HYALURONIC ACID-BASED BIOINKS FOR CELL-FRIENDLY BIOPRINTING

By

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David Ira Shreiber

And approved by

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ABSTRACT OF THE DISSERTATION

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by MADISON GODESKY

Dissertation Director:
David Ira Shreiber

Extracellular matrix (ECM) is an intricate network of proteins, sugars, and proteoglycans that provides critical signaling context to resident cells through mechanical and bioactive properties. As such, the independent control of these features is a frequent target in the development of biomaterials. This research investigates the development of a bioink system based on thiol-modified hyaluronic acid (HA-S) and polyethylene glycol diacrylate (PEGDA) for 3D bioprinting. The objectives of this work were to (1) develop a versatile approach to independently control the mechanical and bioadhesive features of HA-S-PEGDA across multiple time scales and (2) adapt the system to be amenable to 3D extrusion bioprinting.

To this end, we leveraged the distinct dual-crosslinking mechanism of HA-S-PEGDA to control the mechanical properties at different time points. Rheological studies confirmed that two crosslinking reactions occur in HA-S-PEGDA: (1) rapid crosslinking between HA-thiols and PEG-acrylates resulting in
gelation in minutes and (2) prolonged disulfide crosslinking, which dramatically stiffens the network over a period of days-to-weeks. Like native HA, HAS-PEGDA does not support the adhesion of most healthy adult human cells, but the thiol modification provides a convenient target to introduce bioactive ligands. We demonstrated that the steady-state stiffness of the network can be manipulated independently of the initial crosslinking reaction by targeting a percentage of HA-thiols with peptide-ligands or inert spacers. Moreover, we identified ranges in which the mechanical and bioactive properties can be co-modulated in HAS-PEGDA, and we validated the biological functionality in vitro using human mesenchymal stem cells and rat dermal fibroblasts.

To adapt the formulation for 3D cell culture, reaction templates were developed to prioritize bioactive peptide-grafting, initial gelation, latent crosslinking, and network degradation, across time scales of seconds, minutes-to-hours, days, and weeks, respectively. Finally, we demonstrated that the time-dependent rheological features of HAS-PEGDA can be leveraged to formulate printable bioinks for extrusion-based 3D bioprinting. By harnessing the inherent viscoelastic features of HA, we identified a window of printing conditions that resulted in excellent cell viability, mechanical recovery, resolution, and bioink tunability. Taken together, the results presented in this thesis establish a customizable bioink system based on thiol-modified hyaluronic acid for extrusion-based bioprinting.
To my dad, for convincing me to try one semester of engineering.
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CHAPTER 1: INTRODUCTION

1.1 Perspective

Bioengineered tissues offer significant therapeutic potential in regenerative medicine and value as in vitro tools for basic research, product development, and precision and personalized medicine, especially with the advent of 3D bioprinting. With a 3D printer, it is possible to build complex structures with micrometer-level spatial resolution from computer-generated patterns. However, 3D printing tissues requires materials that create an initial signal context for cells to maintain their programmed functions. Bioprinting offers the potential to recreate the complex, inhomogeneous features of native tissues, including the layers of human skin. One industry that is committed to realizing the potential of 3D bioprinting is the cosmetics industry.

The global cosmetics market is valued at an estimated $532B and growing faster than ever before. By 2023, the industry is expected to be worth $806B. Driven in part by globalization, millennials, and the rise of so-called “indie brands”, consumers have begun to push for personalization in skincare. To meet the demand, significant research is required to identify the biological differences that make skin of different tones, ages, and genders unique. In the meantime, both legacy and indie brands have capitalized on this push for
personalization, tapping into specific consumers through digital media and targeted messaging strategies.\textsuperscript{17} Sustainable growth in this space, however, requires consumer trust and therefore congruency between brand messaging and scientific development.\textsuperscript{7,9,18}

Today, the US is the world’s largest cosmetics market, and culturally, the American consumer landscape has begun to shift toward “radical transparency”.\textsuperscript{7,8} In cosmetics, this is exemplified by demands for “clean” ingredients, “sustainable” manufacturing, and “cruelty-free” development processes. In addition to consumers, activist investors have shaped the US market.\textsuperscript{7} In 2017, for example, one of the most well-known animal rights groups, People for the Ethical Treatment of Animals (PETA), acquired a share of Louis Vuitton Moët Hennessy (LVMH), one of the largest global conglomerates operating in the cosmetics space.\textsuperscript{19} Although a single share may seem inconsequential, the strategic move granted PETA access to LVMH’s shareholder meetings, where they have become an increasingly more powerful force behind cruelty-free movement in the cosmetics industry.

While the US government has remained relatively neutral on the issue of animal testing, nearly a decade ago, the EU banned cosmetics testing on animals in cases where alternative methods exist (2010/63/EU).\textsuperscript{20–23} Despite the federal government’s relative inaction, the US market has aligned to these standards through American consumers. For instance, in 2018, the people of California banned cosmetics testing on animals, aligning state laws with the cruelty-free mandates implemented across the EU and 10 additional
California Law SB-1249 will take effect on January 1, 2020, but in the meantime, at least four other states have passed similar legislation.

While there is no doubt that “millennials are obsessed with researching before buying”, as a recent McKinsey report said best, American consumers and activists tend to ignore the reality that technology-driven alternatives to cosmetics testing lag our cultural progression. Moreover, among the FDA, FTC, and FCC, the United States has a more complex regulatory landscape in comparison to the EU for claims made about cosmetics. As a result, new cosmetic products are largely tested on humans prior to launching in the US market. Complicating human testing further is the consumer-driven, industry-wide push for personalization. To represent the target end-user during clinical safety and efficacy testing, human subjects must be recruited on the basis of their ethnicities, genders, and ages, often times disproportionately to the general population for statistical power. For many reasons, human testing has significant risks, costs, and ethical and tactical challenges. Recently, leaders in the cosmetics industry have turned to bioengineered human skin as a technology-driven solution to this billion dollar problem.

Engineered models of skin fabricated from human cells should, at least in theory, recapitulate human physiology more accurately than animal models. Commercially available in vitro models, including MatTec EpiDerm™ and EpiSkin SkinEthic™ offer significant value for product development and screening compounds but do not replace the need for clinical testing on humans. To realize the potential of bioengineered skin and, ultimately, eliminate the need for
human test subjects, *in vitro* skin models should accurately recapitulate the biological features that make human skin unique.\(^{34,35}\) For example, SkinEthic\(^\text{TM}\) recently developed human skin reconstructions that contain pigments to model different Fitzpatrick phototypes. Although this is progress in terms of personalization, currently available *in vitro* models lack the specialized structures of the skin, which are critical to physiology and make us unique.\(^{4,36}\) (Figure 1.1)

### 1.2 Biology of human skin

Skin is a multi-layered protective barrier against external aggressors including UV light, reactive oxygen species, pollution, mechanical injury, pathogenic microbes, moisture loss, and extreme temperature.\(^{37}\) The epidermis is the thinnest, most superficial section of the cutaneous barrier. It is stratified into four layers and undergoes continuous renewal.\(^{11}\) At the cellular level, the epidermis is primarily composed of keratinocytes, which form morphologically distinct layers: the stratum corneum (SC), stratum granulosum, stratum spinosum, and stratum basale.\(^{37-39}\) As keratinocytes migrate from the basal layer outward toward the SC, they become more elongated in shape and differentiate into corneocytes.\(^{11}\) At the end of this migration process, corneocytes die, release proteins, ceramides, and lipids to form the semi-impenetrable barrier of the SC.\(^{12,40}\) A key function of the epidermis is to prevent trans-epidermal water loss, the extent to which influences hydration, penetration of topicals, biomechanical properties of the lower dermal layers, and outermost physical appearance.\(^{15}\)
Skin is a multi-layered protective barrier that undergoes continuous renewal. The epidermis is the thinnest, most superficial section and it is composed primarily of keratinocytes that are stratified into four distinct layers: the stratum corneum (SC), the granular layer (GL), the spiny layer (SL), and basale layer (BL). Between the BL and the dermal layer lies the dermal-epidermal junction (DEJ). The dermis is primarily composed of ECM, but also contains specialized structures of the skin including blood vessels, nerves, glands, and hair follicles.
Other cell types, including melanocytes, Merkel cells, Langerhans, and inflammatory cells, reside within the epidermis, although in fewer numbers than keratinocytes. In the basal layer of the epidermis, melanocytes produce melanin, pigments that protect tissues from photodamage. Melanin is transferred to keratinocytes throughout the upper layers of the epidermis in discrete packets. There are 3 basic types of melanin, and the relative ratios of these pigments and activity of melanocytes determine an individual’s skin color and response to sunlight. The production, transport, and breakdown of melanin is influenced by ethnicity, age, and gender.

Light exposure is believed to be the most significant factor that affects skin health across all populations. Damage from UV exposure is powerful and cumulative and is a significant risk factor to develop skin cancer. In addition to the development of skin cancer, UV light exposure also contributes to the visual signs of skin aging, premature degradation of ECM molecules, hyperpigmentation, and physical and mechanical changes in the epidermis and dermis. Although it is known that longer wavelengths of light penetrate skin more deeply, the role of visible light, specifically blue light, in photodamage remains unclear. Some studies suggest changes in the relative thicknesses of the dermis and epidermis due to pigmentation, but in general, results are incomplete and conflicting. Further research is required to elucidate the features that make human skin unique.

Below the epidermis, lies the dermis, a thicker section of skin composed of connective tissue, blood vessels, nerves, glands, and hair follicles. As an
avascular tissue, the epidermis relies on the dermis for nutrient transport, hydration, and cell-signaling cues. The dermis is primarily composed of extracellular matrix (ECM): an intricate network of bioactive molecules providing mechanical support and critical signaling context to resident cells. Fibroblasts not only produce, but also respond to, remodel, and regenerate dermal ECM. The dermis is divided into papillary and reticular sections. The papillary dermis is a looser meshwork of structural proteins, including type-I and type-III collagen, glycosaminoglycans, including hyaluronic acid, and other soluble signaling molecules, nutrients, and metabolites. The papillary dermis is distinct for its finger-like projections, known as dermal papillae, which increase surface area for nutrient transport across the dermal-epidermal junction (DEJ). The DEJ is a complex barrier to chemicals and cells, and provides mechanical support for adhesion between the dermis and epidermis. During the aging process, mechanical changes in the dermis, for example, due to glycation-mediated collagen crosslinking or UV-mediated stiffening, can stimulate compaction by resident fibroblasts. Compaction progressively flattens the dermal papillae, limiting nutrient transport into the avascular epidermis, and influences skin’s outermost appearance.

The reticular dermis is situated below the papillary dermis and is composed of a dense, irregular matrix of structural proteins including collagen and elastin fibers. The reticular dermis is anchored to loose, areolar, connective and adipose tissue, known as the hypodermis. At the cellular level, hypodermal connective tissue consists of an extensive, interconnected network of
mesenchymal stem cells, neurons, fibroblasts, and endothelial cells, all of which have well-documented responses to mechanical stimuli.\textsuperscript{53,64,65} Human mesenchymal stem cells (hMSCs) produce a wide variety of cell-signaling molecules, including inflammatory mediators, growth factors, pro-regenerative cytokines, and ECM components under applied mechanical loads.\textsuperscript{65–71} Although a common target for regenerative medicine, the role of hMSCs in determining biological differences of skin, specifically in the context of neurogenic inflammatory diseases, including rosacea, remain unclear.\textsuperscript{68,72–75}

Despite the hypodermis being a rich source of hMSCs, the human hair follicle is a more well-established dermal stem cell niche.\textsuperscript{36,40,72,76} The pilosebaceous unit is a specialized structure of human skin composed of the hair follicle (HF), sebaceous gland, arrector pili muscle, and the stem cell bulge.\textsuperscript{36,76} (Figure 1.1) In addition to hair cycling, HF stem cells in the bulge provide signaling cues for regeneration in the surrounding niche.\textsuperscript{40} Interestingly, UV light serves as a negative regulator for proliferation of certain populations of human stem cells in the HF and surrounding niche.\textsuperscript{76} Although this research is emerging, it is hypothesized that cell renewal at night offers a competitive advantage as it decouples DNA replication from exposure to UV and ROS.\textsuperscript{40} Previous research established cross-talk among signaling cues in the HF and regeneration in the surrounding niche.\textsuperscript{36,40,76} For example, empty hair follicles can recruit and de-differentiate interfollicular stem cells in the dermis and epidermis through physical and chemical signaling cues.\textsuperscript{77} Although feedback among light-mediated signaling and melanocyte, fibroblast, and dermal papillary function have been
established, further research is required to recapitulate the HF signaling niche in human skin equivalents. The most obvious differences in human skin with respect to ethnic, age, and gender identities are in differential responses to sunlight. To adequately model human skin regeneration, the precise biological features that make human skin types and tones distinct should be captured.

1.3 Bioprinting human skin

To realize the potential of bioengineered skin and, ultimately, eliminate the need to test new cosmetics products on human subjects, the specialized structures of human skin and surrounding signaling niche must be modeled.\textsuperscript{34,35} Bioprinting is a computer-based additive manufacturing approach that relies on the precise 3D positioning of cells to build complex tissue structures, often with the aid of biomaterials.\textsuperscript{5} Layer-by-layer, cells, biomaterials, and bioactive molecules can be specifically positioned to create the precise anisotropic features of native tissues. Extrusion-based bioprinting is one of the most accessible, customizable, and widely used biofabrication approaches, and it involves extruding cells through nozzles of 100-1000 µm in diameter during the printing process.\textsuperscript{78} Although one of the greatest limitations to extrusion-based bioprinting is the magnitude of shear force on cells at the nozzle,\textsuperscript{79,80} by harnessing viscoelasticity and deformation, hydrogel-forming bioinks offer the potential to dissipate shear forces and print filaments that are finer than the nozzle diameter.\textsuperscript{81}
1.4 Bioinks for extrusion bioprinting

1.4.1 Definition

Bioinks are formulations of cells that are suitable for automated processing and typically include biomaterials and other actives to facilitate fabrication.\textsuperscript{82} To develop workable bioinks, hydrogel precursors are typically included in formulations for their ability to flow as fluids during processing, but provide structural integrity upon gelation.\textsuperscript{5} There are many considerations in the translation of a hydrogel system into a bioink, but in general, \textit{printability} is determined by the quality of printed constructs and biocompatibility.\textsuperscript{80,83,84} Although, by definition, cells are the essential component of a “bioink”,\textsuperscript{82} biomaterial ink components such as polymers, hydrogel precursors, biologics, and other actives provide critical signaling context for cells to maintain their programmed functions and coordinate the formation of tissues.

1.4.2 Bioinks based on native ECM

1.4.2.1 Recapitulating specific adhesive properties

In native tissue, ECM is not a continuum of properties, but a dynamic network in which both mechanical and bioactive properties work in concert to regulate cell fate.\textsuperscript{37,85} ECM spatially guides cell function through specific presentations of mechanical and adhesive properties. Far from static, adhesive interactions generate traction forces that act on the cytoskeleton to regulate gene
expression and on the matrix, providing feedback at multiple time scales.\textsuperscript{85–87} At the tissue level, local variations in matrix traction influence a variety of cellular behaviors including adhesion, migration, and differentiation.\textsuperscript{66,88,89} To recapitulate the complex signaling role of ECM, bioinks should allow for stiffness and adhesive properties to be independently controlled.

1.4.2.2 Non-cell-adhesive ECM materials

While adhesive ligands are critical to the regulatory functions of most ECM molecules, they also complicate the use of ECM or ECM derivatives for bioprinting. From an engineering perspective, non-cell-adhesive materials are advantageous to pattern specific ECM features while limiting non-specific cellular interactions\textsuperscript{90}, which precludes most native biomaterials. One notable exception is hyaluronic acid (HA).\textsuperscript{66,91–95} HA is a non-cell-adhesive glycosaminoglycan (GAG) that is ubiquitous in native connective tissues, including human dermal tissue. Structurally, HA is one of the simplest ECM components: it is a linear, unbranched, and regularly repeating glycosaminoglycan (GAG). \textsuperscript{(Figure 1.2)} Unlike other GAGs, HA lacks thiol-functionality and covalent linkages to specific core proteins.\textsuperscript{96} Furthermore, since HA’s GAG chain is negatively charged, it attracts cations and water and swells to fill large volumes.\textsuperscript{96} HA’s water content also enables it to resist compaction forces from cell movement\textsuperscript{54,96} and thus maintain tissue shape as cells exert traction on the matrix.
HA is a native ECM glycosaminoglycan (GAG) composed of up to n=25,000 repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine. Under physiological conditions, carboxylic acid groups become negatively charged, and result in a polyanionic HA polymer chain. Unlike other GAGs, native HA is non-sulfated, and it does not form proteoglycans.
1.4.3 HA-based biomaterials

Although HA interacts non-covalently with other components of the ECM and plays an important role in stabilizing the matrix, it does not independently assemble into higher-order molecular structures, such as collagen or fibronectin. To reinforce the mechanical properties of hyaluronic acid several crosslinking strategies have been explored.

1.4.3.1 Methacrylate-modified hyaluronic acid

One approach to form HA hydrogels is through UV-mediated crosslinking. Methacrylate modifications to HA have been widely explored as a mechanism to introduce photoreactive sites to the HA backbone. Methacrylate-modified hyaluronic acid (HA-MA) can undergo free radical polymerization when exposed to photoinitiator, such as Irgacure, and UV light. In addition to various uses in regenerative medicine, previous research by Skardal et al. demonstrated that HA-MA offers a key advantage as a bioink for 3D printing for its rapid curing time. Photocrosslinking enables a nearly instantaneous transition between solution and gel states during bioprinting, and methacrylate modifications offer sites to introduce other modified ECM materials for cell adhesion, including collagen methacrylamide. From an engineering perspective, there are many advantages to photocrosslinkable bioink systems, however, the key limitation to this curing approach are the effects that UV light and free radical damage can have on stem cells. In addition to introducing
radical-mediated DNA aberrations, UV light is an important stimulus for photoreactive tissues, including skin.\textsuperscript{40,41}

\subsection{1.4.3.2 Thiol-modified hyaluronic acid}

In addition to HA-MA, thiol-modified HA (HA-S) has been explored as a more cell-friendly derivative of hyaluronic acid for regenerative medicine.\textsuperscript{95,104,108,109} HA-S crosslinked with polyethylene glycol diacrylate (PEGDA) is a hydrogel-forming biomaterial that presents many of the attractive characteristics of native HA with a convenient mechanism to improve its mechanical properties.\textsuperscript{94} In contrast to many hydrogel systems that rely on a single gelation reaction, Ghosh et al. demonstrated that the mechanical properties of HAS-PEGDA are dictated by two crosslinking reactions that occur at different time points.\textsuperscript{108} The first crosslinking mechanism is a nucleophilic, Michael-type addition reaction between HA-thiols and PEG-acrylates and results in the material’s gelation in minutes.\textsuperscript{110} After initial gelation, remaining thiols on HA can then form disulfide crosslinks upon oxidation.\textsuperscript{94} Although this material has a useful crosslinking scheme, the non-cell-adhesive properties of HA-S and PEGDA hydrogels present a challenge for cultured cells.\textsuperscript{91}

To introduce sites for cellular adhesion and degradation, previous research by Prestwich et al. demonstrated that thiol-modified gelatin (gel-S) can be included in the hydrogel network to enable cell growth.\textsuperscript{95,111} Murphy et al. evaluated HA-S, gel-S, and PEGDA hydrogels, among other ECM derivative materials, as bioinks for 3D printing and demonstrated excellent printability of the
HA-S and gel-S system. Although these initial results offer significant value in terms of screening candidate bioink materials, a limitation of this research is that it does not leverage the full extent to which non-cell-adhesive HA-S hydrogels can be programmed to influence cell-matrix interactions. Gel-S is a material with widespread adhesive properties in native tissues, and as such, this template does not allow for matrix adhesivity to be programmed to specific cell types of interest. Moreover, since gel-S contributes to the crosslinking density of the network, the resultant stiffness of the bioink would be affected over multiple time scales by including the thiol-functionalized adhesive component.

### 1.5 Thesis summary

To realize the potential of 3D printing personalized tissues for regenerative medicine, bioinks should allow for the independent control of mechanical and bioactive features across multiple time scales. This thesis investigates the development of a bioink based on HA-S, PEGDA, and functionalized, specific peptide-ligands. (Figure 1.3) The mechanical properties of HAS-PEGDA are dictated by two cytocompatible crosslinking reactions that occur at distinct time points: a rapid, Michael-type nucleophilic addition reaction between HA-thiols and PEG-acrylates; and a prolonged maturation of disulfide crosslinks from remaining thiols. (Scheme 1.1) We hypothesized that these reactions would enable the independent tuning of the mechanical and bioactive features of HAS-PEGDA.
across multiple time scales: initially to the 3D printing process and overtime to tune cell-matrix interactions.

Like traditional printer inks that rely on four pigments to produce the entire color spectrum, the goal of this thesis is to establish a simple bioink system to program a spectrum of signal conditions in printed ECM. In Chapter 2, we establish the proof-of-principle to demonstrate that the HAS-PEGDA system can be used to independently control stiffness and specific bioadhesivity and these features can be tuned to influence dermal fibroblast behavior. In Chapter 3, we address the challenges with this material to include cells in 3D. In Chapter 4, we introduce an approach for additive bioprinting and identify the strengths and weakness of this material as a bioink. Finally, we close with an overall discussion of the thesis research and propose opportunities for future development.
FIGURE 1.3 THIOL-MODIFIED HA (HA-S) CHEMICAL STRUCTURE

Introducing thiol-modifications into the HA polymer backbone maintains many of the attractive characteristics of native HA, while providing convenient targets to enhance the mechanical properties and bioactive properties of the material.
SCHEME 1.1 HAS-PEGDA HYDROGEL CROSSLINKING CHEMISTRY

A. HA-thiols and PEG-acrylates react rapidly through Michael-type nucleophilic addition reactions. Double end anchorage of PEGDA crosslinks HA-S and forms a hydrogel network. B. Following initial gelation, remaining thiols on HA backbone can spontaneously oxidize and form latent disulfide crosslinks.
CHAPTER 2: HYALURONIC ACID-BASED HYDROGELS WITH INDEPENDENTLY TUNABLE MECHANICAL AND BIOACTIVE SIGNALING FEATURES

Note: This chapter is reproduced from the following publication:


2.1 Introduction

Bioengineered tissues offer significant therapeutic potential in regenerative medicine and value as in vitro tools for basic research, product development, and precision and personalized medicine, especially with the advent of 3D bioprinting. However, bioengineered tissues require materials to create an initial signal context for cells to maintain their programmed functions and coordinate the formation of tissues. One promising source or inspiration for materials is extracellular matrix (ECM).

During tissue development and regeneration, cell functions are coordinated through series of fate decisions directed in part by matrix-bound guidance signals. Cells respond to ligands presented in the ECM according to
the type, number, and distribution of receptors they express. Inside the cell, receptor-ligand interactions trigger signal cascades that can regulate gene expression and integrate multiple external cues into a single programmed response. At the tissue level, ECM-bound ligands serve as positional controls and influence tissue shape as their presentations change over time. For instance, the density and specificity of adhesive ligands can recruit certain cell types to precise spatial locations. Additionally, in the absence of adhesive interactions, anchorage-dependent signaling can trigger apoptosis, ensuring only specific cell types can survive in particular anatomical positions.

In addition to ligand-initiated molecular signaling, adhesive complexes transmit mechanical forces between cells and the ECM. Cells generate traction forces between membrane integrins and adhesive ligands as they move. Depending on the stiffness of the ECM and the distribution of adhesion sites, traction forces differentially regulate cell shape and gene expression. Through mechanotransduction, cells interpret changes in matrix stiffness as alterations in adhesive ligand density. Mechanical forces not only act on cells, but they also reorganize the matrix. Through remodeling, ECM can propagate signals from cell-to-cell, dictate tissue orientation, and provide feedback across multiple time and length scales.

While adhesive ligands are critical to the regulatory functions of most ECM molecules, they also complicate the use of ECM or ECM derivatives for tissue engineering. From an engineering perspective, non-cell-adhesive materials are
advantageous to pattern specific ECM features while limiting non-specific cellular interactions,\textsuperscript{90} which precludes most native biomaterials. One notable exception is hyaluronic acid (HA).\textsuperscript{66,91–95} Structurally, HA is one of the simplest ECM components; it is a linear, unbranched, and regularly repeating glycosaminoglycan (GAG). Unlike other GAGs, HA lacks thiol-functionality and covalent linkages to specific core proteins.\textsuperscript{96} Since HA’s GAG chain is negatively charged, it attracts cations and water and swells to fill large volumes.\textsuperscript{96} During morphogenesis, for example, the swelling of HA forces physical changes to the shape and structure of developing tissues. Stem cells produce HA to create open, hydrated, and cell-free spaces for specific progenitors to infiltrate.\textsuperscript{37} HA’s water content also enables it to resist compaction forces from cell movement\textsuperscript{96,124} and thus maintain tissue shape as cells migrate. Once migration ends, such as in the formation of the heart, cornea, and several other organs,\textsuperscript{37} cells degrade HA with hyaluronidase to create space for new ECM molecules.\textsuperscript{91,92,119}

Although native HA has many advantages, bioengineering applications have been restricted because of its weak mechanical properties when isolated from the rest of the ECM.\textsuperscript{94,108} Thiol-modified hyaluronic acid (HA-S) crosslinked with polyethylene glycol diacrylate (PEGDA) is a hydrogel-forming biomaterial that presents many of the attractive characteristics of native HA with a convenient mechanism to improve its mechanical properties.\textsuperscript{94} In contrast to many hydrogel systems that rely on a single gelation reaction, the mechanical properties of HAS-PEGDA are dictated by two crosslinking reactions that occur at different time points.\textsuperscript{108} The first crosslinking mechanism is a nucleophilic,
Michael-type addition reaction between HA-thiols and PEG-acrylates and results in the material’s gelation in minutes. After initial gelation, the remaining thiols on HA can then form disulfide crosslinks upon oxidation. Although disulfide crosslinking is considerably slower than initial gelation, it dramatically stiffens the hydrogel over time. Furthermore, HA-thiols provide convenient targets to introduce bioadhesive ligands. However, consuming thiols will naturally affect the resultant stiffness of the network.

In the current study, we investigate several strategies to decouple and tune the stiffness and adhesive ligand conditions presented in HAS-PEGDA hydrogels. By leveraging the dual crosslinking mechanism, we establish a system to independently control the mechanical and bioactive features across multiple time scales. (Scheme 2.1) Enabling control over stiffness and adhesion would have great value for applications across tissue engineering and regenerative medicine, especially in 3D bioprinting, where different inks may be combined at specific locations to spatially control tissue composition and behavior.
Leveraging the dual-crosslinking system, the HAS-PEGDA hydrogel system offers the potential to control the mechanical properties across multiple time scales by selectively occupying free-thiol sites, which would otherwise form latent disulfide crosslinks.

**SCHEME 2.1 PROPOSED HYDROGEL SYSTEM**

Leveraging the dual-crosslinking system, the HAS-PEGDA hydrogel system offers the potential to control the mechanical properties across multiple time scales by selectively occupying free-thiol sites, which would otherwise form latent disulfide crosslinks.
2.2 Methods

2.2.1 Rheology

Hydrogel samples were prepared in Petri dishes, and the rheological properties were measured over long curing times using a Kinexus Ultra Rotational Rheometer (Malvern Panalytical Ltd., Malvern, UK) equipped with a 3D-printed lower geometry and 20mm parallel upper plate. HA-S (Glycosil®, BioTime, Inc., Alameda, CA) and PEGDA (MW 3.4kDa, Laysan Bio Inc., Arab, AL) were purchased commercially, and stock solutions containing 1.25% HA-S and 4% PEGDA (%w/v) were prepared according to the manufacturer’s instructions. Stock solutions of HA-S and PEGDA were diluted serially and mixed in 4:1 volumetric ratios to achieve the indicated final concentrations of HA-S (0.8% or 1.0%) and PEGDA (0.2% or 0.6%). Circular hydrogel samples of 0.2mL HAS-PEGDA were formed in Petri dishes containing annular molds of PDMS (id=20mm), covered with 0.01 M phosphate buffered saline solution (1x PBS) after 2 hours of gelation, and incubated at 37°C. Gels were removed from the incubator periodically to assess the shear storage modulus \(G'\) by exposing the gels to an oscillatory shear strain of 0.5% at a frequency of 1 rad/s. Unless otherwise stated, results are presented as mean ± SD of n≥4 sample replicates.
2.2.2 Tuning the rate of latent crosslinking

To accelerate latent crosslinking, dimethyl sulfoxide (DMSO), a mild oxidant\textsuperscript{126,127} was used. Briefly, hydrogel samples of 0.8% or 1.0% HA-S and various concentrations of PEGDA crosslinker (0.2-1.0%) were prepared, and $G'$ was measured after 1 day of gelation as described above. After initial testing, the samples were covered with 0.01M phosphate buffer containing 20% DMSO (%v/v) and incubated at 37°C and 5% CO\textsubscript{2}. After 3 days of thiol-oxidation, rheological measurements were repeated. Significant differences in $G'$ at steady-state between gels that had been oxidized for 3 days with the oxidation medium or 30 days with ambient oxygen were identified with t-tests (p<0.05).

2.2.3 Tuning the extent of latent crosslinking

Hydrogels containing 0.8% HA-S and 0.2% PEGDA were prepared as described above and allowed to mature for 1 day of gelation. To selectively block latent stiffening, solutions of 1 mL PEG-monoacrylate (PEGMA) (MW 4.8 kDa, Laysan Bio, Inc., Arab, AL) diluted in PBS to 0.05, 0.1, 0.2, 0.3, 0.6, 1.2, or 12 mM were diffused through the 0.2 mL hydrogels. After 2 days of reaction, PEGMA solutions were replaced with oxidation buffer containing 20% DMSO, and the rheological properties were measured at steady-state. For simplicity, the total amount of PEGMA applied to each hydrogel condition (µmol) is reported as a relative concentration with respect to the volume of the sample (0.2 mL).
In separate experiments, fluorescein-o-acrylate, a thiol-reactive and fluorescent molecule, (Millipore Sigma, St. Louis, MO) was incorporated in place of PEGMA to confirm that the changes in mechanical properties were due to the consumption of free thiols. The mechanical properties of these gels were assessed as described above. In addition, relative fluorescence intensities ($\lambda_{ex}/\lambda_{em}=490/520$ nm) of separate gels that had been cured in 96-well plates (50 $\mu$L/well) were detected with an Infinite® 200 PRO microplate reader (Tecan Group Ltd., Männedorf, CH) after a series of 10 washes with PBS (250 $\mu$L/well) at room temperature for a minimum of 1 hour per wash to remove unbound dye.

2.2.4 Peptide functionalization

A fibronectin-derived bioactive adhesive peptide (GRGDS) and an inactive reverse sequence (SDGRG) were purchased commercially (Bachem Americas, Inc., Torrance, CA) and functionalized with acrylate reactive groups using acrylate-PEG-succinimidyl valerate (PEGMA-SVA) (MW 3.4kDa, Laysan Bio, Inc., Arab, AL) as a molecular scaffold.\textsuperscript{128} (Scheme 2.2) To conjugate the peptides to PEGMA, N-terminal primary amines were reacted with PEGMA-SVA in a 5:4 molar ratio ($\text{NH}_2$:SVA) in 0.1M PBS at pH 8.0. After 4 hours of reaction, conjugation efficiencies were estimated with fluorescamine (MilliporeSigma, St. Louis, MO) to compare primary amine content before and after the reactions.\textsuperscript{129,130} The PEGMA-peptide conjugates were then purified with centrifugal filter units (Amicon™ Ultra-15 3kDa MWCO, Millipore Sigma, St.
Louis, MO) against picopure water, according to the manufacturer’s instructions. Purified PEGMA-peptide solutions were frozen at -80°C and lyophilized to dryness. The lyophilized products, PEGMA-GRGDS and PEGMA-SDGRG, were stored at -20°C under argon until use.

2.2.5 Cell studies

Hydrogels containing 0.8% HA-S and various concentrations of PEGDA (0.2, 0.4, 0.6, or 0.8%) were prepared in 96-well tissue culture plates (50 µL/well). After 1 day of gelation, solutions of 7.5 mM PEGMA-peptide were applied to the gels (50 µL/well) to quench remaining HA-thiols without perturbing the hydrogels’ initial gelation. The ratios of bioactive ligands (PEGMA-GRGDS) and inactive, negative control sequences (PEGMA-SDGRG) were altered to manipulate bioactivity independently of the steady-state crosslinking density. In each solution, the total concentration of peptide (PEGMA-GRGDS + PEGMA-SDGRG) was provided in excess of remaining HA-thiols to block latent stiffening. After 2 days of reaction while rotating, peptide solutions were aspirated, and the gels were washed thoroughly with culture media to remove any unbound ligand, as described above. Type-I bovine collagen hydrogels (Elastin Products Company, Owensville, MO) were prepared at 2.0 mg/mL as previously described \(^{51}\) and served as positive controls for adhesivity.
Rat dermal fibroblasts (RDFs) from transgenic rat pups that constitutively express green fluorescent protein (GFP) were isolated and expanded in tissue culture using complete media: DMEM (MilliporeSigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 1% penicillin-streptomycin, and 1% L-glutamine. At approximately 90% confluency, RDFs were detached with trypsin-EDTA, pelleted, and re-suspended to 25,000 cells/mL in culture media. Cells were seeded on all hydrogels using an automated, 96-channel, high-throughput transfer pipet (VIAFLO 96, Integra Biosciences Corp., Hudson, NH) to achieve a uniform final seeding density of 5,000 cells/well. RDFs were incubated at 37°C and 5% CO₂ prior to adhesion and spreading assays.
Succinimidyl valerate (SVA) is an activated NHS ester that reacts rapidly with primary amines to form stable amide bonds under alkaline conditions. Since unless otherwise modified, every peptide contains a primary amine at its N-terminus, PEGMA-SVA serves as a versatile molecular scaffold to functionalize peptides with thiol-reactive groups. To conjugate PEGMA-SVA to N-terminal amines, 25 µmol of peptide was reacted with 20 µmol of PEGMA-SVA in 5mL of 0.15M, pH 8.0 PBS buffer. The reaction solution was protected from light and rotated overnight at room temperature.
2.2.6 Adhesion and spreading assays

After 2 hours of incubation, cell suspensions were aspirated from the gels, and non-adherent cells were removed with two additional washes with culture media. Concentrations of live, adherent cells were estimated with fluorescence intensity measurements ($\lambda_{ex}/\lambda_{em} = 488/520$ nm) of constitutively expressed GFP. Cells were then incubated overnight to allow for spreading to occur. After 1 day in vitro, adherent RDFs were fixed with 4% paraformaldehyde and permeabilized with Triton-X. Nuclei were stained with DAPI, and F-actin was stained with TRITC-phalloidin (MilliporeSigma, St. Louis, MO). Fluorescence images in each of the three channels were taken with a 10X objective lens from at least three positions in each well using an Olympus IX81 inverted microscope (Olympus, Melville, NY). To determine the number of cells, DAPI-labeled nuclei were identified and counted using the built-in cell counter tool in FIJI (FIJI is just ImageJ, Dresden, DE). To evaluate cell spreading, images of RDF cell bodies (GFP channel) were converted to binary image masks by applying an Otsu threshold filter, and the confluency in each image was estimated with the built-in particle analysis function. The average cell spreading area in each image field ($\mu m^2$/cell) was estimated by normalizing the percent confluency (GFP channel) to the corresponding cell count (DAPI channel) for each image, then scaling this ratio to the image area ($\mu m^2$). Cell morphologies were classified as round, partially spread, or spread for areas of 250-500 $\mu m^2$, 500-750 $\mu m^2$, or >750 $\mu m^2$, respectively.
2.3 Results and Discussion

2.3.1 Mechanical properties evolve across multiple time scales

To characterize the dual crosslinking system, rheological properties were monitored over long curing times in hydrogels containing various amounts of HA-S and PEGDA. In all hydrogel formulation conditions studied, HA-thiols (0.9 µmol thiol/mg HA-S) were presented in molar excess of PEG-acrylates (0.6 µmol acrylate/mg PEGDA). Consistent with previous reports,\textsuperscript{108,110,111} rheological studies confirmed that: gelation occurs in minutes; the initial crosslinking reaction plateaus by 1 day of gelation; and latent disulfide bonding dramatically stiffens the material over time (Figure 2.1). In addition, at 1 day of gelation, $G'$ is primarily controlled by the concentration of PEGDA (Figure 2.1) and thus the extent of thiol-acrylate crosslinking.\textsuperscript{96,108,132} For instance, in gels with the same HA-S concentration (0.8%), $G'$ after 1 day is significantly greater in gels crosslinked with 0.6% PEGDA than in gels crosslinked with 0.2% PEGDA ($p=0.001$, t-test). Moreover, in gels with the same PEGDA concentration (0.2%), $G'$ after 1 day is not significantly different for gels with 0.8% HA-S or 1.0% HA-S ($p=0.765$, t-test). Once disulfide crosslinking occurs, however, $G'$ is significantly greater in hydrogels containing 1.0% HA-S than in samples containing 0.8% HA-S ($p<0.001$, t-test). The results also suggest that disulfide crosslinking evolves to greater levels and over longer time scales than previously reported.\textsuperscript{108,111}
2.3.2 Latent disulfide crosslinking can be accelerated with DMSO

The 30-day time frame for disulfide crosslinking to contribute significantly to the steady-state mechanical properties is far from ideal for practical applications in the laboratory or clinic. To leverage the potential of this system, and guided by the literature, we explored a number of materials that could accelerate disulfide crosslinking in HAS-PEGDA, including cysteine- and serum-free DMEM, trace amounts of H₂O₂, oxidized glutathione, and DMSO. In general, we found thiol oxidation in the presence of 20% DMSO is rapid, relatively mild, and produces consistent crosslinking results. Figure 2.2 demonstrates that G' is nearly identical for gels that were matured for 30 days in PBS or for 3 days in 20% DMSO.
FIGURE 2.1 TWO CROSSSLINKING REACTIONS, OCCURRING AT DISTINCT TIMEPOINTS, DICTATE THE RHEOLOGICAL PROPERTIES OF HAS-PEGDA

The initial gelation reaction between HA-thiols and PEG-acrylates plateaus before 1 day of gel maturation, and over a period of days-to-weeks, HA-thiols undergo secondary disulfide crosslinking. Although disulfide crosslinking matures slowly in PBS, it dramatically stiffens the material over time.
FIGURE 2.2 THE RATE OF SECONDARY DISULFIDE CROSSLINKING CAN BE ACCELERATED WITH DIMETHYL SULFOXIDE (DMSO)

$G'$ at steady-state is not significantly different when thiols mature in PBS for 30 days or in 20% DMSO for 3 days ($p>0.75$, t-tests).
2.3.3 **Mechanical stiffness is controllable at multiple time points**

With a convenient mechanism to accelerate gel maturation, we next examined how the two crosslinking mechanisms could be targeted independently. In agreement with previous reports, G’ is nearly linearly proportional to the PEGDA concentration (R²=0.989) after 1 day of gelation (Figure 2.3). However, at steady-state, G’ is not significantly different among gels of varying concentrations of PEGDA (p=0.449, one-way ANOVA). As Figure 2.3 demonstrates, hydrogels of 0.4%, 0.6%, 0.8%, and 1.0% PEGDA have relatively constant moduli at steady-state, and G’ is only slightly lower in the 0.2% PEGDA formulation condition. These findings suggest that PEGDA controls the initial stiffness of the material, but at steady-state, the mechanical properties are less tunable than previously reported. Perhaps compounding these differences, we also found that the concentration of PEGDA influences the rate of latent stiffening; disulfides mature more rapidly in gels with higher concentrations of PEGDA. Therefore, at time points prior to full equilibrium, differences in G’ are exaggerated by the gels’ differential rates of disulfide crosslinking.

Over time, disulfide bonding significantly increases the stiffness of the hydrogels and offers a broader target range than crosslinking with PEGDA (Figure 2.3). Therefore, for greater control over the equilibrium mechanical properties, we tuned the extent of latent stiffening by introducing PEGMA to occupy the sites that would otherwise form disulfide crosslinks (Figure 2.4). When PEGMA is presented in excess of remaining HA-thiols, the steady-state
stiffness ($G' = 141 \pm 7 \text{ Pa}$) is nearly equivalent to hydrogel stiffness on 1 day of gelation ($p=0.8267$, t-test), indicating that latent disulfide crosslinking can be completely blocked. In the same time frame, negative control gels, which were not grafted with PEGMA (0mM) had significantly higher moduli ($G' = 812 \pm 145 \text{ Pa}$, $p=0.0013$) than gels quenched with PEGMA in excess. These results indicate that the steady-state mechanical properties can be tuned independently of initial gelation by grafting specific concentrations of thiol-reactive molecules after 1 day of gel maturation.

Similar to the rate trends discussed above with PEGDA, we also observed faster rates of disulfide crosslinking when PEGMA was incorporated into the network. Previous studies by Ghosh, et al., report an apparent “zipping effect”\textsuperscript{108} in which the authors suggest that PEGDA concentration controls the relative proximities of free-thiols in the hydrogel network and thus their tendency to form disulfide crosslinks. In addition to a possible proximity effect, our findings suggest that the PEG chain, itself, may accelerate disulfide bonding independently of the initial crosslinking density that is driven by PEGDA. Although the mechanism by which this occurs is out of the scope of this work, non-covalent interactions are known to influence the rate of disulfide bond formation.\textsuperscript{47,134,137,138} In preliminary studies, we observed faster rates of disulfide crosslinking in conditions that lowered the strength of intermolecular forces. Latent stiffening occurred faster in gels hydrated with phosphate buffer (PB) than with PBS, in buffer solutions containing lower salt concentrations (constant pH), and in gels of higher
concentration or molecular weight PEG. Collectively, these observations suggest that electrostatic effects may influence the rate of latent stiffening in HAS-PEGDA, for example, by lowering the energy barrier to disulfide bonding.\textsuperscript{47,134,137}

When fluorescein-o-acrylate was conjugated to the hydrogels in place of PEGMA to block latent disulfide crosslinking, G′ at steady-state also decreased in a dose-dependent manner (Figure 2.4). In addition, the concentration of residual, bound fluorescein was inversely related to G′ when HA-thiols (6 mM) were in molar excess of fluorescein-acrylates. The influence of the fluorescein-o-acrylate appeared to saturate around 7.5 mM, where both the residual fluorescence intensity and equilibrium stiffness plateaued (Figure 2.5). Taken together, these findings indicate that the equilibrium stiffness can be tuned independently of initial gelation while concurrently tethering functional molecules to the hydrogel.
The concentration of PEGDA crosslinker controls G’ after 1 day of gelation, but at steady-state, G’ depends on both the concentration of PEGDA and the availability of HA-thiols. After 1 day of gel maturation, G’ is directly proportional to the concentration of PEGDA \((R^2=0.989)\) when HA-thiols (7.2 mM) are in molar excess of PEG-acrylates (0.6µmol/mg of PEGDA). At steady state, G’ is not significantly different among the different concentration conditions of PEGDA \((p=0.449, \text{ ordinary one-way ANOVA})\) at steady-state.
FIGURE 2.4 THE EXTENT OF LATENT STIFFENING CAN BE CONTROLLED BY GRAFTING PEGMA TO HA-THIOLS AFTER 1 DAY OF GELATION

$G'$ at steady-state inversely relates to the concentration of PEGMA diffused into the hydrogel on 1 day of gelation. When PEGMA is presented in 10x molar excess (60 mM) of remaining HA-thiols (6 mM), $G'$ at steady-state is nearly identical to $G'$ after 1 day of gelation, indicating that disulfide crosslinking is completely blocked.
FIGURE 2.5 ACRYLATE-FUNCTIONALIZED MOLECULES GRAFTED TO HAS-PEGDA SELECTIVELY DISRUPT LATENT STIFFENING

The residual fluorescence intensities ($\lambda_{\text{ex}} = 490 \text{ nm} ; \lambda_{\text{em}} = 520 \text{ nm}$) of hydrogels grafted with fluorescein-o-acrylate inversely relate to $G'$ at steady-state, demonstrating that the final stiffness can be controlled independently of initial gelation while binding functional molecules to the network.
2.3.4 **Introducing controlled bioactivity**

Without the addition of peptide-ligands, HAS-PEGDA is non-cell-adhesive.\textsuperscript{54,93–95,101} Dermal fibroblast adhesion was used as a proof-of-concept to validate that specific adhesivity could be introduced into the hydrogels (Figure 2.6). Various ratios of the functional adhesive ligand (PEGMA-GRGDS) and inactive control peptide (PEGMA-SDGRG) were grafted to tune adhesion at a time point in between the two crosslinking reactions. In all conditions, the total concentration of PEGMA-peptide grafted to the hydrogels after 1 day of gelation was maintained in excess of remaining HA-thiols to completely block latent stiffening. For gels of constant stiffness, the adhesive ligand concentration influenced both the density of adherent cells after 2 hours (Figure 2.6) and RDF morphology after 1 day \textit{in vitro} (Figure 2.7). Without the addition of peptides, virtually no cells attached to gels (Figure 2.7.A), and few cells attached non-specifically to gels quenched with the negative control peptide (Figure 2.7.B). Hydrogels quenched with a 1:3 ratio of active ligand-to-inactive peptide were more adherent, and fibroblasts in these conditions were partially spread after day \textit{in vitro} 1 (DIV 1) (Figure 2.7.C). Cells attached in the greatest numbers on hydrogels quenched with the active ligand (Figure 2.7.D). These cells were also the most spread and exhibited stellate shapes. No significant differences were observed when a greater excess of active ligand (9 mM) was applied to the hydrogels (Figure 2.7.E). This suggests that the wash steps were sufficient to remove unbound ligand, which would otherwise competitively inhibit adhesion.
For hydrogels of constant stiffness, adhesive ligand concentration controls the attachment and spreading of RDFs in a dose-dependent manner. The maximum concentration of bound ligand in the hydrogels (x-axis) depends on both the relative ratio of the active ligand and the availability of HA-thiols in gels of 0.8% HA-S and 0.6% PEGDA after 1 day of gelation (~3.2 mM). For each condition, the average density of adherent cells was normalized to the initial cell seeding concentration of 5,000 RDFs/well and is presented as normalized mean ± SEM for n≥3.
FIGURE 2.7 CELL MORPHOLOGY AFTER 1 DAY IN VITRO

Images taken on DIV 1 with a 10x objective lens visualize RDF cell bodies (GFP, green), F-actin stress fibers (TRITC-phalloidin, red), and cell nuclei (DAPI, blue).

A. There is virtually no cell attachment to gels without grafted peptide. B. Very few cells attached to hydrogels quenched with the negative control peptide PEGMA-SDGRG (0 mM RGD), and of the cells that adhered, none had spread.

C. RDFs partially spread on hydrogels quenched with a 1:3 ratio of active ligand to inactive peptide (~0.8 mM RGD) and D. RDFs exhibited stellate shapes on hydrogels quenched with the adhesive ligand PEGMA-GRGDS (~3.2 mM RGD).

E. No significant differences were observed when a greater excess of active ligand (9 mM) had been applied to the hydrogels F. RDF attachment and spreading on positive control hydrogels of 2.0 mg/mL type-I bovine collagen.
2.3.5 Decoupling mechanical and bioactive signal features

The heat maps in Figure 2.8 demonstrate that stiffness and adhesive ligand concentration work in concert to influence both the adhesion of RDFs and their spreading areas. In each condition, stiffness was controlled by the concentration of PEGDA as HA-thiols were quenched with PEGMA-peptides prior to latent stiffening. Since the peptides target HA-thiols remaining after initial gelation, PEGDA concentration also influences the possible range of bound ligands. In general, the number of adherent cells was affected by both the concentration of PEGMA-GRGDS and the stiffness of the hydrogel (Figure 2.8.A.). For gels of constant stiffness (columns), cell attachment directly relates to the concentration of adhesive ligand. Additionally, on gels of constant ligand density, cell adhesion relates to the stiffness of the substrate (Figure 2.8.A. moving left-to-right across the rows). For instance, in conditions of 2 mM PEGMA-GRGDS, fibroblast density on the stiffest hydrogels (0.8% PEGDA) was more than three times that of the most compliant hydrogels (0.2% PEGDA).

In addition to the number of cells, the concentrations of both PEGDA and the adhesive ligand influenced cell spreading, albeit to different extents (Figure 2.8.B.). For instance, on the most compliant gels (0.2% PEGDA), cell spreading areas were least affected by adhesive ligand concentrations (Figure 2.8.B., column 1), despite these gels having the broadest range to graft bioactives (0-6 mM). On the slightly stiffer hydrogels formulated with 0.4% PEGDA, we observed broader ranges of cell spreading areas (Figure 2.8.B., column 2), despite there
being a narrower range to conjugate adhesive ligands (0-4.5 mM). In general, this trend continued for increasing concentrations of PEGDA. For example, the concentration of PEGMA-GRGDS in the 0.2% PEGDA condition (Figure 2.8.C.) is more than triple that of the 0.8% PEGDA condition, but cell density, spreading area, and stress fiber formation are more pronounced on the 0.8% PEGDA hydrogels (Figure 2.8.D.). These findings demonstrate the underlying concept that stiffness and ligand density work in concert to coordinate traction-based cell behaviors, including cell adhesion and spreading, and serves as a proof-of-principle to demonstrate that the mechanical and bioactive properties in HAS-PEGDA can be independently tuned to influence traction-based cell behaviors.
FIGURE 2.8 RDF ADHESION AND MORPHOLOGY ARE CO-DIRECTED BY MATRIX STIFFNESS AND ADHESIVE LIGAND CONCENTRATION

When latent stiffening is completely blocked, RDF adhesion and morphology directly relate to the ligand concentration for gels of constant stiffness (columns) and to the stiffness for gels of constant ligand concentration (rows). A. Average density of adherent cells (RDFs/well) (DAPI) B. Average cell spreading areas ($\mu$m$^2$/cell) (GFP) C. RDFs on 0.8% HA-S and 0.2% PEGDA hydrogels quenched with ligand (~6mM RGD) D. RDFs on 0.8% HA-S and 0.8% PEGDA hydrogels quenched with ligand (~2mM RGD).
2.4 Conclusion

This study describes several opportunities to manipulate ECM signaling features in HAS-PEGDA hydrogels. By leveraging the hydrogel's two cytocompatible crosslinking reactions, we demonstrate that the mechanical properties can be tuned across multiple time scales while introducing specific bioactivity. Non-cell-adhesive hydrogels are advantageous because they allow for the incorporation of precise densities and specificities of attachment ligands to influence cell-type-specific behaviors. The system described in this paper demonstrates how the initial crosslinking reaction of HA-S can be decoupled from the extent of latent stiffening to enable the full range of bioactives to be immobilized to HA-thiols without perturbing hydrogel gelation. Moreover, cellular interactions with the hydrogels validate that stiffness and ligand density are tunable in relevant ranges to influence cell behavior. Although the focus here was to manipulate mechanotransductive signaling, such as through cell adhesion and spreading this template offers a versatile mechanism to tether other chemical signals including small molecules, biologics, and ECM components through primary amines. Given the time scales of crosslinking and gel maturation, and the potential to introduce different components at different locations with 3D bioprinting, this HAS-PEGDA approach may offer significant value as a bioink system.
2.5 Acknowledgements

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CHAPTER 3: CONTROLLING NETWORK REMODELING: IMPROVEMENTS FOR CELL ENCAPSULATION

3.1 Introduction

With a 3D printer, it is possible to build complex structures with micrometer-level resolutions from computer-generated patterns. However, 3D bioprinting of tissues has unique challenges, as it requires materials to create a structural framework and initial signal conditions for resident cells. Engineered materials allow for precise densities of biologically active molecules, including adhesive ligands and degradation sites, while the mechanical properties can be independently controlled.\textsuperscript{139,140} From a bioprinting perspective, many biological scaffolds allow tissue reorganization through cell-mediated compaction. As such, biomaterials that enable tissue compaction are advantageous to facilitate tissue maturation, \textit{in vitro}.

In many native tissues, hyaluronic acid (HA) forms the initial scaffold of tissue development and regeneration. HA has high water content, which enables it to dissipate compressive forces from cell movement and resist tissue compaction. Previously, we established a biomaterial system to tune the mechanical and adhesive ligand properties of thiol-modified HA (HA-S) and polyethylene glycol diacrylate (PEGDA) hydrogels across multiple time scales. However, to build more complex biological structures from an HAS-PEGDA
framework, the system must be amenable to 3D culture. Despite the fact that
cells survive for weeks inside HAS-PEGDA, the system does not support the
spreading or migration of encapsulated cells.\textsuperscript{101} The current study aims to adapt
the biomaterial system established in Chapter 2 to be amenable to 3D bioink
formulation.

Positional controls in the extracellular matrix (ECM) assist to coordinate
cell fate decisions, and the spatial and temporal changes in their presentation
can have dramatic impacts on tissue architecture.\textsuperscript{37} Cells respond to soluble,
cell-bound, and matrix-bound guidance signals according to the distinct set of
receptors they express and how they integrate these signals over time.\textsuperscript{47} Since
the ECM presents a physical barrier for encapsulated cells, localized degradation
creates space for cell movement and enables the generation of traction forces,
which are critical for cells to probe the 3D environment.\textsuperscript{141} Traction forces not
only act on cells through mechanotransductive signal cascades, but they also
remodel and reorganize the matrix, which cells subsequently re-orient to.\textsuperscript{120} ECM
is highly dynamic, and its constant turnover in native tissues offers an important
signaling mechanism to control and respond to cell behavior. By remodeling,
ECM transmits signals from cell-to-cell,\textsuperscript{141} establishes tissue orientation,\textsuperscript{63} and
provides feedback across multiple time and length scales.\textsuperscript{50,118} For example, in
connective tissues such as the skin, human mesenchymal stem cells (hMSCs)
and fibroblasts actively compact, turnover, and re-organize collagen and
fibronectin fibers.\textsuperscript{37}
Cells can remodel native ECM molecules through a class of highly-conserved degradation enzymes known as matrix metalloproteinases (MMPs). MMPs recognize and cleave specific peptide sequences on matrix macromolecules. During degradation, cryptic binding sites can become exposed and peptides can break away from the matrix entirely, stimulating differential responses from cells. For instance, unbound ligands can compete with matrix-bound ligands for integrin receptors and weaken the strength of adhesive interactions. Both physically and chemically, degraded ECM can encourage cell migration, limit tissue compaction, and stimulate the synthesis of new matrix. Over time, ECM remodeling dramatically impacts the 3D geometric features of tissues.

Covalently crosslinked biomaterials can contain more stable bonds than the dynamic ones present in native ECM and are therefore often more resistant to degradation. Several studies have demonstrated that cell growth inside covalently crosslinked hydrogels depends on the hydrogel’s pore size and the extent to which the network can be remodeled. To improve the HAS-PEGDA system established in Chapter 2, and guided by the literature, we introduced controlled degradation to enable local remodeling by encapsulated cells. (Scheme 3.1) First, we accelerated peptide-conjugation reactions to improve network functionalization with matrix-bound ligands in the presence of cells. Next, we introduced degradable peptides with sites for specific cleavage by MMP-2, a zinc-dependent gelatinase enzyme that is constitutively expressed by hMSCs and fibroblasts. We then characterized the extent to which the
bulk mechanical properties can be preserved when degradable crosslinks are introduced into the gels. Finally, we examined the response of encapsulated cells for their abilities to remodel, grow, and spread inside the degradable hydrogels.
SCHEME 3.1 PROPOSED DEGRADABLE HYDROGEL SYSTEM

Introducing MMP-2-degradable peptides into the PEGDA backbone offers the potential to control the initial crosslinking dynamics and range to tune $G'$, while allowing for local remodeling of the network pore structure by cells. The VPM peptide is degradable by proteolytic enzymes (gelatinases) secreted by cells, including fibroblasts and hMSCs. As degradation occurs, network pore size expands, drawing more water into the hydrophilic network.
3.2 Methods

3.2.1 Hydrogel preparation

Thiol-modified hyaluronic acid (HA-S) (Glycosil®, BioTime, Inc., Alameda, CA), polyethylene glycol diacrylate (PEGDA) (MW: 3000 Da), polyethylene glycol monoacrylate (PEGMA) (MW: 5000 Da), and polyethylene glycol monomaleimide (PEGMM) (MW: 3000 Da) (Laysan Bio Inc., Arab, AL) were purchased commercially and stock solutions were prepared as previously described in 1x PBS. For simplicity, PEGMX denotes mono-functionalized PEG bearing either an acrylate (PEGMA) or maleimide (PEGMM) reactive group. To formulate hydrogels in the 3D context, thiol-reactive components (PEGDA and PEGMX) were first combined into one solution, and then added to HA-S.

3.2.2 Thiol-reactivity determination

To indirectly assess the reactivity of each functional group (acrylate or maleimide), HA-S was reacted with various concentrations of both PEGMA and PEGMM and free-thiol content was measured after 1 hour of reaction using 5,5-dithiobis-2-nitrobenzoate (DTNB), otherwise known as Ellman’s reagent (Thermofisher Scientific, Waltham, MA). Ellman’s Reagent reacts with free thiols and forms a yellow product (TNB) that is detectable by absorbance at 412 nm. Ellman’s reagent was reacted in molar excess of known concentrations of a thiol standard, reduced glutathione (GSH) (Thermofisher Scientific, Waltham, MA) in
pH 8.0 phosphate buffer (PB) according to the manufacturer’s instructions.\textsuperscript{148}

The thiol content of 1 mg/mL HA-S was interpolated from the standard absorbance curve of GSH in replicates of n=6. Next, solutions of 0.8% HA-S and various concentrations of PEGMM or PEGMA (0, 0.5, 1, 2, 4, 6, or 8 mM) were prepared, and after 1 hour of reaction were diluted 10-fold in PB. Remaining thiols on HA-S were reacted with Ellman’s reagent for 10 minutes, and absorbance was read at $\lambda_{\text{max}} = 412$ nm as previously described.\textsuperscript{111,148} The remaining thiol concentrations of 0.8% HA-S after 1 hour of reaction with various concentrations of PEGMM or PEGMA are presented as the mean (interpolated) thiol concentration (mM) ± SD in replicates of at least n=4.

3.2.3 Rheological measurements

The extent to which thiol-reactivity could be leveraged to prioritize peptide-grafting was characterized, rheologically, using a Kinexus Ultra Rotational Rheometer (Malvern Pananalytical Ltd., Malvern, UK). Hydrogels of 0.8% HA-S, 0.4% PEGDA, and various concentrations of PEGMX (0, 0.5, 1, 2, 3, 4, 8, or 10 mM) were prepared in 0.2 mL circular rheology samples (ID: 20 mm) as previously described.\textsuperscript{140} After 1 hour of curing, hydrogels were covered with 0.01 M PBS and incubated at 37°C. To characterize the mechanical properties of PEGMX-functionalized hydrogels in the context of 3D formulation, $G'$ was measured after both initial gelation ($t=1$ d) and disulfide crosslinking (‘steady-state’). Samples were subjected to an oscillatory shear strain of 0.5% and a
frequency of 1 rad/s (0.1592Hz), as previously described. To accelerate disulfide crosslinking after 1 day of gelation, hydrogels were oxidized in PBS containing 20% DMSO (pH 7.4) for 3 days, at which point the steady-state mechanical properties were measured. Results are presented as the average shear storage modulus, G’ (Pa) ± SD in at least n=4 sample replicates. The extent to which either reactive group (acrylate or maleimide) influences network mechanical properties was compared after each crosslinking reaction, and a range to decouple the mechanical properties in each case was identified.

3.2.4 Ligand functionalization

The bioactive adhesion peptide, GRGDS (MW: 490.5 Da, Bachem Americas, Inc., Torrance, CA) was functionalized with either acrylate and maleimide reactive groups according to Scheme 3.2. Heterobifunctional PEGMX-succinimidyl valerate (PEGMX-SVA) was used as a molecular scaffold (MW 3kDa, Laysan Bio, Inc., Arab, AL) to introduce thiol-reactive groups to the peptide’s N-terminus. Briefly, 20 µmol of PEGMX-SVA was reacted with 30 µmol of peptide in 5 mL buffer containing 0.1 M PBS (Millipore-Sigma, St. Louis, MO) and 0.04 M HEPES (VWR International, Radnor, PA) to maintain a reaction pH of 7.4 ± 0.2. The reaction was protected from light and rotated at 4°C for 2 hours. Under these conditions of temperature, pH, and time, reactions between primary amines and thiol-reactive moieties were demonstrated to be efficient and selective to N-terminal amines. (Appendix A.3.C) Conjugation efficiency was
estimated using fluorescamine (MilliporeSigma, St. Louis, MO) to compare primary amine concentrations before and after reactions with SVA.\textsuperscript{129,130} (Appendix A.3) The PEGMX-peptide conjugates (approximate MW: 3500 Da) were then separated from buffer salts and unreacted peptide, which had been provided in molar excess of PEGMX-SVA. The PEGylated-peptide conjugates were purified against deionized water using centrifugal filter units (MWCO: 3000 Da, Amicon™ Ultra-15, EMD Millipore, Burlington, MA) according to the manufacturer’s instructions and subsequently sterile filtered through 0.2 µm syringe filters. Purified reactions were frozen at -80°C, lyophilized, and the dry products, PEGMA-GRGDS and PEGMM-GRGDS, were blanketed in argon and stored at -20°C for up to 3 months until use.
SCHEME 3.2 BIOACTIVE PEPTIDES WERE FUNCTIONALIZED WITH PEGMM REACTIVE GROUPS USING PEGMM-SVA AS A VERSATILE MOLECULAR SCAFFOLD

Succinimidyl valerate (SVA) is an activated NHS ester that reacts rapidly with primary amines to form stable amide bonds under alkaline conditions. To functionalize peptides with PEGMM reactive groups, N-terminal primary amines were targeted with PEGMM-SVA molecular scaffolds. Peptides were reacted in a 3:2 molar ratio (NH₂:SVA) at pH of 7.4 ± 0.2 and 4°C for 2 hours to minimize off-target reactions between amines and maleimide reactive groups. (Appendix A.3)
3.2.6 hMSC culture

Human bone marrow-derived mesenchymal stromal cells (hMSCs) were purchased commercially (Texas A&M Institute for Regenerative Medicine, College Station, TX) and expanded in tissue culture using Minimum Essential Medium (MEM) α (Gibco® GlutaMAX, ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Neuromics, Edina, MN), 1% penicillin-streptomycin (MilliporeSigma, St. Louis, MO), and 1 ng/mL human basic fibroblast growth factor (bFGF) (ThermoFisher Scientific, Waltham, MA). hMSCs were incubated at 37°C and 5% CO₂ and passaged at 90% confluence. Immediately prior to cell studies, hMSCs were incubated with MEM-α containing 2 µM CellTracker™ Green CMFDA fluorescent dye for 30 minutes in accordance with the manufacturer’s instructions (ThermoFisher Scientific, Waltham, MA).150

3.2.7 2D substrate preparation

Stock solutions of 25 mM PEGMM-GRGDS, PEGMA-GRGDS, PEGMM, and PEGMA were prepared in sterile deionized water, and dilutions of biologically-inactive spacer sequences were prepared. Hydrogels containing 0.8% HA-S, 1mM PEGMX-GRGDS, and various concentrations of PEGMX spacer sequences (0, 1, 2, or 3 mM) were prepared in 96-well assay plates (50 µL/well) with n=12 replicates per condition. Hydrogels were incubated at 37°C for 1 hour then hydrated with PBS (100 µL/well). After 1 day of gelation, hydration buffers were aspirated from the gels and replaced with either 20% DMSO to
accelerate disulfide crosslinking in half of condition replicates or 10 mM N-ethyl-maleimide (NEM) (Pierce™, Thermo Fisher Scientific™, Waltham, MA) to quench remaining thiols in the other half of replicates. NEM solutions were aspirated after 2 hours of quenching remaining thiols, and DMSO solutions were aspirated after 3 days of oxidizing remaining thiols. At each end point, 250 μL of wash buffer (1x PBS with 1% penicillin-streptomycin) was added to the gels. Hydrogels were subsequently washed with 5 volumes of wash buffer for at least 1 hour per wash while rotating at 37°C to remove residual NEM or DMSO from the networks. In parallel, 0.8% HA-S, 0.4% PEGDA, 1 mM PEGMX-GRGDS, and various concentrations of PEGDA (0.2, 0.4, 0.6, or 0.8%) were prepared and all gels were quenched with NEM after 1 day of curing as described above.

3.2.8 2D cell adhesion assays

At approximately 90% confluence, hMSCs were detached with TrypLE™ (Thermo Fisher Scientific, Waltham, MA), pelleted, and re-suspended to 25,000 cells/mL in culture media. Cell suspensions were seeded simultaneously (100 μL/well) using an automated 96-channel high-throughput transfer pipet (VIAFLO 96, Integra Biosciences Corp., Hudson, NH) to achieve a uniform final seeding density of 2,500 cells/well. After 2 hours of incubation, cell suspensions were aspirated, and non-adherent cells were removed with two washes of media. Relative concentrations of pre-dyed adherent cells were estimated with fluorescence intensity measurements (λ_ex = 492 nm; λ_em = 520 nm) (Appendix
A.5) and presented as average fluorescence intensities ± SD, normalized to control wells that had been coated with 5 μg/cm² poly-L-lysine (PLL) overnight at room temperature (Millipore-Sigma, St. Louis, MO).

3.2.9 PEGDA-VPM synthesis

A matrix metalloproteinase-2 (MMP-2) cleavable peptide sequence, VPM, flanked with terminal cysteine residues (GCRDVPMMSMRGGDRCG, MW: 1697.0) was purchased commercially (Lifetein, Somerset, NJ) and integrated into the PEGDA crosslinker backbone through terminal cysteine residues. (Scheme 3.3) Briefly, 10 μmol of VPM peptide was reacted with 40 μmol of PEGDA (MW: 2 kDa, Laysan Bio, Arab, AL) in 5 mL of PBS. (Appendix A.4) Reactions were protected from light and rotated at 4°C for 2 hours. The PEGylated-peptide conjugates, were purified against deionized water using centrifugal filter units (Amicon™ Ultra-15, EMD Millipore, Burlington, MA) in multiple steps. First, high molecular weight networks (>10 kDa) were removed from the target product (approximate MW: 6 kDa) using a 10 kDa MWCO filter membrane. Then, low molecular weight species (<3 kDa) were separated from the filtrate solution with a 3 kDa MWCO membrane, as previously described. The purified PEGDA-VPM crosslinker product was sterile-filtered using a 0.2 μm syringe filter, frozen -80°C, and lyophilized to dryness. The dry product, PEGDA-VPM, was blanketed with argon and stored at -20°C for up to 6 months until use.
The MMP-2-degradable peptide sequence GCRDVPMGMRGDCG was reacted with PEGDA (MW: 2 kDa) in a 1:4 molar ratio (cysteine-thiol to PEG-acrylate) at pH 7.4 for 2 hours at 4°C. The reaction chemistry leverages Michael-type nucleophilic addition of cysteine-thiols to PEG-acrylates. (Appendix A.4)
3.2.10 **Enzymatic degradation studies**

MMP-2-degradable hydrogels were formulated by combining 0.8% HA-S and various concentrations of PEGDA-VPM crosslinker (0.4, 0.8, or 1.2%) in circular rheology samples in Petri dishes as previously described. Non-degradable control hydrogels were fabricated in parallel using 0.6% PEGDA as a crosslinker. Samples were hydrated with PBS after 1 hour of curing, and the mechanical properties were monitored periodically with oscillatory strain-controlled rheology (0.5% strain at a frequency of 0.1592 Hz). The extent of thiol-acrylate crosslinking was characterized by measuring G' after 1 day of gelation, and the resultant stiffness was measured after 4 days of curing, following disulfide maturation with 20% DMSO. Following the “steady-state” rheological measurement, samples were thoroughly washed with PBS, and incubated with a 1 mg/mL working solution of Collagenase/Dispase® enzyme (Roche Diagnostics, Indianapolis, IN) in pH 7.4 PBS. After 3 days of incubation at 37°C with the proteolytic enzymes, mechanical properties were re-measured. Results are presented as average G’ (Pa) ± SD in replicates of n=5 samples, and t-tests were used to detect statistically significant differences in network stiffness following enzymatic degradation (p<0.05).
3.2.11 3D cell encapsulation

To demonstrate the potential for controlled network degradation in the cellular context, hMSCs were encapsulated inside HA-S-based hydrogel discs at a concentration of 500,000 cells/mL. Solutions containing 0.8% HA-S were functionalized with 1 mM PEGMM-GRGDS adhesive ligand and various concentrations of PEGMM (0, 2, or 3 mM) to prioritize the incorporation of bioactives and control the extent of latent disulfide crosslinking, respectively.

MMP-degradable hydrogels were formed upon crosslinking HA-S with PEGDA-VPM (0.4 or 0.8%) and negative control hydrogels were formed by crosslinking with PEGDA (0.4%). Cellular hydrogel solutions were pipetted to mix, and 0.2 mL volumes were plated inside of annular PDMS molds (ID: 0.9525 cm) fixed inside 12-well tissue culture plates (Corning™, Fisher Scientific, Hampton, NH) using high-vacuum silicone grease (Dow Corning®, Millipore-Sigma, St. Louis, MO).

To positively control for degradability, collagen hydrogels were prepared in parallel to gelatinase-degradable HAS-PEGDA-VPM networks. Type-I bovine collagen (Elastin Products Company, Owensville, MO) was reconstituted in 0.02 N acetic acid to a concentration of 3.75 mg/mL with gentle rotation over night at 4°C. Collagen was buffered to pH 7.4 and a concentration of 3.0 mg/mL with 1M HEPES, 0.15 N NaOH, and 10X MEM as previously described by Shreiber, et al., and hMSCs were incorporated into pre-warmed solutions at a density of 500,000 cells/mL. Type-I collagen hydrogels self-assembled upon incubation at 37°C. After 1 hour of curing, PDMS molds were removed from all hydrogel conditions,
and 2 mL of complete hMSC culture media was added to each well. When media was added, hydrogel discs floated freely from the molds, enabling unconstrained mechanical remodeling by encapsulated cells.\textsuperscript{89,151,152} Hydrogels were incubated at 37°C and 5% CO\textsubscript{2}, and culture media was replaced every 2-3 days.

3.2.12 Cell-mediated network remodeling

The bulk geometric features of proteolytically-degradable hydrogel discs were monitored over 21 days of tissue culture. At each time point, macroscopic features of the hydrogel discs were captured by imaging under bright field, and embedded cells were visualized with an Axio Zoom.V16 fluorescence microscope (Carl Zeiss, Jena, DE) ($\lambda_{ex} = 492$ nm and $\lambda_{em} = 520$ nm). The cross-sectional area of each disc was estimated in FIJI (FIJI is just ImageJ, Dresden, DE),\textsuperscript{131} and results are presented as mean cross-sectional area (cm\textsuperscript{2}) ± S.D. among n=4 replicates. Statistically significant differences in disc cross-sectional areas relative to their baseline values were detected with t-tests ($p<0.05$).

3.3 Results and Discussion

3.3.1 Prioritizing peptide-grafting

Crosslinking hydrogels in the presence of cells requires that component materials are simultaneously mixed. Since adhesive ligands and crosslinkers compete for the same HA-thiol sites, we prioritized bioactive ligand incorporation
over crosslinking to leverage better control over ECM functional properties in 3D. This is an essential modification because, as thiols saturate, unbound adhesive ligands compete with matrix-bound ligands for adhesive interactions, inhibiting cell attachment to the matrix.\textsuperscript{144} (Appendix A.5.C) To adapt the system for 3D cell culture, maleimide reactive groups were investigated for bioconjugation to HA-S.

In agreement with previous reports,\textsuperscript{111} preliminary thiol quantification assays demonstrated that HA-S contains a thiol concentration of $0.9 \pm 0.01 \, \mu\text{mol/mg}$ of HA-S. In other words, hydrogels of 0.8% HA-S contain a free-thiol content of approximately 7.2 mM, unless otherwise consumed by thiol-reactive conjugates, crosslinkers, or disulfide bond. (Appendix A.1) Maleimide functional groups are frequently used in bioconjugate chemistry for their rapid reactions with thiols (seconds-minutes) under physiological conditions.\textsuperscript{139} During reactions with PEGMM or PEGMA, thiol quantification assays demonstrated that HA-thiols are consumed more rapidly by PEGMM than PEGMA conjugates after 1 hour of reaction. (Figure 3.1) The 1-hour time frame is significant to cell encapsulation since hydrogels must be supplemented with culture media after approximately 1 hour of curing for cell survival. After culture media is added, grafting reactions are diluted, and unbound ligand can competitively inhibit cell adhesion inside the 3D hydrogels.\textsuperscript{144} Nonlinear regression of remaining thiols with respect to grafted PEGMX concentration demonstrated that nearly 100% of targeted thiols were consumed by PEGMM ($R^2=0.99$), while only about 58% of targeted HA-thiols were consumed by PEGMA ($R^2=0.94$).
FIGURE 3.1 HA-THIOL QUANTIFICATION AFTER 1 HOUR OF REACTION

The concentration of remaining HA-thiols after 1 hour of reaction with maleimide is linearly dependent ($R^2=0.99$), and the slope of $-1.1 \pm 0.1$ on the plot of remaining HA-thiols vs. concentration of PEGMM suggests that the grafting efficiency is nearly 100% in this range. On the other hand, the slope of $-0.59 \pm 0.2$ ($R^2=0.94$) on the HA-thiol consumption vs. conjugate concentration curve demonstrates that grafting is less efficient after 1 hour of reaction with PEGMA.
The extent to which each PEGMX conjugate affects HAS-PEGDA network crosslinking was characterized rheologically. After 1 day of curing, hydrogels with increasing amounts of PEGMA had slightly elevated G', suggesting that the presence of the PEG chain influences the stiffness of the network. (Figure 3.2) However, in formulas that included 10-12 mM PEGMA, hydrogel formation was significantly perturbed. At the thiol-saturation point (7.2 mM), double-end anchorage of PEGDA is significantly inhibited by grafted conjugates, and hydrogel networks do not form. In accordance with previous research, there was a concentration-dependent decrease in G' at steady-state when PEGMA was introduced to block disulfide crosslinking in the 3D formulation context.

In hydrogels grafted with PEGMM, G' was not significantly different after 1 day of curing among conjugate concentrations between 0-4 mM PEGMM ($p=0.586$, 1-way ANOVA). (Figure 3.3) This suggests that the initial mechanical properties are preserved within this concentration range. As thiols became saturated between 4-8 mM PEGMM, crosslinking was significantly perturbed, and gels did not form. Since the theoretical remaining thiol concentration of 0.8% HA-S and 0.4% PEGDA hydrogels is 4.5 mM after initial crosslinking, the saturation point after 4 mM demonstrates the efficient incorporation of PEGMM into the network. The concentrations at which each conjugate perturbs initial gelation suggests that maleimide functionalities incorporate more efficiently than acrylates into HAS-PEGDA networks. At steady-state, PEGMM-grafted hydrogels also exhibited a dose-dependent decrease in G', and the results suggest that PEGMM incorporates in a linear manner ($R^2=0.98$).
FIGURE 3.2 CROSSLINKING PROPERTIES OF 0.8% HA-S, 0.4% PEGDA, AND VARIOUS CONCENTRATIONS OF PEGMA IN 3D FORMULATION

At t=1d gelation, PEGMA mixed into the hydrogels increases the stiffness of the material, until it starts to perturb crosslinking as thiols become totally saturated by PEG-acrylates (10 mM of PEGMA). After crosslinking with 0.4% PEGDA, the remaining thiol concentration is theoretically 4.5 mM. However, the thiol-acrylate reaction is not 100% efficient in this 1-hour gelation period before gels are hydrated with PBS.
FIGURE 3.3 CROSSLINKING PROPERTIES OF 0.8% HA-S, 0.4% PEGDA, AND VARIOUS CONCENTRATIONS OF PEGMM IN 3D FORMULATION

After 1 day of hydrogel curing, there are no significant differences among G’ of 0.8% HA-S and 0.4% PEGDA containing between 0-4 mM of PEGMM ($p = 0.586$, 1-way ANOVA). However, between 4-8 mM of grafted PEGMM, HA-thiols become saturated with monofunctionalized conjugates, and hydrogels do not form. In agreement with prior experiments, there is a linear decrease in G’ at steady-state ($R^2=0.98$) due to the targeted blocking of disulfide crosslinking.
In addition to demonstrating that PEGMM reacts more efficiently with HA-thiols than PEGMA with chemical and rheological methods, we demonstrated the efficiency of PEGMM-ligand grafting using 2D cell adhesion. Previous studies have demonstrated that, unless otherwise modified, HAS-PEGDA is distinctly non-cell-adhesive.\textsuperscript{92,140} In addition, cell adhesion and spreading on top of HAS-PEGDA not only depends on the specificities and concentrations of bioactive ligands grafted to the network, but also on the mechanical properties of the hydrogels.\textsuperscript{140} However, this research, which was presented in Chapter 2, relied on a nonspecific, diffusion-based approach to conjugate peptide-ligands to the network.\textsuperscript{140} To leverage more precise formulation methods for 3D bioprinting, all bioink components must be simultaneously mixed into the pre-gel formulation. In this 3D formulation context, monofunctionalized bioactive conjugates (PEGMA or PEGMM) must compete with PEGDA crosslinkers for thiol-conjugation sites.

Cell adhesion to hydrogels with constant ligand concentration (1 mM PEGMX-GRGDS) and various crosslinking densities (0.2, 0.4, 0.6, 0.8, or 1.0% PEGDA) generally followed consistent trends with results presented in Chapter 2, but offered more precise control over bioadhesivity at lower concentrations of ligand. (Figure 3.4) On hydrogels functionalized with 1 mM PEGMM-GRGDS, hMSC adhesion was linearly dependent on the concentration of PEGDA (network stiffness) between 0.2-0.6% PEGDA. As thiols became saturated with ligands and crosslinkers between 0.8-1.0% PEGDA, cell adhesion to PEGMM-GRGDS-functionalized hydrogels followed an expected logarithmic trend as double-end anchorage of PEGDA became less probable.\textsuperscript{96}
Hydrogels of 0.8% HA-S, 1 mM PEGMX-GRGDS, and various concentrations of PEGDA were prepared in 96-well plates, quenched with NEM to block latent stiffening, and thoroughly washed with PBS prior to seeding (pre-dyed) hMSCs at a density of 2,500 cells/well. After 2 hours of attachment, densities of adherent cells were estimated with fluorescence intensity measurements. Results are presented as relative cell density ± SD, within the linear range of the standard.
curve, where fluorescence is proportional to hMSC concentration. (Appendix A.5.A.)

On hydrogels where crosslinkers and bioactive ligands compete equally for remaining thiols (PEGMA-GRGDS), cell adhesion to the network was less predictable and exhibited nonlinear trends. (Figure 3.4) Around 0.6% PEGDA, cell adhesion to the network peaked; after which point, adhesion declined steeply as HA-thiols reached their saturation point. This trend suggests the possibility that, even with the extensive wash steps permitted in 2D, competitive adhesion with unbound ligand may still play a role in weakening adhesive interactions on the substrate. However, unlike the 2D system established in Chapter 2, where gels can be prepared in advance of seeding cells, all components of a 3D bioink system must be formulated simultaneously, and unbound ligands inside the network would competitively inhibit cell adhesion inside the 3D hydrogel.

To leverage the full range to tune the mechanical and bioactive properties in HAS-PEGDA, PEGMX spacer sequences were conjugated to the 3D matrix to control the extent of disulfide crosslinking in the network. Pre-gel formulations of 0.8% HA-S, 0.4% PEGDA, 1 mM PEGMX-GRGDS, and various concentrations of PEGMX were prepared in 96-well plates, and half of the hydrogels were quenched with NEM to block latent stiffening. The relatively consistent adhesion of hMSCs to NEM-quenched hydrogels of constant stiffness (0.4% PEGDA) and adhesive ligand density (1 mM PEGMM-GRGDS) suggest that bioactive ligand incorporation was efficient regardless of the concentration of inactive PEGMM spacer incorporated into the network between 0-3 mM. (Figure 3.5) On the other
hand, hMSC adhesion to hydrogels functionalized with 1 mM PEGMA-GRGDS decreased with increasing concentration of PEGMA spacer, suggesting that ligand incorporation was less efficient as HA-thiols were saturated. (Figure 3.6) In hydrogels that were able to fully mature, hMSC attachment was not as pronounced as expected from rheology and adhesion assays on NEM-quenched hydrogels of varying PEGDA crosslinking densities. (Figures 3.5 and 3.6) Subsequent studies reiterated the inconsistencies in cell attachment on HAS-PEGDA with varying degrees of remaining thiols. These limitations will be discussed in more detail in Chapter 5. Despite the inconsistencies observed in thiol-oxidized hydrogels, adhesion on NEM-quenched hydrogels demonstrated that bioadhesive ligand incorporation is prioritized near the thiol-saturation point when PEGMM is used to tether conjugates to the network in place of PEGMA.

Taken together, these studies suggest that maleimide functional groups improve peptide conjugation efficiency to HA-S. The faster reaction kinetics between maleimides and thiols in comparison to acrylates enable bioactive ligands to be more efficiently incorporated into 3D hydrogel systems when all component materials are combined simultaneously. Chemical, rheological, and biological studies confirmed that PEGMM is more reactive with HA-thiols, and validated that mechanical and bioactive properties of HAS-PEGDA hydrogels are more completely controllable in the 3D formulation context using PEGMM to prioritize bioactive ligand-grafting.
FIGURE 3.5 PEGMM PRIORITIZES THE INCORPORATION OF LIGANDS

Adhesion of hMSCs on hydrogels of 0.8% HA-S, 0.4% PEGDA, 1 mM PEGMM-GRGDS, and various concentrations of PEGMM. The combined concentration of PEGMM (1 mM PEGMM-GRGDS + x mM of PEGMM spacer sequence) is presented on the x-axis, while the relative concentration of adherent hMSCs, as measured with fluorescence intensity, is presented on the y-axis. (Appendix A.5.C) Results are presented as mean ± SD in sample replicates of n=4 wells and normalized to PLL-coated positive control wells.
FIGURE 3.6 PEGMA COMPETES WITH PEGMA-GRGDS AND PEGDA FOR HA-THIOLS IN 3D FORMULATION SYSTEMS

The combined concentration of PEGMA (1 mM PEGMA-GRGDS + PEGMA) is presented on the x-axis, while the relative concentration of adherent hMSCs is presented on the y-axis. Cell densities are normalized to PLL-coated control wells and presented as mean ± SD in sample replicates of n=4 wells. In the NEM-quenched case, hMSC adhesion decreases near the thiol saturation point, suggesting the incomplete incorporation of PEGMA-GRGDS ligand when all hydrogel components are simultaneously combined.
3.3.2 Introducing controlled network degradation

Previous research involving a variety of different cell types demonstrated that cells encapsulated inside HAS-PEGDA hydrogels survive, but do not spread or migrate in 3D; however, bioinks must be amenable to 3D cell culture for use in bioprinting applications. We hypothesized that network pore structures inside HAS-PEGDA hydrogels were too small for cells to navigate and probe the 3D ECM environment. To tune the porosity of the HA-S matrix while maintaining the ranges to manipulate the mechanical and bioactive properties established in Chapter 2, MMP-2-sensitive peptides (VPM) were incorporated into the otherwise non-degradable PEGDA crosslinker backbone to allow for local remodeling by encapsulated cells.

To characterize the extent to which the initial mechanical properties could be maintained while introducing sites for controlled network remodeling, the rheological properties of proteolytically-degradable hydrogels were monitored across multiple time scales. Previous rheological characterizations with PEGDA demonstrated that rapid nucleophilic addition reactions between HA-thiols and PEG-acrylates dictate the material’s mechanical stiffness after 1 day of hydrogel curing, while the prolonged maturation of disulfide crosslinks from free-thiols control the steady-state crosslinking density. Previous studies also established that $G'$ is linearly proportional to the concentration of PEGDA crosslinker in hydrogels after 1 day of curing for 0.8% HA-S and 0.2-1.0% PEGDA. The ability to decouple the initial and final stiffnesses is a key feature for bioprinting.
To confirm that the mechanical properties are maintained when HA-S is crosslinked with PEGDA-VPM in place of PEGDA, rheological properties were measured after 1 day of hydrogel curing for various concentrations of degradable crosslinker. Consistent with previous studies, Figure 3.7 demonstrates that $G'$ increases linearly with the concentration of PEGDA-VPM crosslinker ($R^2 = 1.00$) incorporated into the formula after 1 day of curing. This trend parallels previous results in non-degradable HAS-PEGDA hydrogels, however, for equimolar concentrations of acrylates, the slope is greater in networks crosslinked with PEGDA-VPM.

Somewhat surprisingly, separate studies (results not shown) indicate that longer PEG chains produce hydrogels with higher $G'$ after 1 day of crosslinking. Non-covalent intermolecular forces from the PEG chain, itself, contribute to the stiffness of the network on 1 day of gelation. Additionally, previous results demonstrated that thiol-thiol crosslinking is accelerated in the presence of PEG,\textsuperscript{140} which to some extent, may be captured in rheological measurements taken after 1 day of hydrogel curing. Also consistent with previous studies, Figure 3.8 demonstrates that, at steady-state, $G'$ of fully matured hydrogels does not significantly differ among 0.6% PEGDA, 0.8% PEGDA-VPM, or 1.2% PEGDA-VPM ($p=0.766$, 1-way ANOVA). These findings support the possibility that longer PEG chains in PEGDA-VPM (MW: 6 kDa) contribute to faster disulfide crosslinking reactions in equimolar concentrations of PEGDA (MW: 3 kDa).
FIGURE 3.7 G’ IS LINEARLY RELATED TO THE CONCENTRATION OF PEGDA-VPM CROSSLINKER AFTER 1 DAY OF CURING

Hydrogels of 0.8% HA-S were crosslinked with various concentration of PEGDA-VPM and G’ was measured after 1 day of hydrogel curing as previously described. Results are presented as average G’ (Pa) ± S.D. in n=8 sample replicates. Nonlinear regression indicates that G’ is proportional to the concentration of PEGDA-VPM after 1 day of curing (R^2 = 0.999).
When gelatinase enzymes were applied to MMP-degradable hydrogels, $G'$ decreased significantly ($p < 0.001$, t-tests), whereas no significant differences were detected in control hydrogels, which had been crosslinked with PEGDA, before and after incubation with solutions of proteolytic enzyme ($p = 0.892$).

(Figure 3.8) After degradation with proteolytic enzymes, the drop in $G'$ was proportional to the densities of PEGDA-VPM that had been initially crosslinked into the hydrogels. For instance, fully matured hydrogels containing 0.8% PEGDA-VPM degraded from $G' = 959 \pm 200$ Pa at full equilibrium to $G' = 553 \pm 106$ Pa after incubation with gelatinase enzyme for 3 days, while 1.2% PEGDA-VPM hydrogels degraded from $G' = 1035 \pm 164$ Pa at full maturation to $G' = 524 \pm 208$ Pa after incubation with gelatinase. In hydrogels of 0.8% and 1.2% PEGDA-VPM, the mean changes in moduli after proteolytic degradation were proportional to $G'$ on 1 day of curing. After 1 day of curing, 0.8% PEGDA-VPM hydrogels had an average modulus of $G' = 505 \pm 66$ Pa, and the mean change after degradation was $\Delta G' = -406$ Pa; while 1.2% PEGDA-VPM hydrogels had an average modulus of $G' = 756 \pm 174$ Pa after 1 day of curing, and the mean change after enzymatic degradation was $\Delta G' = -511$ Pa. In negative control hydrogels, which had been crosslinked with non-degradable PEGDA, $G'$ remained stable after incubation with proteolytic enzymes ($\Delta G' = +16$ Pa). These results suggest that the mechanical changes in hydrogels crosslinked with PEGDA-VPM were due to the sensitivity of the peptide-crosslinker to enzymatic degradation.
Rheological testing demonstrates that PEGDA-VM degrades in the presence of gelatinase enzyme. The relative drop in $G'$ after incubation with proteolytic enzymes depend on the density of MMP-degradable crosslinks in the network. Results represent average $G'$ (Pa) ± S.D. in sample replicates of at least $n=4$, and suggest that controlled degradation can be introduced into the HA-S network by incorporating MMP-degradable peptides into the PEGDA backbone.
To control for the degradation of PEGDA-VPM, stock solutions of crosslinkers were pre-degraded with proteolytic enzymes for 3 days in a 37°C water bath prior to formulation. When 1.2% PEGDA-VPM crosslinker was reacted with 0.8% HA-S after pre-incubation with gelatinase enzyme, the stiffness after 1 day of curing was only $G' = 6$ Pa. This suggests that the crosslinker had been almost completely degraded prior to reaction with HA-S.

3.3.3 Cell-mediated network remodeling

To validate the potential for network remodeling by encapsulated cells, we monitored changes in the bulk geometric features of free-floating hydrogel discs over 21 days of culture without the addition of exogenous proteolytic enzymes. Like spreading and migration, cellular compaction of the ECM is a traction-based cell behavior that is directed by ECM stiffness, adhesive ligand density, and network pore structure in 3D. Although local remodeling enables the potential for encapsulated cells to exert traction and spread, morphological assessments are difficult to quantify accurately in 3D. In this study, the bulk geometric features of degradable HA-S hydrogels were monitored over time to indirectly assess cell-mediated remodeling of the ECM in 3D. Since ECM presents a physical barrier for encapsulated cells, localized degradation creates space for cell movement and enables the generation of traction forces necessary for cell spreading, migration, and matrix remodeling in 3D.
Figure 3.9 illustrates the geometric changes of type-I collagen positive control hydrogel discs due to matrix compaction by encapsulated hMSCs. After 48 hours of culture, the mean cross-sectional area of collagen control hydrogels decreased by 82.4% as hMSCs remodeled the network. On the other hand, compaction did not occur in degradable HA-S-based hydrogel discs. (Figure 3.10) In fact, outward swelling forces controlled the bulk geometric changes of HA-S-based hydrogel networks. Swelling is a parameter that directly relates to network pore size, and thus, the extent of network degradation.

Hydrogel disc cross-sectional area measurements demonstrated that networks crosslinked with 0.4% PEGDA-VPM and quenched with PEGMM dramatically swell over time in the presence of hMSCs. For instance, after 21 days in culture, hydrogels completely disintegrated and reverted back to viscous polymers. (Figure 3.10) The cross-sectional areas of negative control hydrogels, on the other hand, which had been crosslinked with non-degradable PEGDA, remained virtually unchanged over the course of 21 days in culture. (Figure 3.11) In alignment with previous reports, HA’s outward swelling forces dominated inward contraction forces by encapsulated hMSCs. These results also align with HA’s physiological functions; for instance, as a major constituent of joint fluid, HA dissipates compressive forces during mechanical loading.
FIGURE 3.9 COMPACTION OF TYPE-I COLLAGEN HYDROGELS BY ENCAPSULATED HMSCS

The 3D geometric features of free-floating, type-I collagen discs 500,000 hMSCs/mL were monitored as positive controls for ECM compaction. Hydrogels were cast in annular molds (ID: 0.95 cm) and disc cross-sectional areas (cm$^2$) were tracked over time as encapsulated hMSCs compacted and remodeled the gels.
FIGURE 3.10 DEGRADATION OF HAS-PEGDA-VPM HYDROGELS BY ENCAPSULATED HMSCS

Hydrogels containing 0.8% HA-S, 0.8% PEGDA-VPM, 1 mM PEGMM-GRGDS, 3 mM PEGMM, and 500,000 hMSCs/mL were cast in annular discs (ID: 0.95 cm), and disc cross-sectional areas were monitored over time as cells degraded the gels. No exogenous enzyme was added to the cellular hydrogels.
Annular hydrogel discs (ID: 0.95 cm) of 0.2 mL were prepared, and disc cross-sectional areas were monitored over time as encapsulated hMSCs (500,000 cells/mL) remodeled the networks. Bulk geometric changes are dictated by a balance of both outward swelling and inward compacting forces.
The morphological changes of encapsulated hMSCs paralleled degradation rates of PEGMM-quenched, 0.4% PEGDA-VP hydrogels. In hydrogels crosslinked with non-degradable PEGDA, cells remained round, while hMSCs inside hydrogels crosslinked with gelatinase-degradable PEGDA-VP generally spread over time. (Figure 3.12) Although cell migration was not the purpose of this experiment, hMSCs were observed along the bottom surfaces of tissue culture wells containing degradable hydrogel discs. When the hydrogels were transferred into new tissue culture plates, cells again migrated out of the degradable hydrogels and proliferated along the interior surfaces of the fresh tissue culture wells. No hMSC migration occurred in wells containing hydrogel discs that had been crosslinked with non-degradable crosslinkers (PEGDA) or where remaining thiols had not been quenched with PEGMM.

These results demonstrate that encapsulated hMSCs can degrade the network pore structure of hydrogels crosslinked with PEGDA-VP without supplementation of exogenous proteolytic enzymes, and that controlled network degradation allows cells to exert traction, spread, and migrate in 3D inside HA-S-based hydrogels. Although hydrogel swelling limited the extent of network compaction, the gradual swelling of 0.4% PEGDA-VP hydrogels quenched with PEGMM enabled indirect monitoring of network degradation over time.
FIGURE 3.12 HMSC MORPHOLOGY

3D hydrogels containing 1 mM PEGMM-GRGDS and 500,000 hMSCs/mL were visualized with an Olympus IX81 inverted microscope equipped with a 10X objective lens and cross-polarized light filters to visualize hMSC spreading inside hydrogels on DIV 9. hMSCs remained round inside hydrogels crosslinked with (A) non-degradable PEGDA and (B) 0.4% PEGDA-VPM (unquenched), but spread inside hydrogels crosslinked with (C) 0.4% PEGDA-VPM and quenched with 3 mM PEGMM and (D) 0.8% PEGDA-VPM quenched with 2 mM PEGMM.
In hydrogels crosslinked with 0.8% PEGDA-VPM and quenched with PEGMM, cell spreading and migration were observed; however, changes in the bulk geometric features were generally insignificant over time. One potential explanation for these observations is that outward swelling forces and inward compacting forces were relatively balanced inside the higher stiffness, MMP-degradable hydrogels. This suggests that higher initial crosslinking densities (mechanical stiffnesses) may be necessary for hMSCs to generate enough traction to compact HA-S-based hydrogels in 3D. With respect to 3D bioprinting, HA-S networks with tunable swelling properties provide an opportunity to harness HA-S degradability to manipulate the geometric features of 3D-printed structures over time. However, further research is required to leverage site-specific degradation for patterning 3D cellular geometries over time; recommendations for future work will be discussed in more detail in Chapter 5.

Finally, this study demonstrated a more complicated picture of HAS-PEGDA networks with respect to remaining HA-thiols. In contrast to the swelling observed in 0.4% PEGDA-VPM hydrogels quenched with PEGMM, otherwise identical hydrogels with unquenched free-thiols had remained unchanged over 21 days of culture. Although this trend could be attributed, in part, to stiffer networks formed during disulfide crosslinking, unlike the 0.8% PEGDA-VPM hydrogels, hMSCs remained round inside of the unquenched 0.4% PEGDA-VPM hydrogels. Additionally, no cell migration out of the unquenched hydrogel discs was observed. These findings parallel the trends demonstrated in 2D adhesion assays, where cell attachment to unquenched networks had been unexpectedly
low and inconsistent in comparison to NEM-quenched hydrogels of higher PEGDA concentration. Although the specific reasons for these trends were not directly investigated in this research, we expect that both covalent and non-covalent interactions among HA-thiols and MMPs, growth factors, and other proteins influence interactions with HA-S-based networks. Suggestions for future studies will be discussed in more detail in Chapter 5.

### 3.4 Conclusion

The continuous degradation and synthesis of ECM are essential processes in both native tissues and 3D culture systems. To leverage better control over 3D signal contexts, peptide-conjugation chemistry was adapted in HAS-PEGDA hydrogels to prioritize ligand binding over hydrogel crosslinking. We demonstrated that crosslinkers can be rendered proteolytically-degradable by incorporating MMP-sensitive peptide sequences into the PEGDA backbone, and we validated that the initial structural integrity of the network can be preserved while providing sites for local remodeling by cells. 3D cell encapsulation experiments demonstrated that HA-S resists compaction forces from hMSCs, and the hydrophilic networks swell as they degrade. The complete disintegration of degradable, cellular hydrogels demonstrated that endogenous enzymes secreted by encapsulated hMSCs allowed for sufficient degradation and ECM remodeling.
Along the same lines as receptor-ligand specificity and selective adhesion on 2D hydrogels, introducing specific sites for cell-mediated remodeling demonstrated the potential to tune 3D network permissivity to cell types of interest and the MMPs they constitutively express. Taken in context with rheological studies, these results confirm that, PEGDA-VPM crosslinkers are degradable over longer time scales, while over shorter time scales (seconds-to-days), which are relevant to 3D printing, the mechanical and structural properties of HA-S can be tuned. While this work establishes a template to control the extent of network remodeling by encapsulated cells, further research will be required to elucidate the complex regulatory roles among thiol-modified HA and its proteoglycans, adhesive ligand combinations, and interactions with cell-secreted signaling molecules, including MMPs.

3.5 Acknowledgements

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CHAPTER 4: HYALURONIC ACID-BASED BIOINKS FOR CELL-FRIENDLY BIOPRINTING

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4.1 Introduction

Bioprinting is a computer-based additive manufacturing approach that relies on the precise 3D positioning of cells and extracellular matrix (ECM) materials to build complex tissue structures. Bioinks are formulations of cells that are suitable for automated processing and typically include biomaterials to facilitate the fabrication process, as well as other actives to pattern the extracellular signaling niche. Extrusion-based bioprinting involves the extrusion of cells through narrow nozzles of 100 to 1000 µm in diameter during printing. It is one of the most accessible, customizable, and widely used biofabrication approaches. To develop workable bioinks, hydrogel precursors are typically included for their abilities to flow as fluids during processing, but provide structural integrity upon gelation.
One of the greatest limitations to extrusion-based bioprinting is the magnitude of shear force that cells are subjected to at the nozzle. Hydrogel-forming bioink components offer a potential solution to dissipate shear forces on cells, and print filaments finer than the printer nozzle by harnessing viscoelasticity. Hyaluronic acid (HA) is a native ECM molecule that offers significant potential for bioink development due to its inherent physical, chemical, and rheological properties. This study investigates the printability of a tunable bioink system based on thiol-modified HA for extrusion bioprinting.

In native tissue, extracellular matrix (ECM) is not a continuum of properties, but a dynamic network in which both mechanical and bioactive properties work in concert to regulate cell fate. To recapitulate the complex signaling role of native ECM, bioinks should allow for stiffness and adhesive properties to be independently controlled and, ultimately, spatially patterned. In addition to controlling the printability of a bioink, hydrogel systems with tunable rheological features can provide ECM signaling cues to resident cells, provided that the mechanical signaling features are recoverable.

Natural components of ECM are biologically relevant materials for their ability to be remodeled, however, many ECM materials, such as collagen and fibronectin, form higher-order molecular structures that cannot recover from the shear forces of extrusion. Hyaluronic acid (HA) is one exception. In native tissue, HA is a ubiquitous ECM glycosaminoglycan composed of disaccharide repeats that form linear, high-molecular weight chains. HA has excellent swelling properties and is permeable to small molecules, electrolytes, nutrients,
and gas exchange. Distinctly non-cell-adhesive, it mediates cell-matrix traction forces, ECM remodeling, and microbial contamination.

Although native HA is an attractive building block for tissue engineering, it does not have lasting mechanical integrity. Thiol-modified hyaluronic acid (HA-S) crosslinked with polyethylene glycol diacrylate (PEGDA) is a platform hydrogel system with tunable mechanical and bioactive properties. Two cell-friendly cross-linking mechanisms dictate the mechanical properties of HAS-PEGDA. Thiol-acrylate crosslinking occurs at very short time scales and results in gelation, and disulfide bonding matures gradually over time. Previous work established strategies to control the mechanical and bioadhesive properties across multiple time scales. By altering the composition of HA-S, PEGDA, and PEGylated peptides, traction-based cell behaviors, including cell adhesion, spreading, and degradation can be programmed into the otherwise non-cell-adhesive hydrogels.

There are a variety of biomaterials that leverage mechanotransductive signaling to direct cell behavior, and recapitulating the features of native ECM is a frequent target of bioink development. However, adapting tunable hydrogel systems into ‘printable’ bioinks can be a challenge. There are many considerations in the translation of a hydrogel system into a bioink, but in general, printability is determined by the quality of printed constructs and biocompatibility. The current study investigates the rheological features of HAS-PEGDA as a bioink for extrusion printing. Using rheology as a starting point, we assess the trade-offs among resolution, cell viability, and mechanical recovery and identify a window of opportunity for bioink printability.
4.2 Methods

4.2.1 Rheology

HA-S (Hystem®, Ascendance Biotechnology, Medford, MA) and PEGDA (MW 3kDa, Laysan Bio Inc., Arab, AL) were purchased commercially, and stock solutions containing 1.25% HA-S and 4% PEGDA (%w/v) were prepared in deionized water according to the manufacturers’ instructions. Serial dilutions of each stock solution were prepared and mixed in 4:1 volumetric ratios (HA-S to PEGDA) to achieve hydrogel concentrations of HA-S (0.8 or 1.0%) and PEGDA (0.2, 0.4, 0.6, or 0.8%), as indicated. Rheological measurements were performed on a Kinexus Ultra Rotational Rheometer (Malvern Instruments, Malvern, UK) equipped with a 20mm parallel upper plate. Unless otherwise stated, 0.2 mL samples were analyzed with a gap height of 0.6 mm at 25ºC in replicates of at least n=3 samples.

4.2.1.1 Viscometry

The extent to which crosslinking influences the apparent viscosity of the material over time was compared between formulations of 1% HA-S and either 0% or 0.4% PEGDA. Each formulation was first monitored under a constant shear rate of 1 s⁻¹ and then with a continuous shear rate ramp from 0.01 to 10 s⁻¹ (60 seconds/decade ramp time). To quantify the extent of shear-thinning prior to bioink crosslinking, the apparent viscosity of 1% HA-S was characterized across constant shear rates of 0.01 to 1000 s⁻¹ with 10 rates per decade. For
each shear rate, apparent viscosities were averaged over 30 s of constant shear, with a sampling rate of 10 samples/second. Results are presented as the average apparent viscosity (Pa s) at each shear rate (s⁻¹) among n=3 sample replicates. Additionally, shear stress ramps between 0.01-1000 Pa (10-minute ramp time) were performed on 1% HA-S. Results are presented as the average apparent viscosity (Pa s) at each shear stress (Pa) for n=3 sample replicates. The yield stress was defined as the mean stress at which the apparent viscosity peaks.

4.2.1.2 Oscillation

To approximate the linear viscoelastic region (LVER) of pre-gel bioink formulations, solutions of 1% HA-S were subjected to oscillatory shear strain-controlled amplitude sweeps (0.01-10% strain, f=1Hz) and frequency sweeps (f=0.1-100Hz, 0.5% strain). To characterize gelation time as a function of PEGDA concentration, formulations of 0.8% HA-S and 0.2%, 0.4%, or 0.6% PEGDA were subjected to an oscillatory shear strain of 0.5% and a frequency of 1 rad/s (0.1592Hz). G' and G'' were monitored during gelation for multiple concentrations of PEGDA crosslinker. Gelation time was considered to be the point that the elastic modulus G' crosses the viscous modulus G''. Results are presented as average G' (Pa) at each time point.
4.2.1.3 Mechanical recovery

Although dictating the PEGDA crosslinker concentration enables the time-dependent mechanical properties to be controlled, gelation complicates the characterization of mechanical recovery. Therefore, to analyze the structural recovery of pre-gel formulations under high-shear, 1% HA-S was first characterized in the absence of PEGDA. Briefly, G’ and G” were monitored within the LVER for 1 minute to determine the quasistatic viscoelastic properties (shear strain=0.1% and frequency=1 Hz). The samples were then subjected to a steady shear rate of 100 s\(^{-1}\) for 1 minute to simulate conditions of high-shear during bioprinting. Structural recovery (G’) was then monitored as a function of time by applying controlled oscillatory shear in the LVER (strain=0.1% and frequency=1 Hz). The rebuild time was defined as the time to recover 90% of the initial elastic moduli.
4.2.2 **Bioink-dispensing system**

A multi-channel, programmable syringe pump system (Harvard Apparatus, 71-2109, Holliston, MA) was implemented to separate the HA-S component from the crosslinker until immediately prior to printing. In this system, pre-filled syringes are positioned in parallel and a single piston drives extrusion at a constant displacement rate across all channels. Under conditions of steady-flow, the relative cross-sectional area of each syringe determines the volume ratio of each component in the bioink formulation (Table 4-1).

From the individual syringes, the ink components met at a Y-junction, combined, and flowed out of a single silicone tube (ID: 0.08cm; L: 20cm) (Masterflex L/S 13, Cole-Parmer, Vernon Hills, IL) through a 27 Gauge syringe nozzle (ID=0.21mm; L: 3.1cm) (Becton, Dickinson and Company, Franklin Lakes, NJ). The nozzle was fixed to a modified ADIMLab Gantry 3D printer head (ADIMLab, Wan Chai, HK), and printer movements were programmed with Repetier-Host (Hot-World GmbH & Co. KG, Knickelsdorf, DE).

The bioink extrusion rate (Q) (mL/min) was defined as the combined volume flow rate of all ink components from the y-junction. Given the tube geometry and gelation times, bioink extrusion rates of 0.05, 0.1, 0.2, and 0.5 mL/min were chosen to limit premature ink gelation at the nozzle. In addition to (bulk) gelation time (Equation 3), extrusion rate also influences the bioink’s viscosity at the nozzle through shear rate-dependent rheological behaviors.
### 3D Printer Specifications:

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Build dimensions</td>
<td>310 x 310 x 410 mm</td>
</tr>
<tr>
<td>Position accuracy</td>
<td>XY 0.01 mm, Z 0.04 mm</td>
</tr>
<tr>
<td>Printing speed</td>
<td>0-720 cm/min</td>
</tr>
<tr>
<td>Nozzle size</td>
<td>27G (ID: 0.21 mm)</td>
</tr>
</tbody>
</table>

### Bioink-Dispensing System Specifications:

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syringe cross-sectional area</td>
<td>1mL: 0.179 cm²</td>
</tr>
<tr>
<td></td>
<td>3mL: 0.589 cm²</td>
</tr>
<tr>
<td>Dilution factor</td>
<td>1mL: 4.282</td>
</tr>
<tr>
<td></td>
<td>3mL: 1.305</td>
</tr>
<tr>
<td>Piston rate</td>
<td>0.00018-190 mm/min</td>
</tr>
<tr>
<td>Volume flow rates (1mL/3mL)</td>
<td>0.0000138-14.6 mL/min</td>
</tr>
<tr>
<td>Tubing</td>
<td>ID: 0.8 mm; length: 20 cm</td>
</tr>
</tbody>
</table>

**TABLE 4-1 3D BIOPRINTER SPECIFICATIONS**
\[ u_{\text{avg}} = \frac{Q}{60} = \frac{Q}{60} \]

**EQUATION 4-1: AVERAGE FLUID VELOCITY**

Q: volume flow rate (mL/min)

\[ u_{\text{avg}}: \text{average fluid velocity (cm/s)} \]

R: radius of pipe (cm)

A: cross-sectional area (cm²)

\[ u(r) = 2 \cdot u_{\text{avg}}(1 - \frac{r^2}{R^2}) \]

**EQUATION 4-2: FLUID VELOCITY**

u: fluid velocity (cm/s)

\[ u_{\text{avg}}: \text{average fluid velocity (cm/s)} \]

r: distance from center (cm)

R: radius of pipe (cm)

\[ t_{\text{ex}} = \frac{L_t}{u_{\text{tube}}} \]

**EQUATION 4-3: EXTRUSION TIME**

\[ t_{\text{ex}}: \text{extrusion time (s)} \]

L_t: tube length (cm)

\[ u_{\text{avg}}: \text{fluid velocity inside tubing (cm/s)} \]
\[
\dot{\gamma}_{\text{avg}} = \frac{4 \cdot \left(\frac{Q}{60}\right)}{\pi \cdot R^3}
\]

**EQUATION 4-4: AVERAGE SHEAR RATE**

\(\dot{\gamma}\): shear rate (s\(^{-1}\))

Q: volume flow rate (mL/min)

R: radius (cm)

\[
\text{Re} = \frac{\rho \cdot u \cdot 2 \cdot R}{\mu}
\]

**EQUATION 4-5: REYNOLD’S NUMBER IS A DIMENSIONLESS QUANTITY TO PREDICT FLUID FLOW PATTERNS FROM COMPETING VISCOUS AND INERTIAL FORCES**

\(\rho\): density (g/cm\(^3\))

\(u\): fluid velocity (cm/s)

R: radius of pipe (cm)

\(\mu\): dynamic viscosity (g/[cm\(\cdot\)s] = 10\(^{-1}\) Pa\(\cdot\)s)
4.2.3 Resolution

A solution containing 2.6% PEGDA and 0.005% acrylate-PEG-Rhodamine, a thiol-reactive fluorescent molecule (MW: 2kDa, Creative PEGWorks, Chapel Hill, NC), was prepared in phosphate buffered saline and loaded into a 1 mL syringe (Becton, Dickinson and Company, Franklin Lakes, NJ). This syringe was fit in parallel to a 3 mL syringe containing 1% HA-S in the bioink-dispensing system described above. A G-code was created in Repetier-Host (Appendix A.6) to extrude bioink “filaments” of 2 cm in length on glass cover slips (75 x 38 mm, Ted Pella, Inc., Redding, CA) with controlled bioink extrusion rates of 0.05, 0.1, 0.2, or 0.5 mL/min and variable print speeds of 100, 250, 500, and 700 cm/min. The nozzle was zeroed to the glass coverslips and line segments were printed with a z-stand-off distance of 2 mm. Upon printing, glass slides were immediately transferred into Petri dishes, covered, and placed inside of a humidity chamber to prevent dehydration.
4.2.4 Imaging

Line segments were imaged with an Axio Zoom.V16 fluorescence microscope (Carl Zeiss, Jena, DE) under 7x magnification ($\lambda_{ex} = 558$ nm; $\lambda_{em} = 575$ nm). Images were stitched together in FIJI (FIJI is just ImageJ, Dresden, DE) using a grid stitching algorithm, and scaling for each reconstructed image was confirmed by calibrating the image length (pixels) to the known geometry of the glass coverslip. Images were converted to 8-bit binary inverting image masks using Huang thresholding, particles <50 px were filtered from the images, and holes were filled. Each printed line was sectioned into a rectangular image area of 1500 x 300 px, and average signal intensities down each column (1 x 300 px) were interpolated with the plot profile function. Average signal intensities were normalized to 255 to calculate the diameter at each pixel along the length of the printed filament. Histograms were plotted to interpret filament uniformity, and average diameters were calculated for each combination of bioink extrusion rate and printing speed. Average filament diameters were calculated among n=4 replicates per extrusion rate, and print speed combination and plotted as mean ± SD. Statistically significant differences in filament diameter with respect to bioink extrusion rate were detected with multiple t-tests with $p=0.05$ indicating statistical significance.
4.2.5 Cell culture

Human bone marrow-derived mesenchymal stromal cells (hMSCs) were purchased commercially (Texas A&M Institute for Regenerative Medicine, College Station, TX) and expanded in tissue culture using Minimum Essential Medium (MEM) α (Gibco® GlutaMAX, ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Neuromics, Edina, MN), 1% penicillin-streptomycin (MilliporeSigma, St. Louis, MO), and 1ng/mL human basic fibroblast growth factor (bFGF) (ThermoFisher Scientific, Waltham, MA). hMSCs were incubated at 37°C and 5% CO₂ and passaged at 90% confluence.

4.2.6 Cell viability

Immediately prior to cell viability assessments, hMSCs were incubated with MEM-α containing 2 µM CellTracker™ Green CMFDA fluorescent dye in accordance with the manufacturer’s instructions (ThermoFisher Scientific, Waltham, MA) to illuminate live cells. Pre-dyed hMSCs were then detached with trypsin-EDTA (TrypLE™, Gibco®, Thermofisher Scientific, Waltham, MA), pelleted, and resuspended to 1x10⁶ cells/mL in complete culture medium. The entry of a membrane-impermeant dye, ethidium homodimer (Invitrogen Live-Dead Cell Viability Assay, ThermoFisher Scientific, Waltham, MA) was used to demonstrate excessive shear force during extrusion. A bioink containing hMSCs, ethidium homodimer, and HA-S was loaded into a 3 mL syringe, and a 1 mL syringe containing PEGDA crosslinker was situated in parallel in the bioink-
dispensing system. Based on the syringes' cross-sectional areas, the final concentration of each component in the bioink formulation was 0.8% HA-S, 100,000 hMSCs/mL, 1 µM ethidium homodimer, and 0.6% PEGDA.

To assess cell viability under various extrusion rates, a G-code was developed to print 50 µL bioink aliquots directly into a 96-well tissue culture plate (Corning®, Corning, NY). (Appendix A.7) The bioink was extruded through a 0.02 cm (diameter) nozzle (27 G) as previously described using extrusion rates of 0.05, 0.1, 0.2, 0.5, and 1.0 mL/min, and conditions were replicated in sets of at least n=10 samples. As controls for cell viability, hydrogels were formulated and plated manually with pipettes in identical concentrations as printed bioinks. After 15 minutes of crosslinking, each well was covered with 50 µL of PBS. As negative controls for cell viability, manually formulated control wells (n=10) were covered with 50 µL of 1x Triton-X solution to permeabilize the cell membrane, allowing ethidium homodimer dye inside the cell. Fluorescence intensities were read after 1 hour on an Infinite® 200 PRO microplate reader (Tecan Group Ltd., Männedorf, CH).

To investigate the viability of cells within the bioink suspension, relative concentrations of live cells were estimated with fluorescence intensity of Green-CMFDA dye (λex = 492 nm; λem = 520 nm) and compared among sample replicates. Concentrations of dead cells were estimated with fluorescence intensity measurements of ethidium homodimer (λex= 528 nm; λem= 645 nm) scaled between average intensities of positive and negative control wells.
4.2.7 Mechanical recovery

Solutions of HA-S and PEGDA ink components were extruded through the bioprinting system described above with a controlled bioink extrusion rate of 0.2 mL/min. Circular hydrogel samples (d=20 mm) of 0.2 mL of 0.8% HA-S and 0.4% PEGDA were printed into Petri dishes and samples of identical concentrations were prepared manually, as previously described. After 2 hours of curing, samples were hydrated with PBS and incubated overnight at 37°C. In preparation for rheological testing, Petri dishes were fit into a 3D-printed lower geometry with an annular PDMS mold to prevent slipping under oscillatory shear. The parallel upper plate was lowered to a normal force of 100 mN, and the gap was maintained at this constant height for the remainder of the experiment (approximately 0.4-0.8 mm). Compressive stresses were allowed to relax for until the normal force decayed to 0 mN or for 10 minutes prior to oscillatory strain measurements (strain=0.5% and frequency=1 rad/s). G' is presented as mean ± SD (Pa) for n=5 sample replicates per formulation condition after 1 day of curing. Statistically significant differences between extrusion-printed and manually-formulated samples were detected with a t-test (p=0.05).
4.3 Results and Discussion

4.3.1 Bioink gelation time

A rheological assessment of a bioink can help predict its printability, but time-dependent increases in viscosity due to crosslinking complicate the flow profile. (Figure 4.1) The viscosity profile of 0.8% HA-S + 0.4% PEGDA measured over a constant shear rate of 1 s⁻¹ indicates that the dynamic viscosity exponentially increases after about 120 seconds of crosslinking, suggesting this formulation has appropriate gelation dynamics to minimize lag time between printing subsequent layers. However, these results also indicate that HA-S must be mixed with the final concentration of crosslinker immediately prior to printing to avoid premature gelation.

Previous studies examined the rheological behavior of HA-S and PEGDA hydrogels across multiple time scales and demonstrated that crosslinking occurs in two distinct phases. Crosslinking between PEG-acrylates and HA-thiols dictates initial gelation (minutes-to-hours), and prolonged maturation of disulfide crosslinks from HA-thiols (days-to-weeks) significantly increases the steady-state stiffness over time. In addition to influencing cell behavior over longer time scales, we hypothesized that HAS-PEGDA’s dual-crosslinking mechanism would allow for the initial mechanical properties to be tuned to the 3D bioprinting process. We examined the rate of initial gelation to identify a workable PEGDA concentration range to allow for bioink printability.
FIGURE 4.1 DURING INITIAL GELATION, THE VISCOSITY OF HAS-PEGDA IS TIME-DEPENDENT

For constant shear rate of 1 s⁻¹, the apparent viscosity of 1% HA-S + 0.4% PEGDA increases dramatically during crosslinking. The exponential increase in viscosity over the first 5 minutes of gelation suggests that the flow profile of HAS-PEGDA and the apparent viscosity at the nozzle will be time-dependent due to crosslinking.
With respect to extrusion-based bioprinting, bioink gelation rate must be tuned to maintain enough fluidity to avoid nozzle clogging and withstand the shear forces of extrusion, but also generating enough resistance to flow, through crosslinking, to hold shape once the material has been deposited. However, even in the absence of crosslinking, the component bioink materials are complex, viscoelastic fluids. Viscoelastic bioinks have inherently unsteady flow, characterized by sinusoidal storage and loss properties.\textsuperscript{132} Rheological properties have both an elastic (instantaneous) component (G’) and a viscous (time-dependent) component (G’’) under applied shear loads.\textsuperscript{132}

To characterize gelation time, oscillatory strain-controlled mechanical tests were performed on pre-gel formulations in the linear viscoelastic region (LVER), (Figure 4.2), and gelation time was defined as the time at which G’ permanently exceeded G’’. Pre-gel formulations of HA-S and PEGDA were subjected to controlled oscillatory strain (0.5%) at constant frequency (0.1592 Hz) to monitor G’ and G’’ with crosslinking time. (Figure 4.3) During initial gelation, G’ increased with crosslinking time, and by altering the concentration of PEGDA between 0.2%-0.6%, gelation time can be tuned within the timescale of seconds-to-minutes. (Figure 4.4) Given the bioink-dispensing system’s geometric features (Table 4-1) and the workable range of PEGDA concentration previously established, we identified a lower limit of printable extrusion rates of 0.05 mL/min with an upper limit of 0.6% PEGDA to prevent nozzle clogging. This is consistent with previously established ranges to tune G’ across each reaction time scale.\textsuperscript{140}
Oscillatory shear strain-controlled amplitude sweeps of 1% HA-S at frequency of 1 Hz demonstrates 0.5% strain falls within the linear viscoelastic region (LVER), where complex shear stress is proportional to complex shear strain. Nonlinear regression determined a linear fit between 0.01-3.2% strain ($R^2=0.995$).

**FIGURE 4.2 LVER – OSCILLATORY SHEAR STRAIN AMPLITUDE SWEEP**
FIGURE 4.3 TIME-DEPENDENT CROSSLINKING

Gelation profile of 0.8% HA-S + 0.2% PEGDA demonstrates the time-dependent mechanical properties during crosslinking. Oscillatory shear strain and frequency were controlled at 0.5% and 1 rad/s (0.1592 Hz), respectively, and \( G' \) and \( G'' \) were monitored during gelation. The gelation time is considered to be the point that \( G' \) crosses \( G'' \), which is approximately after 5 minutes of curing.
FIGURE 4.4 GELATION RATE IS DICTATED BY PEGDA CONCENTRATION

For gels of constant HA-S concentration (0.8%), the concentration of PEGDA crosslinker controls the rate of initial gelation. The mechanical properties can be tuned within time scales relevant to standard 3D printing times (seconds-to-minutes) by adjusting the amount of PEGDA.
4.3.2 Viscometry

To be printable, crosslinking must be timed such that the bioink gels rapidly upon printing, but maintains enough fluidity to avoid clogging the 3D printer nozzle.\textsuperscript{79,84,158} This presents a trade-off between the ability to flow through the nozzle, critical for cell viability and mechanical recovery, and maintaining shape with high-fidelity, essential to resolution and adequate printing speeds. To identify a window of opportunity for resolution and cell viability to coexist, an ideal bioink would have a low apparent viscosity inside the nozzle and high apparent viscosity immediately following extrusion.\textsuperscript{79,84} Therefore, printable bioinks tend to exhibit shear-thinning, yield stress, and quick recovery kinetics.\textsuperscript{37,56}

Shear-thinning is a non-Newtonian rheological behavior characterized by decreasing apparent viscosity with increasing shear rate. In terms of bioprinting, shear-thinning suggests that a material will behave more fluid-like under the forces of extrusion, but will resist flow at rest. At time points prior to gelation, the apparent viscosity profiles of 1\% HA-S and 1\% HA-S + 0.4\% PEGDA exhibit similar shear rate-dependent trends. (Figure 4.5) However, once gelation begins around 2 minutes, viscosities begin to deviate. Therefore, to approximate shear flow behavior prior to gelation, 1\% HA-S was characterized in the absence of PEGDA. (Figure 4.6) The apparent viscosity of HA-S decays from approximately 250 Pa s to 0.02 Pa s between 0.01-1000 s\(^{-1}\), indicating that HA-S is highly shear-thinning in a relevant range for 3D printing.
FIGURE 4.5 APPARENT VISCOSITY PROFILES OF HA-S AND HAS-PEGDA

Shear rates were swept from 0.01-100 s\(^{-1}\) with a ramp time of 1 minute/decade. Pre-gel solutions of 1% HA-S + 0.4% PEGDA and 1% HA-S follow consistent viscosity profiles over shear rate, but after approximately 2 minutes of gelation (1 s\(^{-1}\)), apparent viscosities begin to deviate.
The apparent viscosity of 1% HA-S was measured at constant shear rates between 0.01-10,000 s\(^{-1}\). Each data point represents the mean dynamic viscosity (Pa s) among n=3 samples averaged over 30 s of constant shear rate. The apparent viscosity decays 4 orders of magnitude between 0.01-10,000 s\(^{-1}\), indicating that HA-S is highly shear-thinning in a relevant range for 3D printing. Infinite apparent viscosity approaching zero shear also suggests a yield stress.
4.3.3 Yield stress

Yield stress implies that the material behaves more solid-like at rest, but flows as a fluid once a critical stress is applied. At a molecular level, yield stress materials form networks that must either be broken or untangled for the material to flow. At the yield point, the network either deforms in the direction of flow or irreversibly breaks down.\textsuperscript{157} Shear stress ramps between 0.01 to 1000 Pa demonstrate that an apparent yield stress of approximately 5 Pa exists for 1\% HA-S. (Figure 4.7) One limitation to this method, however, is that the influence of time is not captured in the measurement.\textsuperscript{159} To demonstrate that HA-S has a true yield stress, oscillatory shear was controlled at 0.5\% strain while frequency was swept from 0.1 to 100 Hz. (Figure 4.8) demonstrates that $G'$ exceeds $G''$ under quasistatic strain, which suggests the presence of an intrinsic yield stress.
FIGURE 4.7 HA-S HAS AN APPARENT YIELD STRESS

Shear stress ramp between 0.01-1000 Pa (10-minute ramp time) demonstrates HA has an apparent yield stress at approximately 5 Pa.
FIGURE 4.8 OSCILLATORY STRAIN-CONTROLLED FREQUENCY SWEEP

Oscillatory frequencies were swept from 0.1-100 Hz with a controlled complex shear strain of 0.5%. Average storage (G') and loss (G'') moduli are presented on the left y-axis (Pa) while phase angle is presented on the right y-axis (°). At low frequency, 1% HA-S behaves more solid-like (G'>G''), suggesting that 1% HA-S has a true yield stress. A LVER exists between 0.1-1.5 Hz, where G' is constant.
4.3.4 Cell viability

There are many parameters that influence shear forces on bioinks during extrusion bioprinting including (1) nozzle geometry, (2) bioink extrusion rate, and (3) apparent viscosity.\textsuperscript{161} Although finer nozzles, faster extrusion rates, and higher viscosities improve resolution,\textsuperscript{81,158} Reynolds’s number predicts these features increase viscous forces on cells.\textsuperscript{5,161,162} (Equation 4-5) In addition to the advantage that shear-thinning offers in terms of bioprinting resolution, it also offers a benefit to cell viability. Shear-thinning bioinks can help protect cells from extrusion through the nozzle by altering the velocity profile of the bioink across the inner radius of the nozzle. During pipe flow, no slip boundary conditions create a graduated velocity profile with the fastest fluid rates at the center of the nozzle.\textsuperscript{162} Shear-thinning fluids help protect bioinks during extrusion by limiting regions of high strain near the nozzle wall.\textsuperscript{79,80,158} Smaller diameter nozzles create steeper velocity gradients, which are believed to stretch cells and disrupt the integrity of the membrane to higher degrees.\textsuperscript{161}

Cell viability was assessed during bioprinting for multiple bioink extrusion rates: 0.05, 0.1, 0.2, 0.5, and 1 mL/min. (Figure 4.9) Formulations containing 0.8% HA-S and 0.6% PEGDA, the upper limit of PEGDA concentration for printability, were used to exaggerate differences in bioink viscosity while enabling materials to flow as fluids through the nozzle. Cell viability was biphasic for 0.8% HA-S and 0.6% PEGDA for extrusion rates of 0.05-1 mL/min. As predicted rheologically, 0.6% PEGDA bioinks were extrudable at rates as low as 0.05
mL/minute without clogging the nozzle. Due to the balance of PEGDA gelation time and flow time prior to extrusion (crosslinking time, 120s), low extrusion rates resulted in high viscosities at the nozzle. (Equation 4-3) As a result, 0.6% PEGDA bioinks extruded at 0.05 mL/min had significantly lower cell viability (47.8% ± 13.8) than manually pipetted control formulas (75.7% ± 7.7) (p=0.0018).

At the opposite end of the spectrum, the fastest extrusion rate of 1 mL/min also limited cell viability (54.6% ± 6.1) (p=0.047). Although fast extrusion rates reduce apparent viscosity at the nozzle through shorter crosslinking times and exaggerated shear-thinning, fluid speed also directly increases shear forces on cells and materials inside the nozzle. (Equation 4-4) For fast flow times, HAS-PEGDA crosslinking is minimal, shear-thinning plateaus, and extrusion rate directly increases shear rate inside the nozzle. (Equation 4-3) With that, steep velocity gradients exist between the wall and center of the nozzle. (Equation 4-2)

However, between these two extremes exists a window of optimal cell compatibility. For extrusion rates of 0.1, 0.2, and 0.5 mL/min, no significant differences in cell viability were detected between manually-formulated control wells and wells that had been bioprinted. Bioinks that were printed with an extrusion rate of 0.2 mL/min had nearly equivalent viability (70.1% ± 10.4) as wells that had been manually-formulated and pipetted (75.7% ± 7.7) (p=0.981). These results demonstrate that bioinks of 0.8% HA-S and 0.6% PEGDA are printable within the extrusion rate range of 0.1-0.5 mL./min, where hMSC membrane integrity is preserved.
Bioinks of 0.8% HA-S, 0.6% PEGDA, 1 µM ethidium homodimer, and 100,000 pre-dyed hMSCs/mL were extruded through 0.02 cm (ID) nozzle at various extrusion rates. Bioinks were printed into a 96-well plate (50 µL/well) in replicates of n=10 wells. Concentrations of dead cells were approximated by measuring the relative fluorescence intensities ($\lambda_{ex} = 528$ nm; $\lambda_{em} = 645$ nm) and scaling between positive control wells, which had been formulated manually, and negative control wells, where cells were permeabilized with Triton-X.

(continued…)
(…continued)

Results demonstrate that cell viability is biphasic with respect to extrusion rate. No significant differences between manually-formulated, positive control wells and bioprinted wells for extrusion rates of 0.1-0.5 mL/min were detected (t-tests, \( p=981 \)). Statistically-significant differences in cell viability were detected between control wells and both the lowest (0.05 mL/min) and highest (1 mL/min) extrusion rates. Significance levels are indicated (*) for \( p<0.05 \) and (**) for \( p<0.001 \).
4.3.5 Mechanical recovery

Leveraging shear-thinning and intrinsic yield stress features, previous studies by Yuk, et al., have demonstrated that bioink droplets can be stretched from their spherical shapes to extrude filaments that are even finer than the nozzle diameter.\textsuperscript{81} Yield stress, however, is only advantageous to bioink printability if shear forces do not impart irreversible damage to the network structure.\textsuperscript{79,83} Mechanical rebuild analyses demonstrated that HA-S can rapidly recover from high shear in the absence of PEGDA crosslinker. The rebuild time, or time to recover 90% of the initial storage modulus after high shear was applied (100 s\textsuperscript{-1} for 1 minute) was determined to be approximately 33s. (Figure 4.10) Fast recovery kinetics suggest that the structural integrity of HA-S is not irreversibly disrupted under these conditions.

The extremely short time scales that bioinks are exposed to high shear during extrusion are not practical to model on the rheometer. Therefore, supplementary mechanical tests were performed to compared G’ after 1 day of gelation between samples of 0.8% HA-S and 0.4% PEGDA that were either formulated manually or extruded through the bioprinting system. (Figure 4.11) No significant differences were detected between G’ in hydrogels extruded through the bioprinter nozzle at a rate of 0.2 mL/min (G’ = 250.1 ± 82.6 Pa) or formulated manually (G’ = 236.9 ± 85.6 Pa) (p=0.954). These results suggest a window of bioink printability where both cells remain viable and materials are recoverable.
FIGURE 4.10 THE VISCOSITY OF HA-S REBUILDS AFTER HIGH SHEAR

Mechanical rebuild analyses demonstrate that HA-S has quick recovery kinetics after being subjected to conditions of high shear. **A)** $G'$ and $G''$ were monitored for 1 minute in LVER (shear strain=0.1% and frequency=1 Hz), **B.** high shear rates (100 s$^{-1}$) were applied for 60s, and **C.** structural recovery of the elastic component of the shear modulus ($G'$) was monitored as a function of time within LVER (strain=0.1% and frequency=1Hz). The rebuild time was defined as the time to recover 90% of the initial modulus, and was calculated to be approximately 33s ($G' = 1.20$ Pa)
FIGURE 4.11 HYDROGEL RECOVERY

$G'$ after 1 day of hydrogel curing is not significantly different when 0.8% HA-S + 0.4% PEGDA samples are formulated manually or extruded through a 3D bioprinter nozzle (ID: 0.2 mm) at a controlled extrusion rate of 0.2 mL/min ($p=0.954$). $G'$ was measured using oscillatory shear strain tests (strain=0.5% and frequency=0.1592 Hz) in sample replicates of $n=5$. 
4.3.6 Resolution

In terms of resolution, the rate of bioink extrusion must be sufficient to dispense a continuous filament for a given printing speed. However, when extrusion is too fast, or printing speed is too slow, excess material will be deposited, resulting in printed strands that are thicker than the nozzle diameter. In general, a higher bioink viscosity at the nozzle allows for faster printing speeds to resist defects caused by rapid stage movements.

Additionally, between the printer nozzle and the printer bed, surface tension competes with viscosity and fluid speed to print continuous bioink strands. The height of the printer nozzle relative to the printer bed, or z stand-off distance, must be tuned with the bioink’s extrusion rate and viscosity to counterbalance surface tension and limit the formation of droplets. Although the current study investigates resolution for a constant nozzle height, previous research has demonstrated that, by harnessing viscoelasticity, shear-thinning, and yield stress, extruded bioink droplets can be deformed from their spherical shapes to print filaments that are even finer than the nozzle diameter.

In the current study, a constant z stand-off distance of 0.2 mm was used to print bioink filaments with speeds between 100-700 cm/s (xy) at constant extrusion rates in the range of 0.05-0.5 mL/min. (Figure 4.12) With an average diameter of $362 \pm 12 \mu m$, the finest bioink strands were printed at the printer’s maximum transversal (xy) print speed (700 cm/s) and the minimum extrusion rate (50 $\mu$L/min). (Figure 4.14) For constant bioink extrusion rates (mL/min), slower
print speeds (cm/min) significantly increased strand diameter due to the accumulation of excess material (p<0.0001, 2-way ANOVA), as predicted by volume conservation. Following this trend, these results suggest that print speeds > 700 cm/s may produce even finer bioink strands.

The results presented in Figure 4.14 also demonstrate that the rate of bioink extrusion is a significant determinant of the diameter of printed strands (p<0.0001, 2-way ANOVA); however, the relationship is more complex than between resolution and print speed. In addition to volume conservation, bioink extrusion rate dictates the fluid speed (Equation 4-2), crosslinking time prior to printing (Equation 4-3), and shear rate inside the nozzle (Equation 4-4). These factors impact the apparent viscosity through both crosslinking time (Figure 4.3) and shear-thinning behavior (Figure 4.6).

Given the gelation rate of 0.8% HA-S and 0.6% PEGDA, a lower extrusion rate limit of 0.5 mL/min was required to maintain bioink flowability. Plot profiles of printed strands demonstrated that the extrusion rate and print speed influenced the tendency of bioink droplet formation. In general, droplet formation was more pronounced at higher bioink extrusion rates and lower printing speeds. The oscillations due to droplet formation resulted in higher standard deviations in bioink filament diameters. Although these results present strand resolutions with a constant z stand-off distance, we also found that the extent of droplet formation was influenced by the height of the printer nozzle in accordance with previous research. (Equation 4-5)
FIGURE 4.12 BIOPRINTING RESOLUTION – 0.1 ML/MIN EXAMPLE

Line segments of 0.8% HA-S and 0.6% PEGDA of 2 cm length were printed with a controlled extrusion rate of 0.1 mL/min and various (xy) printing speeds: (A) 700 cm/min, (B) 500 cm/min, (C) 250 cm/min, and (D) 100 cm/min. At the fastest printing speed, the finest lines were produced, (A), while at the slowest printing speed, excess material accumulated, resulting in less-resolved and less-consistent diameters (D).
FIGURE 4.13 RESOLUTION PLOT PROFILES

(continued…)

A.

B.

C.

Gray Value

Distance (x) (mm)
Inverted binary image masks were generated from fused greyscale images, and relevant line sections of 1 cm in length, corresponding to each print speed, were sectioned from the masks. 

Plot profiles of average signal intensities down 1 px wide columns (x-axis) of known pixel heights (y-axis) were generated, and the filament diameter at each point along the length of the printed strand was interpolated by normalizing the average (inverted) signal intensity to the known column heights (px). From these data, filament diameters at each point across the horizontal axis of each 1 cm segment were calculated, averaged, and distributions were generated.
Bioink filaments of 0.8% HA-S and 0.6% PEGDA were printed with constant extrusion rates using a G-code to vary the print speed between 100-700 cm/min. (Appendix A.6) Data represent mean diameter (mm) ± S.D. at each point along the length (1 cm) of printed strands (Figure 4.13.C.) in replicates of n=4 strands.
4.4 Conclusion

This study validates the use of HAS-PEGDA as a workable bioink for extrusion-based bioprinting. Using rheology as a starting point, we demonstrate that HAS-PEGDA exhibits viscoelastic, shear-thinning, and yield stress properties in relevant ranges for bioprinting. Leveraging these rheological features, we identify a window of bioink printability where both cell compatibility and bioprinting resolution coexist. We demonstrate that the gelation dynamics and initial mechanical properties can be tuned to the 3D printing process by altering the concentration of PEGDA in the formula. Given the concentration ranges previously established for tuning HAS-PEGDA mechanical properties over multiple time scales, we identify a workable range to tune the apparent viscosity at the printer nozzle, allowing for both hMSC membrane integrity to be preserved and for materials to recover from extrusion. Rebuild analyses indicate that the mechanical integrity of HA-S rebuilds following extrusion, and the shear stiffness of the bioink is recoverable. Taken together, this study translates the tunable hydrogel system based on thiol-modified hyaluronic acid previously established into a printable bioink for extrusion bioprinting.
4.5 Acknowledgements

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CHAPTER 5: CONCLUSIONS AND OUTLOOK

5.1 Thesis summary

Bioprinting is an on-demand approach to engineer living replacement tissues from human cells for basic research, product development, clinical testing, and personalized medicine. Like other forms of 3D printing, bioprinting is attractive for the specificity it affords, however, 3D printing tissues has unique challenges as it requires materials to create an initial structural framework and signaling context for resident cells. Bioinks are formulations of cells that are suitable for automated processing and typically include biomaterials and other actives to facilitate fabrication. Recapitulating the features of native ECM is a frequent target for biomaterial development; however, adapting a tunable hydrogel into a printable bioink system can be a challenge.

This thesis investigated the development a bioink system based on thiol-modified hyaluronic acid (HA-S) and polyethylene glycol diacrylate (PEGDA) for extrusion-based 3D bioprinting. The research presented here establishes versatile approaches to present specific bioactive ligands, degradation sites, and physicochemical features inside HA-S-based bioinks while offering independent control over the network’s mechanical properties over time. By targeting HA-thiols across multiple time scales, the mechanical, chemical, and bioactive properties were adapted to extrusion-based 3D bioprinting.
Chapter 2 examined the dynamic, dual-crosslinking mechanism inside HAS-PEGDA hydrogels. The extent to which each crosslinking reaction contributes to the resultant stiffness was characterized rheologically, and several strategies were identified to manipulate the mechanical properties. After 1 day of hydrogel curing, the shear storage modulus was linearly proportional to the concentration of PEGDA crosslinker. However, over a period of days-to-weeks, disulfide crosslinks matured from remaining HA-thiols, and the resultant stiffness depended on both the thiol-acrylate and thiol-thiol crosslinking densities. Additionally, the rate and extent of network maturation were manipulated by selectively targeting remaining HA-thiols after initial gelation reactions with PEGDA. Working ranges with which hydrogel mechanical (0-1000 Pa) and bioactive (0-6 mM) properties could be controlled were established, and these features were co-modulated to direct traction-based cell behaviors, including the adhesion and spreading of rat dermal fibroblasts, on 2D HAS-PEGDA substrates.

In Chapter 3, we adapted the hydrogel system to be amenable to 3D cell culture. Crosslinking hydrogels in the presence of cells requires each component of the bioink to be simultaneously combined. Therefore, to leverage better control over ECM functional properties in the 3D culture context, bioactive ligand-grafting was prioritized over crosslinking by tethering peptides to PEG-(mono)-maleimide (PEGMM) functional groups. Chemical, rheological, and biological assays confirmed that PEGMM reacts more efficiently than PEGMA or PEGDA with HA-thiols under physiological conditions, and as such, allowed for the mechanical and bioactive properties to be more completely decoupled.
When proteolytically-degradable peptides were introduced into PEGDA, rheological studies confirmed the initial crosslinking dynamics and range to tune G' had been preserved, and a percentage of network crosslinks were degraded when gels were exposed to corresponding MMP enzymes. Furthermore, 3D cell studies demonstrated that encapsulated hMSCs could remodel the network pore structure and spread inside the proteolytically-degradable hydrogels. Over 21 days of cell-mediated degradation, network pore sizes expanded dramatically, and outward swelling forces dominated inward compacting forces exerted by hMSCs encapsulated inside the MMP-degradable hydrogels. By introducing sites for proteolytic degradation, the system became amenable to cell encapsulation. In terms of a bioink for 3D printing, controlled network degradability presents an opportunity to manipulate bulk geometric features of cellular, 3D structures over time by balancing swelling and compacting forces with the bioink's composition.

In Chapter 4, the HA-S-based bioink system was adapted for printability. Targeting the hydrogel’s initial crosslinking reaction, gelation dynamics were tuned to the extrusion-based 3D printing process. Extensive rheological characterizations demonstrated that HA-S exhibits viscoelastic, shear-thinning, and yield stress properties in relevant ranges to facilitate bioink printability. Leveraging these rheological features, we defined a workable range to tune the apparent viscosity of the bioink at the 3D printer nozzle, and we identified a window of printability where excellent cell viability, mechanical recovery, and 3D printing resolution coexist. While this thesis established a versatile platform bioink system based on HA-S, PEGDA, and PEGylated peptides, future work is
necessary to augment the system for patterning 3D-printed signal contexts to specific applications, such as the development of bioengineered human skin. With that, the limitations of this research and recommendations for the future development of this project are detailed below.

5.2 Opportunities for future development

5.2.1 Non-covalent interactions

Reflecting on the development of this bioink system more holistically, various experiments over the course of this thesis have suggested more complex interactions than can be explained by covalent crosslinking reactions alone. Although non-covalent interactions were not directly investigated in this research, the significant impacts of intermolecular forces on the physicochemical properties of HA-S networks had become repeatedly apparent throughout this work. For example, we observed that the inclusion of amphiphilic, monofunctionalized PEG chains had (1) improved the solubility of peptide-conjugates, (2) increased non-specific, electrostatic interactions between cells and HA-S-based substrates, (3) accelerated latent disulfide crosslinking reactions, and (4) influenced the degree of network swelling in 3D. Along the same lines, in addition to the concentration and length of amphiphilic PEG chains, we also found that buffer strength, salt concentration, pH, and the presence of metal ions imparted differential biophysical effects on HA-S-based hydrogels. Collectively, these observations
suggest that non-covalent interactions play an important role in determining the biophysical features of HA-S-based networks.

While various approaches were implemented to control for non-covalent interactions in the system, the extent that physicochemical features could be leveraged for bioink patterning was not directly investigated. Supramolecular chemistry exploits hydrogen bonding, metal chelation, hydrophobic interactions, pi-pi stacking, and dispersion forces to create tunable biomaterials with complex, reversible, and directional biophysical features.\textsuperscript{138} Intermolecular forces are highly dynamic by nature, and although relatively weak on an individual basis, their combined anisotropic effects can be dramatic.\textsuperscript{137,163} Thiol chemistry is particularly useful for the rational design of supramolecular biomaterials, and further investigation into this emerging area of research is a promising direction for the future development of this tunable HA-S-based bioink system.\textsuperscript{79}

5.2.2 Biological signaling in the presence of thiols

Thiols are physiologically-relevant reactive groups that are critical to biomolecular structure and function in native signal contexts.\textsuperscript{137} Thiols undergo many important reactions in native tissues: (1) redox reactions of biological thiols provide antioxidant capacity;\textsuperscript{133–135} (2) disulfide bonding determines the tertiary structures of biological proteins;\textsuperscript{136} (3) thiol/disulfide exchange reactions regulate protein activities,\textsuperscript{164} the formation of proteoglycans,\textsuperscript{163} and the self-assembly of
supramolecular structures;\textsuperscript{165} and (4) thiols chelate biological metal ions through non-covalent, electrostatic interactions.\textsuperscript{166}

Extracellular thiols are typically presented in their oxidized form, as disulfide crosslinks (cystine), while intracellular thiols are generally presented in their “free” or reduced states (cysteine).\textsuperscript{37} The relative ratios of intra- and extracellular concentrations of cysteine-thiols and cystine-disulfides establish the redox potential across the cell membrane.\textsuperscript{133} As such, one limitation to the research presented is this thesis is that redox potentials of HAS-PEGDA networks were not directly measured. A delicate balance of thiols and disulfides exists in native tissues of interest, and exploiting these reactions for bioink crosslinking may have differential effects on cells.\textsuperscript{164,167} Additionally, the ability to tune the redox potential in HA-S-based bioinks would offer the possibility to recapitulate appropriate oxidant signaling contexts in both healthy and diseased states. For example, ROS- and antioxidant-signaling pathways are of critical interest to wound healing,\textsuperscript{48,135} traumatic brain injury,\textsuperscript{168} aging,\textsuperscript{61,169} and tissue fibrosis.\textsuperscript{167,170}

In addition to establishing redox potential, biological thiols regulate protein structure, function, and activity through disulfide bonding.\textsuperscript{37} Although proteoglycan formation was not directly investigated in this thesis, we expect that like other sulfated glycosaminoglycans (GAGs), thiol-modified HA can form proteoglycans with certain cell-secreted biological proteins. Through proteoglycan formation, HA-S could potentially (1) limit the length scales that cell-secreted biologics can act, (2) enhance local presentations of cell-signaling
molecules for more efficacious concentrations, (3) block integrin-binding sites to sequester biological activity, or (4) block MMP-degradation sites to prolong its signaling action. Further research is required to not only understand, but also to leverage HA-S proteoglycan formation in bioengineered signaling contexts.

In addition to covalent reactions, cysteine-thiols regulate protein activity in native tissues through non-covalent interactions, including with metal ions. For example, through interactions with catalytic metal ion centers, thiols can sequester protein activities, including Zn-dependent MMP enzyme functions. In biological systems, Na\(^+\), K\(^+\), Mg\(^{2+}\), Ca\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\), Fe\(^{2+/3+}\), Co\(^{2+/3+}\), Ni\(^{2+}\), and Cu\(^{+2+}\) metal ions support a variety of critical biological structures including: (1) receptor-ligand complexes, (2) catalytic enzyme centers, and (3) self-assembled ECM hydrogels. The research presented in Chapter 3 of this thesis demonstrated that cell-mediated degradation and matrix traction were limited in formulations that presented remaining HA-thiols for identical concentrations of PEGDA-VM crosslinkers and PEGMM-GRGDS adhesive ligands. The extent to which remaining thiols may sequester the action of MMPs requires further investigation.
5.3 Conclusion

While future work will be required to untangle, simplify, and program the initial signal contexts of 3D printed tissues, the HA-S-based bioink system established in this thesis demonstrates significant advantages for bioprinting applications. (1) Distinctly non-cell-adhesive, HA-S bioinks can be patterned for specific cell permissivity, (2) and thiols can be targeted at multiple time scales to incorporate bioactives independently of crosslinking reactions. Additionally, HA-S retains many of the advantageous features of native HA including: (3) non-immunogenicity, (4) excellent swelling properties, (5) a high degree of shear-thinning, and (6) the ability to recover its structural integrity after high shear. (7) The bioink system based on HAS-PEGDA also has mechanical properties that can be tuned to the 3D printing process, and independently, to influence cell fate over longer time scales through controlled network remodeling. Importantly, the crosslinking reactions leveraged in this thesis are (8) cytocompatible, and unlike most other bioinks for 3D printing, (9) enable ECM patterning without light exposure. This feature offers significant benefits, especially in 3D printing applications involving photoreactive cells, such as engineering dermal or ocular tissues. Along these lines, (10) HAS-PEGDA hydrogels have excellent optical properties, enabling encapsulated cells to be precisely stimulated with light and the use of convenient optical analytical methods. Taken together, this thesis establishes a promising platform bioink system based on HA-S, PEGDA, and PEGylated peptides as versatile building blocks for 3D bioprinting.


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A.1 Thiol-quantification with Ellman’s reagent

Ellman’s Reagent (5,5-dithiobis-2-nitrobenzoate, also known as DTNB) reacts with free thiols (-SH) and forms a yellow product (TNB) that is detectable by absorbance $\lambda_{\text{max}}=412$ nm. Ellman’s reagent was reacted in excess with known concentrations of reduced glutathione (GSH) to generate a standard absorbance curve, and the thiol concentration of 1 mg/mL HA-S was interpolated from the standard curve to be 0.9 mM, or $0.90 \pm 0.01 \mu$mol thiol per mg of HA-S ($n=6$).
A.2  **PEGMM-peptide conjugation protocol**

**Note:** Substitute PEGMM-SVA starting material with PEGMA-SVA for PEGMA-peptide conjugates then follow the rest of the protocol. Maleimides are more electrophilic than acrylates. Temperature, pH, and reaction time are reduced to minimize maleimide hydrolysis.

1. Calculate net peptide content and the net SVA content in PEGMM-SVA
2. Reconstitute the lyophilized peptide(s) to 40 mM (NH$_2$) in sterile DI H$_2$O.
   **Note:** If peptides contain primary amines in addition to the N-terminus (i.e. contain lysine) then protocol may need to be adjusted to avoid multiple sites of PEGylation.
3. Add 0.765 mL of the peptide stock (30.60 µmol peptide, in total) into a glass reaction vial.
4. Add 3.115 mL of 10x DPBS, pH 6.8 to the peptide solution.
5. Add 0.150 mL of 1M HEPES, pH 9.0 and 0.05 mL of 1M HEPES, pH 6.0 (i.e. reaction should now contain 4.08 mL of 7.5 mM peptide, in total)
6. Check that the pH is 7.2-7.4
7. Sample 80 µL of the peptide solution from the reaction vial to use later in the fluorescamine assay. (Appendix A.3) Label the sample “initial NH$_2$” and dilute with 20 µL of DI H$_2$O.
8. Weigh out at least 20 µmol of PEGMM-SVA. Store unused PEGMM-SVA under Argon, -20°C.
9. Reconstitute PEGMM-SVA to 20 mM in DI H₂O. Protect the vial from light, vortex, and work quickly from this point on.¹²⁸

10. Drop-wise, while vortexing, add 1 mL of PEGMM-SVA stock to the reaction vial containing the peptide. Vortex for 30s.

11. Rotate the reaction vial for 2h at 4°C. Vortex every half hour.

12. After 2h, sample 100 µL from the reaction vial, and store in a new, labeled, 2 mL MCT tube.

13. Dilute both samples (from step 7 & 12) with 1.9 mL of 1x PBS, pH 7.2-7.4, and vortex. The samples each have a peptide concentration of 600 µM.

14. Pipet the ~5 mL reaction solution into a 50 mL conical tube. Rinse the reaction vial with 5 mL of sterile DI H₂O, and add to the tube. Repeat the rinse 1x for a total volume of 15 mL.

15. Filter the solution using a 30 mL syringe and 0.2 µm syringe filter. Collect the filtered solution in the top of a 15 mL centrifugal filter unit with a MWCO between the peptide and the conjugate product, i.e. 3 kDa MWCO for GRGDS peptide.

16. Centrifuge for 30 minutes at 4000 RCF, or until the volume above the filter is less than 1 mL.

17. Working under sterile conditions, discard the solution below the filter.

18. Fill the top of the filter unit to the 15 mL fill-line with sterile-filtered DI H₂O. Repeat steps 16-17.
19. Repeat step 18, for a total of 3 spin cycles. If the top solution was <1 mL per cycle, the remaining salts will be <0.1% of the dry weight.

20. Pipet solution remaining in the top of the filter into a clean, dry, and pre-weighed 7 mL glass vial. Rinse filter membrane extensively with sterile-filtered H$_2$O and combine the rinses with the rest of the rxn product. Keep the total volume =<~5 mL to avoid cracking the glass when frozen.

21. Freeze the product overnight at -80°C.

22. Prepare the frozen product vials for lyophilization: parafilm the top and poke holes with a syringe needle. Return the product to the -80°C freezer for at least a few minutes or refreeze in LN$_2$.

23. Lyophilize for 4 days, or until dryness, while protecting from light.

24. Weigh the product vial and calculate the mass and % recovered.
A.3 Peptide-conjugation efficiency determination: Fluorescamine assay\textsuperscript{129,130}

1. Calculate the peptide concentration in reaction samples and dilute the sample with 1x PBS to a peptide concentration of 100 µM.

2. Confirm pH is 7.4 ± 0.2.

3. Optional (see step 6): From 100 µM reaction samples, prepare (1:1) step-wise dilutions containing 50, 25, 12.5, 6.25, and 0 µM peptide.

4. Weigh out ~2 mg of peptide standard and dilute in reaction buffer to 20 mM, then with 1x PBS to 100 µM. This maintains consistent buffer salts.

5. Confirm pH is 7.4 ± 0.2.

6. From 100 µM stock solution, prepare (1:1) step-wise dilutions of 50, 25, 12.5, 6.25, and 0 µM peptide standard.

7. Plate conditions 80 µL/well in a 96-well black assay plate.

8. Dissolve fluorescamine 3 mg/mL in neat acetone and plate 20 µL/well.

9. Protect the plate from light and rotate for 10 minutes on the plate rocker.

10. Measure the fluorescence intensity at $\lambda_{\text{ex}} = 400$ nm and $\lambda_{\text{em}} = 460$ nm.

11. Plot a standard curve from the intensity readings of the known concentration of peptide standard. Use a linear interpolation to estimate the unknown amine concentration of the reaction solution. This represents the concentration of non-grafted GRGDS. Estimate conjugation efficiency:

$$\text{Conjugation Efficiency (\%)} = \frac{[\text{NH}_2]_{\text{initial}} - [\text{NH}_2]_{\text{final}}}{[\text{SVA}]_{\text{initial}}} \times 100$$
Fluorescamine was used to quantify known initial GRGDS concentration and interpolate residual primary amine content following conjugation with PEGMX-SVA. Peptides (NH$_2$) were reacted with PEGMX-SVA in a 3:2 molar ratio, with the peptides in excess of SVA. The residual peptide content (diluted to 100 µM) were compared to the known peptide standards by reacting with fluorescamine and measuring fluorescence intensity. Grafting efficiencies were calculated from the most concentrated reaction solution (100 µM initial) and interpolating the residual primary amine content from the known standard intensity curve.

**FIGURE A.3.A. GRGDS PRIMARY AMINE STANDARD CURVE: PEPTIDE CONJUGATION EFFICIENCY DETERMINATION WITH FLUORESCAMINE**
Fluorescamine reactivity with N-terminal primary amines is pKa-dependent. The pKa of the N-terminus is influenced by the first amino acid in the peptide sequence. The pKa of a peptide with glycine at the N-terminus is approximately 8.5, while a peptide with an N-terminal serine residue is approximately 7.6. This suggests that at pH 7.4, fewer primary amines are available to react with SVA (and fluorescamine) in GRGDS peptides than compared to SDGRG. However, the lower the pKa, the stronger the acid and the weaker the conjugate base. Weak bases are stable bases, and under these conditions, weak bases are also weak nucleophiles. Therefore, conjugation efficiency is two-fold in that both the availability of primary amines, dictated by the reaction pH, and the relative reactivity, dictated by pKa, influence nucleophilic reactions with primary amines.
Peptides with various N-terminal amino acids were reacted with fluorescamine across multiple reaction buffer pH. We demonstrated that fluorescence intensity (fluorescamine reactions) peak at approximately 1 pH unit above the pKa of the N-terminus. At high pH, hydrolysis of fluorescamine (and SVA) competes with primary amine reactions,\textsuperscript{128,129} as demonstrated by the characteristic decrease in fluorescence intensity.
FIGURE A.3.C. CONDITIONS TO MINIMIZE OFF-TARGET REACTIONS BETWEEN MALEIMIDE REACTIVE GROUPS AND PRIMARY AMINES (NH$_2$)

(continued…)
Maleimides are significantly more electrophilic than acrylates and can react with primary amines.\textsuperscript{139} To control for off-target reactions during peptide-conjugation experiments, various conditions of pH, temperature, and time were studied. Fluorescamine was used to quantify primary amine concentration at baseline, 1 hour, 2 hours, 4 hours, and 24 hours during reactions with PEGMX-SVA, where X denotes acrylate or maleimide reactive groups, and PEGMX as positive controls for off-target reactions. The results are presented as mean fluorescence intensities $\pm$ SD (n=4). Fluorescamine intensity is proportional to primary amine concentration and is presented as a normalized mean to the baseline intensity for each reaction solution.
A.4 PEGDA-MMP-degradable peptide-crosslinker preparation

1. Calculate the net peptide content and net acrylate content in PEGDA.

2. Weigh out 10 µmol of the VPM peptide: GCRDVPMRSMRGGDC (MW: 1697.0, Lifetin). Store any extra peptide under Argon at -20°C.

3. Weigh out at least 40 µmol per reaction of PEGDA (MW: 2 kDa, Laysan) in a conical tube.

4. Reconstitute the peptide to 10 mM in 10x PBS.

5. Reconstitute PEGDA to 10 mM in DI H₂O. Vortex.

6. Transfer 4 mL of the PEGDA solution into an amber reaction vial.

7. Dropwise while vortexing, add 1 mL of the peptide solution to the reaction vial containing PEGDA. Vortex for 30s.

8. Protect from light and rotate at 4°C for 2h.

9. Pipet the ~5 mL reaction solution into a 50 mL conical tube. Rinse the reaction vial with 5 mL of sterile DI H₂O, and add to the tube. Repeat the rinse 1x for a total volume of 15 mL.

10. Filter the solution using a 30 mL syringe and 0.2 µm syringe filter, and collect the filtered solution into the top of a 15 mL centrifugal filter unit (Amicon-Ultra15) with a MWCO of 10kDa.

11. Centrifuge for 30 minutes at 4000 RCF, or until the volume above the filter is less than 1mL.

12. Working under sterile conditions, collect the solution below the 10 kDa MWCO filter, and transfer into a centrifugal filter unit with a 3 kDa MWCO.
13. Fill the top of the 10k Da filter unit to the 15mL line with sterile-filtered DI H₂O. Centrifuge both conical tubes for 30 minutes at 4000 RCF, or until the volume above the filter is less than 1 mL.

14. Discard the solution below the 3 kDa MWCO filter.

15. Discard the solution on top of the 10 kDa MWCO filter, and transfer the solution below the 10 kDa MWCO filter membrane into the 3 kDa MWCO filter unit. At this point the reaction solution unit should be purified of high MW species (>10 kDa).

16. Centrifuge the 3kDa MWCO filter unit for 30 minutes at 4000 RCF, or until the volume above the filter is less than 1mL.

17. Fill the top of the filter unit to the 15 mL line with sterile-filtered DI H₂O.

18. Repeat for a total of 3 spin cycles with pure water.

19. At this point, the solution in the 3kDa MWCO filter unit should be purified of both high MW (>10kDa) and low MW (<3kDa) species. Pipet the solution remaining in the top of the 3kDa MWCO filter into a clean, dry, and pre-weighed 7 mL glass vial. Rinse the filter membrane extensively with sterile-filtered DI H₂O, keeping the total volume =<5 mL in the 7 mL vial for sufficient headspace. (If overfilled, the glass might crack.)

20. Freeze the product overnight at -80°C.

21. Lyophilize for 4 days, or until dryness, while protecting from light.

22. Weigh the product vial and calculate the mass and % recovered.
A.5 **Competitive adhesion assay protocol**

**Note:** This protocol was created for the following publication:


1. Coat 96-well plate with 0.1 mL/well of 50 µg/mL of human type-III collagen (Advanced BioMatrix, San Diego, Ca) in 10mM acetic acid (0.01 N) overnight at 4°C.

2. Aspirate collagen solution, then add 0.2 mL/well of 5% BSA solution in 1x PBS (sterile-filtered) to each well. Incubate for 1-2 hours at 37°C.

3. Rinse plates 3x with 0.2 mL/well of sterile-filtered wash buffer: 0.1% BSA with 5 mM MgCl in 1x PBS.

4. Pre-dye cells with 2.5 µL/mL CMFDA-Green (1 mM stock) in MEM-alpha. Use 10 mL of the (0.0025 mM) solution for T75 flask or 25 mL for T225 flask. Medium must be serum- and cysteine-free.

5. Incubate the cells with the dye for 40 minutes at 37°C. Aspirate the dye and replace with complete culture media.
6. In the meantime, prepare each peptide dilution in wash buffer (Mg+).

7. To positively control for cell adhesion and competitive binding, respectively, prepare solutions of wash buffer (Mg+) containing no peptide or protein and dilutions of soluble type-III collagen (25, 50, and 100 µg/mL) to competitively inhibit cell interactions with (adsorbed) collagen-III.

8. To negatively control for ligand-mediated interactions, prepare peptides and proteins in sterile buffer without Mg: 0.1% BSA and 1 mM EDTA in 1x PBS.

9. After 1 hour of incubation with 1% BSA, aspirate and rinse plates with 0.2 mL/well sterile complete cell media. Aspirate and set plate aside in hood.

10. Re-suspend cell pellet in complete media to 10x the final cell seeding density.

11. Add 0.1 mL 10x cell suspension to 0.9 mL peptide solution. Invert to mix.

12. Plate 0.1 mL/well and incubate for 2h at 37°C.

13. After 2h, look at the adhesion under the light scope. Make sure cells have attached to positive control wells (no soluble peptide), then aspirate.

14. Very carefully, rinse with 1 volume of PBS.

15. Fix cells with 100 µL/well 4% PFA.

16. Read fluorescence intensity on plate reader (λ_{ex}: 488, λ_{em}: 520)

17. Stain for DAPI and/or phalloidin, then image and count number of cells/well.
FIGURE A.5.A HMSC STANDARD FLUORESCENCE INTENSITY CURVE
FIGURE A.5.B. RAT DERMAL FIBROBLAST STANDARD FLUORESCENCE INTENSITY CURVE

\[ R^2 = 0.9996 \]
FIGURE A.5.C. UNBOUND RGD LIGAND COMPETITIVELY INHIBITS CELL ADHESION TO MATRIX-BOUND RGD

(Caption continued to following page…)
Hydrogels of 0.8% HA-S, 0.4% PEGDA, and various concentrations of PEGMA-GRGDS (0, 1, 2, 4, or 8 mM) were prepared in 96-well plates (50 µL/well). Hydrogels were thoroughly washed with PBS prior to seeding cells. After 2 hours of hMSC attachment on top of the hydrogels, the density of adherent cells increases with increasing concentration of grafted ligand until the thiol-saturation point (approximately 4.5 mM). In positive control wells for competitive adhesion, vitronectin (5 µg/well) was adsorbed to the tissue culture plate, and cells were incubated either in the presence or absence of soluble GRGDS peptide. In the absence of soluble ligand, hMSCs adhere strongly to the wells, while 1 mM of soluble GRGDS virtually eliminates adhesion to plates. Results are presented as mean fluorescence intensities of pre-dyed hMSCs ± SD in replicates of n=4.
A.6  Resolution G-code

; Start code

G90 G21; absolute coordinates, mm units

G28

G01 X0 Y0 Z20 F1000

G01 X19 Y52

G91

G01 X20 Y20

G4 S60; purge headspace volume

; tubing ID: 0.08cm, headspace vol.: 0.00503mL/cm x 25cm = 0.125mL.

; purge time = headspace vol/(extrusion rate 1mL/min) x 60s

; Resolution Lines

G91

G01 X30 Y30 F5000

G01 X5 Y-10

G01 X10 Z-19 F7000; change z depending on offset 1

G01 Y-20

G01 X10 F5000

G01 Y20

G01 X10 F2500
G01 Y-20
G01 X10 F1000
G01 Y60

;End code
G90
G01 Z20 F7000
G01 X-75
G01 Y-60
A.7 Cell viability G-code

;Start code

G90 G21; absolute coordinates, mm units

G28

G01 X0 Y0 Z20 F1000

G01 X19 Y52

G91

G01 X20 Y20

G4 S60; purge headspace volume; tubing ID: 0.08cm, vol.: 0.00503mL/cm x 25cm = 0.125mL; purge time = headspace vol/(extrusion rate 1mL/min) x 60s

;96-well plate

G91

G01 X30 Y80 F5000 ; move to position (100,60) - TC plate set top left corner (60,20)

G01 X14.53 Y-11.23 ; A1

G4 S60; time depends on extrusion rate and volume/well - here: 0.05mL/min, 50ul/well

G01 Y-9.02 ; B1

G4 S60

G01 Y-9.02 ; C1

G4 S60
G01 Y-9.02 ; D1
G4 S60
G01 Y-9.02 ; E1
G4 S60
G01 Y-9.02 ; F1
G4 S60
G01 Y-9.02 ; G1
G4 S60
G01 Y-9.02 ; H1
G4 S60
G01 X9.02; H2
G4 S60
G01 Y9.02; G2
G4 S60
G01 Y9.02; F2
G4 S60
G01 Y9.02; E2
G4 S60
G01 Y9.02; D2
G4 S60
G01 Y9.02; C2
G4 S60
G01 Y9.02; B2
G4 S60
G01 Y9.02; A2
G4 S60
G01 X9.02; A3
G4 S3; 0.1mL/min.
G01 Y-9.02; B3
G4 S30
G01 Y-9.02; C3
G4 S30
G01 Y-9.02; D3
G4 S30
G01 Y-9.02; E3
G4 S30
G01 Y-9.02; F3
G4 S30
G01 Y-9.02; G3
G4 S30
G01 Y-9.02; H3
G4 S30
G01 X9.02; H4
G4 S30
G01 Y9.02; G
G4 S30
G01 Y9.02; G4
G4 S30
G01 Y9.02; F4
G4 S30
G01 Y9.02; E4
G4 S30
G01 Y9.02; D4
G4 S30
G01 Y9.02; C4
G4 S30
G01 Y9.02; B4
G4 S30
G01 Y9.02; A4
G4 S30
G01 X9.02; A5
G4 S15; 0.2mL/min.
G01 Y-9.02; B5
G4 S15
G01 Y-9.02; C5
G4 S15
G01 Y-9.02; D5
G4 S15
G01 Y-9.02; E5
G4 S15
G01 Y-9.02; F5
G4 S15
G01 Y-9.02 ; G5
G4 S15
G01 Y-9.02 ; H5
G4 S15
G01 X9.02; H6
G4 S15
G01 Y9.02; G6
G4 S15
G01 Y9.02; F6
G4 S15
G01 Y9.02; E6
G4 S15
G01 Y9.02; D6
G4 S15
G01 Y9.02; C6
G4 S15
G01 Y9.02; B6
G4 S15
G01 Y9.02; A6
G4 S15
G01 X9.02; A7
G4 S6; 0.5mL/min.
G01 Y-9.02 ; B7
G4 S6
G01 Y-9.02 ; C7
G4 S6
G01 Y-9.02 ; D7
G4 S6
G01 Y-9.02 ; E7
G4 S6
G01 Y-9.02 ; F7
G4 S6
G01 Y-9.02 ; G7
G4 S6
G01 Y-9.02 ; H7
G4 S6
G01 X9.02; H8
G4 S6
G01 Y9.02; G8
G4 S6
G01 Y9.02; F8
G4 S6
G01 Y9.02; E8
G4 S6
G01 Y9.02; D8
G4 S6
G01 Y9.02; C8
G4 S6
G01 Y9.02; B8
G4 S6
G01 Y9.02; A8
G4 S6
G01 X9.02; A9
G4 S3; 1mL/min.
G01 Y-9.02; B9
G4 S3
G01 Y-9.02; C9
G4 S3
G01 Y-9.02; D9
G4 S3
G01 Y-9.02; E9
G4 S3
G01 Y-9.02; F9
G4 S3
G01 Y-9.02; G9
G4 S3
G01 Y-9.02; H9
G4 S3
G01 X9.02; H10
G4 S3
G01 Y9.02; G10
G4 S3
G01 Y9.02; F10
G4 S3
G01 Y9.02; E10
G4 S3
G01 Y9.02; D10
G4 S3
G01 Y9.02; C10
G4 S3
G01 Y9.02; B10
G4 S3
G01 Y9.02; A10
G4 S3

;End code
G90
G01 Z20 F7000
G01 X-75
G01 Y-60