fiber, and aggrecan, the predominant proteoglycan in cartilage tissue. Expression of collagen II and aggrecan in IHC is a visual confirmation of chondrogenic differentiation. ASCs cultured in HAGM (Figures 4.6c,d) and in the positive control (Figure 4.6a,b) both express fluorescence from antibodies stained against collagen II and aggrecan. No cell clusters are formed for adequate IHC staining in the negative controls.

Figure 4.6 IHC of cultured aggregates from ASCs cultured with chondrogenic media (a,b) and ASCs seeded in HA and cultured in chondrogenic media (c,d). Fluorescence is from Texas Red secondary antibodies bound to primary antibodies for (a,c) type II collagen and (b,d) aggrecan. (scale bars: 200µm)
The presence of aggrecan and collagen II and the increase in s-GAGs over 11 days of culture demonstrate that ASCs are undergoing chondrogenesis while cultured in high cell density (2×10^7 cells/mL) and that cartilage tissue is forming. Since the ASCs can be evenly distributed within degradable HAGM gels and HAGM-PEGDA scaffolds have a compressive modulus near that of native articular cartilage, a reasonable next step is the implantation of infused HAGM scaffolds in an in vivo model.

Prior to implantation in articular cartilage sites, we sought to confirm the biocompatible nature of the printed scaffolds and the ability to promote tissue growth in porcine mandibular bone defects, a simpler and more practiced procedure for our collaborators (Wheeler group) in Animal Sciences at UIUC. Both scaffold treatments and controls in 10mm defects on one mandible are treated with ASCs because of their demonstrated ability to promote increased levels of bone formation.[111] The pig experiences no adverse effects or setbacks from the implanted scaffolds during the 12 week period and is able to resume consumption of soft foods after just 24 hours. Computed tomography (CT) scans of the pig at 3 and 7 weeks show bone growth in the defects during the 4 week period (Figure 4.7), but no significant difference are noted between ASC treated (Figures 4.7 a,c) and non-ASC treated (Figures 4.7 b,d) defects.
Figure 4.7 Computed tomography (CT) scans of pig containing mandibles with implanted HAGM-PEGDA scaffolds at (a,b) 3 and (c,d) 7 weeks. (a,c) Left mandible contains defects treated with ASCs. (b,d) Right mandible defects had no ASCs.

When the mandibles are harvested after 12 weeks, micro-CT scans are possible on cut defects. Two defects experienced excellent healing, showing complete infilling of bone within the 10mm defect (Figures 4.8 a,d). No negative controls without scaffolds completely healed (Figures 4.8 c,f), though two negative controls were rejected from the results analysis, because they were drilled too far into the marrow space of the mandible. The stem cell rich environment of the marrow space and the widely varied structure of the bone in this region do not permit logical comparisons to be made to other defect sites. In two of the defects with scaffold treatments, bone formation was present, but complete healing did not occur (Figures 4.8 b,e). No significant differences between ASC treated (Figures 4.8 a-c) and non-ASC treated (Figures 4.8 d-f) can be determined from the micro-CT scans.
Bone growth in defect sites is quantified by bone mineral density (BMD) with dual energy x-ray absorptiometry (DXA). Representative DXA scans of a well healed (Figure 4.9a, top) and poorly healed (Figure 4.9b, bottom) are given, and surrounding healthy bone is used to produce a normalized BMD. Defects in which scaffolds are implanted have higher normalized BMDs (0.70 +/- 0.12 and 0.68 +/- 0.16) than non-scaffold treated defects (0.54 and 0.58) (Figure 3.9). Due to low sample size (n=2) and one defect in each non-scaffold treatment being removed from the analysis, no errors can be calculated for treatments without scaffolds. No significant differences are found between ASC and non-ASC treated defects. Increasing the number of pigs for future experiments will allow for increased statistical power and analysis.
Figure 4.9 DXA of bone defect sites after 12 weeks. (a) Representative images of well healed (top) and poorly healed (b) defects. (b) Normalized bone mineral densities (BMD) of defect sites.

4.4 Conclusions

We demonstrated the formation of cartilage on HAGM-PEGDA scaffolds seeded with ASCs pushed towards chondrogenic differentiation. Cartilage had more spread out morphologies on scaffolds versus controls and was able to best infiltrate scaffolds with the incorporation of an RGD peptide. The possibility of infusing scaffolds with cells encapsulated in a dilute HAGM hydrogel was explored for tissue engineering applications. Compressive moduli of printed HAGM-PEGDA scaffolds containing 2% HAGM nearly match that of native articular cartilage, and cells encapsulated in the HAGM were distributed evenly in 3D and underwent chondrogenesis to form cartilage tissue. HAGM-PEGDA scaffolds were implanted in vivo in a porcine model, were biocompatible, and permitted bone growth in defects. This initial in vivo experiment coupled with confirmed cartilage growth in HAGM to be used for encapsulation are promising preliminary findings and point to the need for future articular cartilage tissue regeneration experiments with cell infused HAGM scaffolds.
CHAPTER 5
CONCLUSIONS

3D scaffolds composed of hyaluronic acid, a natural, biocompatible, and biodegradable polysaccharide, were created using direct write-assembly for cell culture and tissue engineering applications. The mechanical properties of HAGM and HAGM-PEGDA scaffolds were explored, and their ability to promote cartilage growth from ASCs and serve as substrates for \textit{in vitro} and \textit{in vivo} implantations were studied. The main conclusions of the thesis are:

- Hyaluronic acid was functionalized with UV reactive glycidyl methacrylate at 8.2%, 19.8% and 29.8% degrees of methacrylation, as confirmed by NMR spectroscopy.
- Viscoelastic inks for direct-write assembly were formulated from 8.2% and 19.8% DM HAGM, PEGDA, glycerol, photoinitiator, and PBS. 3D hydrogel scaffolds were patterned with filament diameters ranging from 10\(\mu\)m to 250\(\mu\)m, and various pitch sizes, and overall dimensions. They were UV cured during printing to ensure long-term structural stability.
- The compressive moduli of printed HAGM-PEGDA scaffolds were related to their composition and 3D geometry. Specific parameters were identified that produced scaffolds with mechanical properties akin to native tissues of interest, i.e., cartilage.
- The injectable nature of HAGM-PEGDA scaffolds was demonstrated by flowing them through tapered microchannels, whose characteristic diameter was reduced by 16-fold to one-third of scaffold diameter, without evidence of structural damage.
- Degradation of HAGM scaffolds in hyaluronidase was shown to be affected by PEDGA concentration, allowing for possible tailoring of degradation times \textit{in vivo} by altering methacrylate cross-linker density.
- Cartilage growth on seeded HAGM scaffolds was shown by staining of glycosaminoglycans, and cartilage was better able to infiltrate 3D scaffold with incorporated cell-adhesive RGD peptide.
- ASCs were seeded in 3D 2% bulk HAGM as a method for even cell distribution in printed 3D HAGM-PEGDA scaffolds. Cells seeded in dilute HAGM demonstrated proliferation, mobility, and chondrogenic differentiation.
- HAGM-PEGDA 3D scaffolds were implanted in porcine mandibles and proved to be biocompatible and able to support growth of new bone.
REFERENCES


