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Engineering Novel Materials for Stem Cell Culture and Expansion

By

Barbara Louise Ekerdt

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Chemical Engineering

in the

Graduate Division

of the

University of California, Berkeley

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Professor David V. Schaffer, Co-chair
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Barbara Louise Ekerdt

Abstract

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By

Barbara Louise Ekerdt

Doctor of Philosophy in Chemical Engineering

University of California, Berkeley

Professor David V. Schaffer, Co-Chair
Professor Rachel A. Segalman, Co-chair

Stem cells are a unique class of cells residing in the body which can replicate indefinitely and develop into more specialized types of cells, a process known as differentiation. Specifically, pluripotent stem cells have the ability to turn into all types of cells found in the body. Embryonic stem cells (ESCs) are the only natural pluripotent stem cell, however much progress in the last decade has expanded the potential of generating induced pluripotent stem cells (iPSCs) from cells as basic as human skin cells. Stem cells, especially iPSCs, could be utilized for many potential therapeutic purposes including tissue engineering, cell replacement therapies, disease modeling, and drug screening. These technologies are not currently feasible for general use due to large amounts of cells required for each application, extremely large patient populations, and few cells surviving implantations. Current stem cell culturing methods do not generate cell numbers at a scalable rate or provide the appropriate biochemical and mechanical cues to aid in proliferation or differentiation to the desired cell types. Therefore, this dissertation focuses on designing and developing novel tunable systems to expand stem cell culture in a scalable manner.

This work investigates two different material systems to culture stem cells in three-dimensional (3D) hydrogels. Stem cells exist in very complex microenvironments, receiving inputs from other cells, the extracellular matrix, as well as soluble factors. The systems described in this work aim to create *in vitro* environments which support cell viability, growth, replication, and potential differentiation by tuning the mechanical and biochemical properties of the hydrogels. Substrate modulus affects stem cell behavior in two-dimensional systems and could play a large role in stem cell behavior and differentiation in 3D systems as well. Therefore, both systems were optimized for the appropriate modulus to support iPSC growth, with the potential to generate gels of varying moduli for enhanced proliferation or differentiation of multiple types of stem cells. Both systems were also designed with specific chemical functionality in order to attach biochemical cues such as proteins or peptides, which may help to better mimic the *in vivo* environments and improve cell viability.

These systems were both designed to easily encapsulate and recover cells by incorporating a thermoreversible component poly(N-isopropylacrylamide) (PNIPAAm). Thermoreversibility and encapsulation overcome the limitations of harsh recovery steps and shear stress associated with many current 3D cell culture methods. One hydrogel system used poly(ethylene glycol) (PEG) as the hydrophilic component due to its versatility, biocompatibility, and hydrophilicity. The other hydrogel system attached PNIPAAm to hydrophilic hyaluronic acid (HA), a natural polysaccharide found in the extracellular matrix which plays an important role in cell signaling and matrix organization. Both of these hydrogel systems successfully supported the growth and expansion of hPSCs, which maintained expression of pluripotency markers after growing within the gels.

In summary, this dissertation presents work on designing and developing novel hydrogel materials to encapsulate and grow stem cells to aid in the production of stem cells for future therapeutics.

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Chapter 1 - Introduction

1.1 Stem cell background

Stem cells are a unique class of cells found in the mammalian body which have the abilities to replicate indefinitely and to turn into more specialized types of cells, a process known as differentiation. Embryonic stem cells (ESCs) are the first types of cells forming the embryo during development [1]. These cells can therefore differentiate into all types of cells found in the body and are defined as pluripotent [2-4]. During development, the embryo separates into three different layers which give rise to specific types of tissues. The endoderm, mesoderm and ectoderm are the innermost, middle, and outermost layers of the embryo, respectively. The endoderm turns into tissues such as pancreatic cells and lung cells, mesoderm generates muscle, bone, and blood cells, and ectoderm produces cells such as skin cells, pigment cells, and neurons. Pluripotent cells can give rise to multipotent cells which are tissue specific and can only differentiate into cells of that tissue [5]. Multipotent cells include neural stem cells, mesenchymal stem cells, and hematopoietic stem cells which can turn into neurons, muscle cells, and blood cells, respectively [6-8].

Since embryonic cells are pluripotent and can turn into all types of cells found in the body, they are a very desirable cell type to start most kinds of therapies or research, however there are many ethical concerns with using embryonic cells. Thankfully there has been much progress in the field within the last decade to generate induced pluripotent stem cells (iPSCs) from cells as innocuous as adult human skin cells (fibroblasts) [9, 10]. In 2006 Takahashi and Yamanaka published on successfully reprogramming adult mouse fibroblasts into induced pluripotent stem cells from the overexpression of a defined set of genes [11]. This discovery started a whirlwind of research and optimization of determining the most ideal chemicals or proteins necessary to reprogram adult human cells into iPSCs [12, 13]. iPSCs are not only useful as an essentially limitless source of pluripotent cells but also for studying basic stem cell biology concepts and are a promising source for human therapeutics.

Stem cell microenvironment

Stem cells live in very complex environments *in vivo*, receiving cues from many types of factors in their surroundings. External physical, chemical, and biological cues help to regulate stem cell behavior, specifically maintenance, proliferation, self-renewal and differentiation [14]. Cells receive inputs from neighboring cells, soluble cues, as well as the biomechanical and biochemical cues of the extracellular matrix (ECM) [15]. Replicating this multifaceted environment *in vitro* has been attempted through the use of novel surfaces and biomaterials, specific soluble and immobilized biochemicals, and with co-culture of other cell types [14, 16-18]. Simplifying the microenvironment to attain the desired cell behavior is difficult but could lead to a better understanding of stem cell biology and faster or more efficient generation of stem cells for future therapeutic purposes.

Stem cell therapies

Some potential therapeutic uses for stem cells (and particularly iPSCs) include cell replacement therapy, tissue engineering, high-throughput drug screening, and disease modeling [13, 19-24].

Cell replacement therapy

Cell replacement therapy has been a promising route to potentially treat patients with neurological disorders or neurodegenerative diseases (such as Parkinson's disease and Alzheimer's disease) for many years [25, 26]. The principle behind the technology is essentially to replace or repair damaged or diseased tissues with healthy cells, supporting cells, or stem cells which will turn into the cell type which is damaged or dead [19]. Cell replacement therapies have expanded to include a range of target conditions or diseases including heart attacks [27, 28], diabetes [29-31], and liver failure or disease [32, 33]. Stem cells are very useful for this therapeutic purpose, whether as part of a mix of implanted cells or as the starting point to generate cells of a desired lineage [24]. Cell replacement therapies can also benefit from the advancement of iPSC technology by using iPSCs generated from the patient's own cells, thus eliminating the issues of immune reaction [9].

Tissue engineering

Tissue engineering is another important use of stem cells for therapeutic purposes, also referred to as regenerative medicine [22, 34-36]. Tissue engineering utilizes novel materials, cells, and biochemical cues to generate a portion of or entire tissues. Tissue engineering was initially performed by using cells isolated and expanded from donor tissue, whether from a different individual or from a biopsy of the affected patient [34]. This route did not always provide an appropriate amount of cells, either from a lack of viable healthy cells in the diseased tissue or an inability to culture cells extracted from the tissue [34]. Immune reactions were also a large concern when the engineered tissue utilized cells that did not come from the patient [37]. iPSCs can minimize these concerns since they can be reprogrammed from the patient's own cells and expanded to a suitable amount before developing into the tissue-specific cell types needed [35].

Drug discovery and toxicology screening

Stem cells can also be harnessed for drug discovery and toxicology screening [10, 38, 39]. iPSCs generated from patients with a range of diseases can be used to produce the specific cells with the diseased phenotypes and can potentially be used as *in vitro* models of the disease as well as a way to screen for drugs that may treat the disease or disorder [10, 40-42]. These cells overcome the issues of difficult access to affected tissues as well as the minimal amounts of cells recoverable from patients with degenerative diseases [13]. In addition, drug screening could be greatly improved with the utilization of iPSCs instead of animal models or immortalized cell lines [39]. For example, animal models for Amyotrophic lateral sclerosis (ALS) prove ineffective when transitioned into human patients, potentially due to the difference in the disease between species [43]. Teratogen screening to find drugs or chemicals which affect human embryo development also does not translate well between species, as seen with thalidomide malformities in humans and no reactions with mice or rats [44]. Toxicity studies could also be improved by initially testing for organ-specific toxicity and eliminating those drugs from the next round of screening [39]. Important tissues to analyze drug side effects include the heart, liver, and central nervous system, all of which can be generated from iPSCs [39].

Large numbers of stem cells needed

All of these therapeutic uses of stem cells require very large numbers of cells. A myocardial infarction would require approximately 50g of heart muscle, which corresponds to approximately $1\text{--}2 \times 10^9$ myocytes [45]. Similarly, treatment of a 70kg patient with Type I diabetes would require $\sim 1.3 \times 10^9$ insulin-producing β cells for insulin independence [46] and an artificial liver would require $\sim 1 \times 10^{10}$ hepatocytes [47]. Parkinson's disease would require $\sim 1 \times 10^5$ surviving dopaminergic neurons to help treat the disease [21]. In addition, very few cells survive transplantation with reports of less than 10% survival for injected cardiomyocytes or dopaminergic neurons [48, 49]. Large patient populations which could benefit from these therapies exponentially increase the demand for stem cells. For instance, more than $\sim 500,000$ people suffer from cardiac arrest each year in the United States alone [50] and approximately 21 million people in the United States are diagnosed with diabetes with over 8 million people estimated to be undiagnosed [51]. Another alarming statistic is that almost 10 million people in the United States are living with Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis and/or spinal muscular atrophy [52]. Generating enough cells to treat even just the newly affected patients each year is currently unfeasible. Toxicology screens would also require on the order of $\sim 2 \times 10^6$ cells per 5,000 compounds, however there are millions of drugs and chemicals that need to be analyzed [53]. Therefore a scalable system which can generate large amounts of stem cells and their progeny is necessary for stem cell technologies and therapeutics to become more of a reality.

1.2 Stem cell culturing methods

Current standard culture methods

The current standards for culturing hPSCs utilize flat, two-dimension (2D) surfaces in combination with either Matrigel (a gelatinous mixture of proteins secreted from mouse sarcoma cells) or a layer of animal cells underneath [54]. These methods are not scalable for the amount of stem cells that need to be produced for human therapeutics due to the potential for immunogenic reactions, low yield, large surface area required, and labor-intensive and time-consuming procedures [54]. Some synthetic or protein surfaces have been developed to create a more defined and xenogenic-free environment, however these can become cost-prohibitive and do not overcome the problem of scaling up in 2D [55-57]. Three-dimensional (3D) systems are viewed as a more promising alternative to 2D culture because they can better mimic the *in vivo* developmental environment and significantly increase the yield and cell viability in a manner much more conducive to current biomanufacturing practices [58].

Culturing as aggregates in suspension

Culturing stem cells as aggregates in a spinner flask or bioreactor is the most basic way to culture in 3D as there is no recovery step from a solid substrate or surface. Single cells or small aggregates will grow into aggregates over the course of multiple days in spinner flasks with appropriate agitation speeds [54, 59]. Aggregate size, seeding density, and agitation speed are all very important parameters for growing aggregates in suspension [59]. When aggregates become too large (either from a fast growth rate or aggregates merging together), the diffusion of nutrients and oxygen to the middle is more difficult and the cells in the middle tend to die or

differentiate, whereas if the agitation speed is too fast the cells on the outside of the aggregates may experience harmful shear forces and also die or differentiate [54].

Culturing with microcarriers

Microcarriers have been utilized to control the size of cell aggregates grown in suspension and to provide a surface for stem cells to anchor [60]. Microcarriers can provide a similar adhesive area for cells when coated in Matrigel, laminin, vitronectin, or mouse feeder layers and they have a high surface area to volume ratio, producing a higher cell yield than 2D culture [60-62]. Using macroporous microcarriers can increase the surface area even more but may not improve the cell viability or proliferation rate [63]. Microcarriers improve the culture conditions and yield over 2D and suspension culture, however they also suffer from clumping in suspension, shear forces on outermost cells, and potentially harmful separation steps [54].

Cell encapsulation and culture

Cell encapsulation is another route to scale-up stem cell production without the harmful effects of shear stress. Different materials including alginate, hyaluronic acid, polyethylene glycol, and agarose can be used to generate environments for enhanced proliferation or differentiation of stem cells [58, 64-66]. Cell encapsulation can provide a more similar environment to what cells experience *in vivo* than 2D methods and also offers high surface to volume ratios for higher yields [54]. Significant progress has been made in developing systems to encapsulate cells, however most systems which encapsulate cells require potentially harsh recovery steps using enzyme or chemical treatments [58, 67-69]. Therefore, there is a need for cell microencapsulation technology which can be used to culture stem cells and recover them in a gentle manner. Developing a thermoreversible microencapsulation system, or hydrogel, would provide a way to easily recover the cells in a fast and simple manner.

1.3 Hydrogels

Hydrogels are three-dimensional networks of hydrophilic synthetic or natural polymers which have a high affinity for water and can swell in the presence of water or physiological fluids [70, 71]. Depending on the composition and nature of the hydrogel many properties can be varied including biodegradability, water uptake, stimuli responsiveness, and mechanical durability or stiffness [70, 72, 73]. Hydrogels are especially useful for biomedical applications including drug delivery, cell encapsulation, and tissue engineering because of their biocompatibility, high water content, and porous nature, making diffusion of nutrients or drugs into or out of the hydrogel generally uninhibited [73, 74]. They are especially promising for cell encapsulation and tissue engineering when materials are selected for their specific biological roles such as proliferation or differentiation [64, 66, 74, 75]. There are two major categories of hydrogels and their routes of formation. Chemically crosslinked hydrogels are formed when a crosslinker reacts with chemical groups on polymer chains generating a “permanent” hydrogel, while physically-linked hydrogels are formed from secondary interactions including hydrophobic or ionic interactions between polymer chains due to an environmental stimulus like pH or temperature [70, 73]. One very important route to generate physically-linked hydrogels is with the incorporation of thermoresponsive polymers.

Thermoresponsive hydrogels

Thermoresponsive polymers change their state from a solution to a gel upon increasing or decreasing the environment temperature. Polymers that experience this sol-gel transition upon temperature elevation have a lower critical solution temperature (LCST), below which the polymer is soluble and above which the polymer becomes insoluble and either crashes out of solution or forms a gel depending on what it is attached to [70]. This temperature-induced phase transition is attributed to the change in free energy upon temperature elevation, making the polymer-polymer and water-water interactions more favorable than polymer-water interactions, also referred to as the hydrophobic effect [70, 76].

Poly(N-isopropylacrylamide) polymers

Poly(N-isopropylacrylamide) (PNIPAAm or PNIPAM) is one of the most common thermosensitive polymers used for biomedical applications because of its biocompatibility, LCST $\sim 32^{\circ}\text{C}$, and nonbiodegradability [70, 77]. The LCST can also be increased or decreased by incorporating hydrophilic or hydrophobic components within the polymer, therefore making a tunable system for different biomedical or therapeutic needs [78-80]. PNIPAAm has been attached to many types of natural and synthetic polymers including acrylic acid [81], hyaluronic acid [82, 83], polyethylene glycol [84, 85], and gelatin [86] to produce thermosensitive hydrogels. Almost all thermosensitive polymers to date are designed for tissue engineering, implantation, or drug delivery, as opposed to cell culture and expansion [70, 77, 87-89]. We previously reported on a thermoreversible PNIPAAm-poly(ethylene glycol) (PNIPAAm-PEG) hydrogel for hPSC culture and expansion which provided a 10^{72} -fold expansion over 280 days with high yield and high purity cells [90]. Building on this novel technology we wanted to develop additional thermoreversible hydrogels which could be modularly tuned and biochemically functionalized.

Polyethylene glycol hydrogels

One hydrogel system we developed utilized PEG as the hydrophilic component, based on the original PNIPAAm-PEG hydrogel which supported cell culture and expansion. PEG (also referred to as polyethylene oxide) is an FDA-approved material for many biomedical applications including implantations and is not immunogenic or toxic [91, 92]. PEG is a useful material for hydrogels because of its biocompatibility, non-specific protein adsorption, hydrophilicity, and versatility [93]. PEG was originally used to prevent protein adhesion and ultimately bacterial and eukaryotic cell adhesion [94-97]. PEG has also been attached to proteins or peptide drugs in order to increase the half-lives of these components and shield them from damage or degradation in the body [92]. PEG has been incorporated into numerous types of hydrogels, whether as a crosslinker between other types of polymer chains or as the main hydrophilic component [75, 98].

Thermoresponsive PEG-PNIPAAm hydrogels

Thermoresponsive hydrogels composed of PEG and PNIPAAm have been developed previously with the intention of implanting into the body. PNIPAAm-PEG block copolymers were used to culture chondrocytes for 7 days to show potential as a cartilage tissue replacement [84]. Mesenchymal stem cells were encapsulated in PNIPAAm-co-PEG methacrylate hydrogels and remained viable after 7 days in culture, showing a proof of concept for use in tissue engineering [85]. Recently, a PNIPAAm-PEG hydrogel was used to maintain MSC viability for 31 days in a

scaffold designed for spinal cord injury repair [99]. Crosslinked PNIPAAm-PEG hydrogels have also been designed for drug and protein delivery [100-102]. Our group published recently on the only PNIPAAm-PEG hydrogel used specifically for cell culture and expansion, successfully encapsulating hPSCs as single cells or aggregates and achieving ~ 10-20-fold increase in cell number over the course of 4-5 days reaching 10^{72} -fold expansion over 280 days with ~95% Oct4 positive cells [90].

Hyaluronic acid hydrogels

Hyaluronic acid (HA) (also known as hyaluronan or hyaluronate) is another very common material for hydrogels for many biomedical applications. It is a natural polysaccharide made of repeating disaccharide units and can retain large amounts of water, thus making it essential in tissues and the extracellular matrix [103]. HA can bind specific cell-surface receptors including CD44 and RHAMM (receptor for hyaluronic acid-mediated motility) [104] and therefore plays an important role in cell signaling as well as matrix organization and morphogenesis [103, 105, 106]. HA is degraded by hyaluronidase within the body and can be easily functionalized with different chemical groups by utilizing the carboxylic acid or hydroxyl moieties within the disaccharides, proving extremely useful for many biomedical applications including tissue engineering and regenerative medicine [105, 107]. Some of the most common uses of HA hydrogels include cell implantation, drug or protein delivery, and tissue engineering [105, 108-111]. Disulfide-linked HA-PEG hydrogels have been generated for cell culture and recovery by the addition of N-acetyl-L-cysteine, though these were not successively expanded [69]. hESCs were also shown to proliferate and remain undifferentiated after 20 days encapsulated in an HA hydrogel, suggesting an HA hydrogel from which cells can be easily recovered may prove advantageous for the culture of hPSCs [67].

Thermoresponsive HA-PNIPAAm hydrogels

Since HA hydrogels are very useful in tissue engineering, cell delivery, and drug delivery applications, there are some HA hydrogels designed to be thermoresponsive for these specific purposes [112]. HA-PNIPAAm hydrogels used as injectable carriers of nucleus pulposus cells to the spine [113], retinal pigment epithelial cells to the retina [114], and adipose-derived stem cells to subcutaneous region [82] prove the usefulness of thermosensitive HA polymers for cell and tissue replacement and wound repair. HA-PNIPAAm has also been used as a tissue adhesion prevention material during surgery [115] and for drug delivery to the bladder when incorporated with gelatin [116]. There have not been any thermoreversible HA polymers developed specifically for (stem) cell culture and expansion.

Tunable hydrogels

Stem cells experience many different types of physical, chemical, and biological signals in their native microenvironments. Adult stem cells are found in multiple parts of the body from the bone marrow to parts of the brain while embryonic stem cells are only in the developing embryo. Because of the diverse biomechanical inputs from these unique tissue environments, hydrogel systems designed to culture multiple types of stem cells and their progeny need to be tunable to reflect these different tissue microenvironments. In an ideal system, mechanical and biochemical properties such as stiffness and protein concentration can be tuned for specific cell responses.

Hydrogel mechanical properties

There has been much work in the stem cell field focusing on how mechanical properties of the stem cell environment can alter stem cell fate [117]. For instance, mesenchymal stem cells differentiate into neurons on soft substrates (0.1-1 kPa), bone on stiff substrates (25-40 kPa), and muscles on substrates with a medium stiffness (8-17 kPa) [118]. In addition, neural stem cells also respond to stiffness in different ways, with softer substrates (<1 kPa) generating more neurons while stiffer substrates (>1 kPa) generating more astrocytes [119]. Therefore, mechanical properties of the hydrogels need to be taken into account when trying to optimize proliferation or differentiation of stem cells.

Biochemical cue incorporation

Biochemical cues play a large role in cell behavior and can influence the proliferation or differentiation of stem cells as well as provide signals for other supporting cells. For instance, morphogen gradients can regulate stem cell fate and the development of tissues [120], certain growth factors (proteins) stimulate or control the growth of blood vessels [121], and others may enhance stem cell survival, proliferation, or differentiation capabilities [122-124]. Incorporating biochemical cues into materials in which the cells are cultured can help to enhance their signaling effects on the stem cells. Immobilizing biochemical ligands to a hydrogel or scaffold can increase local concentration, increase protein potency, and reduce the possibility of cell-mediated endocytosis [125-127]. Many proteins have heparin-binding domains which contain positively charged protein residues that interact with negatively-charged sulfate groups on heparin [128]. Conjugating heparin to materials will indirectly bind these proteins thus releasing in a controlled manner. Multiple hydrogel systems have incorporated heparin specifically for the controlled release of a growth factor or to aid in cell growth, survival, or differentiation [121, 129-133]. Therefore, incorporating biochemical cues directly or indirectly to a hydrogel designed for cell culture and expansion could help improve the proliferation and viability of the stem cells.

1.4 Scope of the dissertation

This dissertation was motivated by the tremendous need to develop materials to produce stem cells of various types in a scalable manner in order to meet the demands for therapies stemming from current stem cell research. We developed two different hydrogel systems to make this process more attainable while utilizing fully-defined animal-component free materials. These systems are thermoreversible and tunable via mechanical, rheological, and biochemical properties in order to enhance the environment for cell proliferation or differentiation. Chapter 2 discusses the development of a tunable thermoreversible PNIPAAm-PEG hydrogel for the culture of hPSCs. Chapter 3 discusses the development of another tunable thermoreversible hydrogel composed of HA-PNIPAAm for the culture of hPSCs and neural stem cells. In Chapter 4 a review of biochemical cue patterns for cell culture is explored in detail. This dissertation work focuses on developing novel materials to recapitulate parts of the stem cell microenvironment to enhance stem cell proliferation and ultimately differentiation for large scale expansion of stem cells.

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Chapter 2 - Functionalizable Thermoreversible PNIPAAm-PEG Hydrogels for 3D Stem Cell Culture

2.1 Introduction

Stem cells have the hallmark capacities to replicate indefinitely in an immature state and to differentiate into various types of cells found in the body. In particular, human embryonic stem cells (hESCs) [1] and induced pluripotent stem cells (hiPSCs) [2] – collectively known as human pluripotent stem cells (hPSCs) – can be harnessed for cell replacement therapies and tissue engineering approaches [3]. For instance, patients who suffer from a broad range of disorders involving tissue degeneration – including Parkinson’s disease, macular degeneration, myocardial infarction (heart attack), or liver failure – could potentially benefit from implantation of cells or engineered tissues to replace damaged or diseased ones [4-6]. However, such approaches require large quantities of specific cell types, a very small percentage of cells typically survives following implantation [7, 8], and thousands to millions of patients are afflicted with each such condition [5, 9-12]. As a result, extremely high quantities of cells are needed. Furthermore, even applications such as pharmacology/toxicity screening and preclinical animal studies require cell numbers that tax current culture systems [13-15]. For all of these considerations, there is a compelling need to scale-up stem cell manufacturing, particularly towards processes amenable to good manufacturing practices (GMP).

Current methods to produce stem cells in large quantities are limited by several factors. First, standard, two-dimensional (2D) stem cell culture requires excessive surface area, which is impractical for large implants and/or for disease indications affecting large patient numbers [16]. In addition, stem cell culture commonly uses poorly defined and highly variable animal-derived cells or proteins (e.g. Matrigel and laminin), which pose risks of immunogenicity, pathogen transfer, and lack of reproducibility and scalability. Innovative 2D synthetic cell culture surfaces have been developed [16, 17], though they also have challenges with scalability and are resource intensive.

Three-dimensional (3D) stem cell culture systems generally have considerably higher yields than 2D systems [9]. However, cell cluster or microcarrier systems are prone to large scale aggregation, which can lead to necrosis and/or nonspecific cell differentiation. Fluid shear can reduce such aggregation, but the accompanying mechanical disruption of cell function can limit cell potency and growth [9]. Embedding cells in a 3D matrix that shields them from mechanical disruption can address these concerns [18]; however, it has been challenging to identify a 3D system with biochemical and mechanical properties that can support cell growth, and minimize the transport barriers that limit cell viability and function [9, 19]. Moreover, 3D systems for stem cell culture also typically involve poorly-defined components, and possess challenges associated with cell recovery [19]. As a result, there is a strong need for biochemically defined and functionally versatile 3D systems for scalable stem cell expansion and differentiation.

We recently reported a system based on a thermoreversible hydrogel that addresses several of these challenges [20, 21]. The thermoreversible component of this hydrogel is poly(*N*-isopropylacrylamide) (PNIPAAm or PNIPAM), which phase separates in water or aqueous buffers above its lower critical solution temperature (LCST) of approximately 32 °C [22]. In solution, the acrylamide groups hydrogen bond with water, but above the LCST it becomes more thermodynamically favorable for these groups to hydrogen bond with each other than with water, yielding hydrophobic microdomains that constitute physical crosslinks in a polymer hydrogel [23] (Figure 2.1a). Hydrogels are generally classified into two categories based on the nature of the bonds existing at the crosslinks. The most common method to synthesize gels utilizes chemical crosslinks between polymer chains resulting in an irreversible gel, while secondary interactions such as hydrogen bonds, ionic, or hydrophobic interactions induced from an environmental stimulus such as temperature or pH form physically-linked gels [24-26]. Generating physically-linked gels is specifically advantageous for biomedical applications in which cells or tissues grown within a hydrogel need to be recovered and easily separated. Incorporation of PNIPAAm to other synthetic polymers [25], proteins [27, 28], and natural polymers or glycosaminoglycans [29, 30] can render the resulting material thermoreversible.

We previously showed that a fully-defined synthetic poly(ethylene glycol)-poly(*N*-isopropylacrylamide) (PEG-PNIPAAm) based hydrogel system together with defined medium (Essential 8) enabled 3D hPSC culture with an expansion rate of ~20 fold per 5 day passage, resulting in a 10^{72} fold expansion over a 280 day culture study [20]. In this system, cells can be mixed with polymer solution at lower temperatures, encapsulated within a hydrogel to protect them from aggregation and shear stress by heating above the LCST, and readily recovered by cooling and centrifugation. However, the system suffers from several limitations. First, the polymer solution is highly viscous at cold temperatures, making it difficult to homogeneously encapsulate cells during scale-up. In addition, the relatively high LCST can result in partial liquefaction and cell loss during media changes. Finally, it lacks the synthetic tunability to further incorporate proteins and peptide biochemical cues that are well-known to control cell behavior.

To address these concerns, we molecularly designed and optimized a new family of materials for hPSC culture and expansion. These thermoreversible statistical copolymers are based on PEG, PNIPAAm, and hydrophobic methacrylate groups; their gelation temperature, stiffness, and viscosity were modulated by tuning the monomer fractions and copolymer composition (Figure A.1). Optimal materials gelled below room temperature around 11-17°C and had a low viscosity of 9.1 Pa·sec or less around 0°C. Furthermore, several hydrogels maintained stiffnesses of ~1 kPa at 37°C for multiple days and could then be reliquified upon cooling. The modular chemistry of the material presented herein will enable conjugation of bioactive molecules. Finally, several hPSC lines survived, expanded, and expressed pluripotency markers after growing within these gels, making them a highly promising platform for defined and scalable stem cell culture.

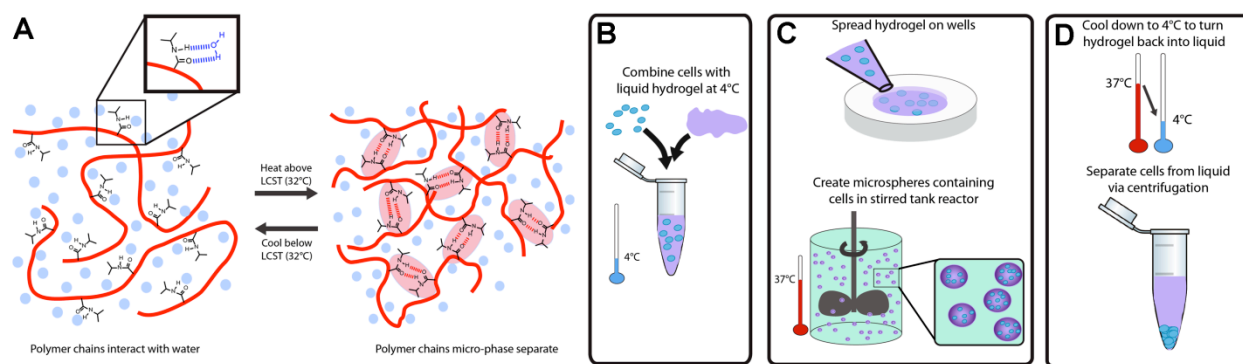


Figure 2.1. PNIPAAm hydrogel LCST schematic and encapsulation procedure

A. In polymers composed of PNIPAAm grafted to a hydrophilic polymer, the PNIPAAm hydrogen bonds with water below the LCST. Above the LCST the PNIPAAm amide groups microphase separate into hydrophobic domains, thereby creating physical links between polymer chains and causing the polymer solution to transition to a hydrogel. B-D Cell culture steps to encapsulate and recover cells. B. Combine cells with liquid hydrogel at 4°C to mix. C. Pipette hydrogel solution onto wells of tissue culture plate, or extrude or disperse hydrogel solution into warm media in a stirred tank reactor. D. Recover cells after growing in hydrogels by cooling down below LCST to turn hydrogel into a liquid and centrifuge to collect cell pellet and repeat.

2.2 Results

Hydrogel properties and optimization

Polymers were systematically optimized to yield thermoresponsive materials that met the following design criteria. First, the gelation temperature was required to lie below room temperature to minimize reliquification during media changes or imaging. Second, material stiffness at 37°C, which affects hydrogel durability and cell function, was tuned to enable high viability cell encapsulation and growth for multiple days. Finally, materials were optimized to undergo reliquification after multiple days at 37°C and to have a low viscosity at cold temperatures.

PEG concentration in PNIPAAm-PEG polymers modulates gelation and LCST

PEG incorporation into the polymer results in hydrophilic domains that support cell culture upon PNIPAAm microphase separation and gel formation above the LCST. As the precursor material, poly(N-acryloxysuccinimide) (PNASI) homopolymer was synthesized in toluene and subsequently functionalized with varying ratios of 3.4 kg/mol (3.4kDa) diamino-poly(ethylene glycol) (diaminoPEG) and isopropylamine to determine the range of PEG concentrations in which the polymer solution would gel upon heating to 37°C. The results are summarized in Table 2.1 and reveal that a range of ~15-25 wt% is appropriate for thermoreversible hydrogel formation at 37°C. The copolymer with 9 wt% PEG did not transition to a gel-like state when heated, but instead turned into large white solids with excreted water. The polymers with 15, 21, and 26 wt% PEG had LCSTs between 33-37°C. The PNIPAAm-PEG made with 30 wt% PEG did not microphase separate until >40°C proving to be unsuitable for 3D stem cell culture at 37°C. Increasing the PEG content also decreased the stiffness (as measured by storage modulus) and made the gels more transparent. None of the PNIPAAm-PEG polymers exhibit LCSTs below room temperature which introduces issues with the material reentering the solution phase during media changes or imaging. Once we created thermoreversible hydrogels composed of

just PNIPAAm and PEG the next optimization step focused on lowering the LCST below room temperature and making stiffer hydrogels.

Table 2.1. PNIPAAm-PEG hydrogel LCSTs as a function of PEG content using 3.4 kDa PEG.

PEG wt%	LCST (°C)
9	28.0*
15	33.0
21	37.1
26	35.7
30	43.6

*The polymer with ~9 wt% PEG exhibited an increase in both storage and loss modulus around 28°C though moduli curves were overlapping and storage modulus was never higher than loss modulus.

Incorporation of hydrophobic methacrylate groups to decrease LCST

Previous work investigating polymeric materials for drug delivery and other biomedical applications have incorporated hydrophobic (e.g., methyl methacrylate) and hydrophilic comonomers into PNIPAAm to control the polymer-solvent intermolecular interactions and ultimately tune the LCST [31-34]. We therefore initially generated a series of PNIPAAm based polymers with a fixed content of hydrophobic butyl acrylate (5 mol%) copolymerized with PNASI and variable 3.4kDa PEG content (9-30 wt%). The resulting polymers still exhibited LCSTs above room temperature, between approximately 30-40°C (Figure 2.2a). An increase in butyl acrylate content from 5 mol% to 10 mol% in PNASI-butyl acrylate copolymer only lowered the LCST by approximately 5°C for a given PEG concentration.

We next investigated whether different methacrylates would result in lower LCSTs. Incorporation of methyl, ethyl, butyl, and isobutyl methacrylate did in fact lower the LCST below room temperature (Figure 2.2a). Introduction of 5 mol% methyl methacrylate into the PNASI-methacrylate copolymer decreased the LCST to 12-15°C when the PEG concentration was 22-27 wt%, but at a PEG concentration of ~30 wt% the LCST was similar to just PNIPAAm-PEG and PNIPAAm-PEG-butyl acrylates at ~39°C. Ethyl methacrylate incorporation yielded gels with similar LCSTs between 10-15°C. Isobutyl and butyl methacrylates reduced the LCST to a range of ~7-10°C for PEG concentrations ranging 20-30 wt% PEG. It is evident that incorporation of methacrylates lowers the LCST below room temperature and allows us to achieve a crucial design goal for these polymer systems envisioned for stem cell culture.

The hydrogels presented herein are designed for operation in aqueous conditions at 37°C. Thus, understanding the mechanical properties of these materials is also critical for making 3D stem cell culture a feasible manufacturing technology. The storage modulus at 37°C was determined via oscillatory rheology. The results reveal that increased alkyl chain length not only decreased the LCSTs of the resulting polymers but also increased the storage modulus at 37°C (Figure 2.2a, b). Incorporation of isobutyl methacrylate generated hydrogels with higher and more stable storage moduli than methyl and ethyl methacrylate. Thus isobutyl and butyl methacrylate were the appropriate hydrophobic groups that exhibited both the mechanical properties and LCSTs required for 3D stem cell culture. Thus, we further investigated their structure-property relationships.

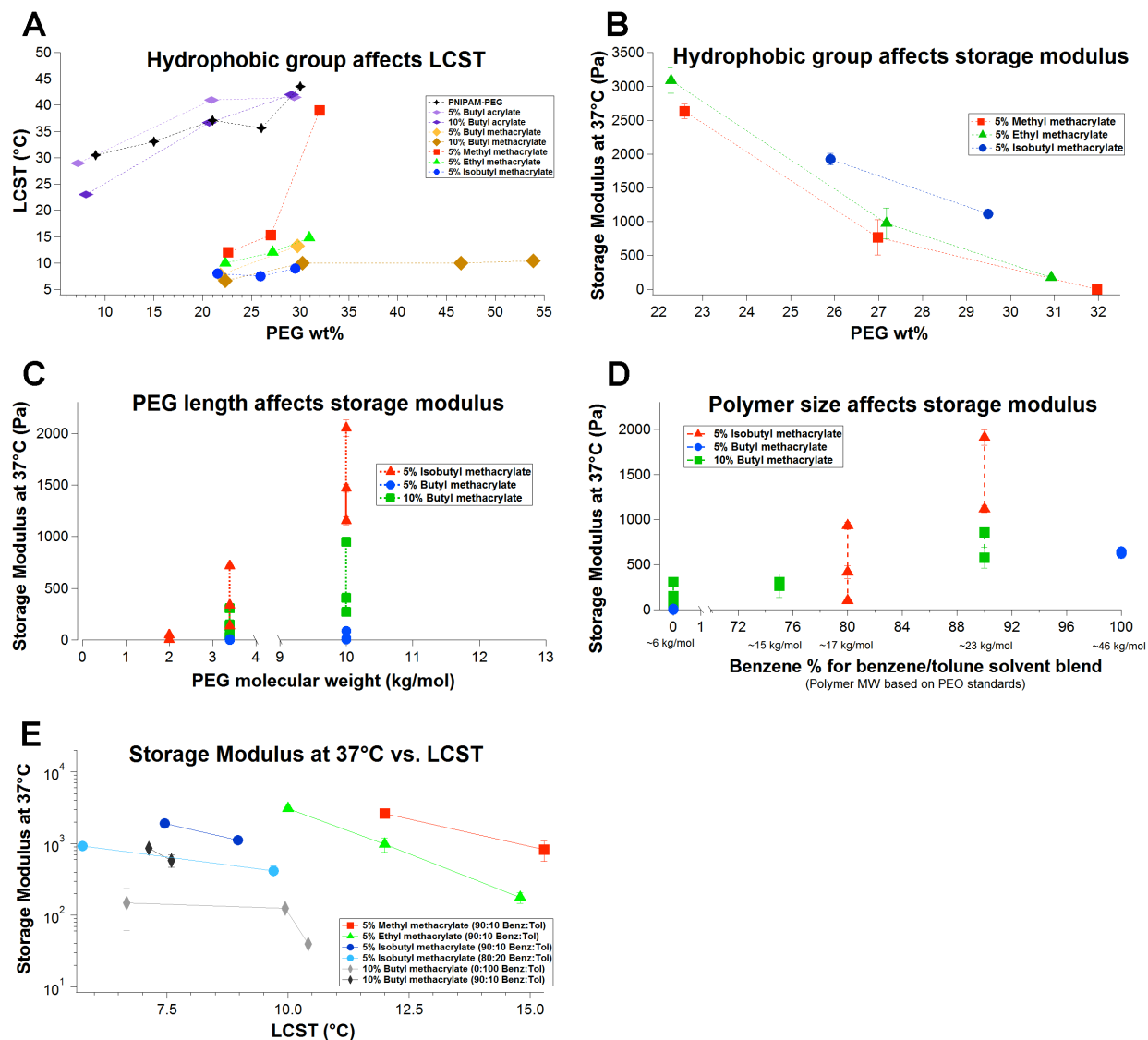


Figure 2.2. Rheology data summaries for PNIPAAm-PEG-methacrylate hydrogels.

A. Addition of hydrophobic methacrylate groups lowered the LCST of polymers: LCSTs versus PEG wt% for PNIPAAm-PEG-(methacrylate) polymers using 3.4k PEG; PNIPAAm-PEG (black diamond) and PNIPAAm-PEG-(meth)acrylate polymers: 5 and 10 mol% butyl acrylate (light and dark purple rhombus), 5 and 10 mol% butyl methacrylate (light and dark gold diamonds), 5 mol% methyl methacrylate (red square), 5 mol% ethyl methacrylate (green triangle), and 5 mol% isobutyl methacrylate (blue circle). All polymers were made with 3.4 kDa PEG and all polymer concentrations between 16-20 wt/vol%. PNIPAAm-PEG, butyl acrylate and butyl methacrylate polymers shown here made in 100% toluene; methyl, ethyl, and isobutyl methacrylates made in 90/10 benzene/toluene blend.

B-E Storage modulus at 37°C based on hydrophobic group (B), PEG length (C), polymer size (D), and LCST (E). Each set of connected markers represent different PEG wt% concentrations for the same polymer size and methacrylate concentration.

B. 5 mol% methyl (red squares), ethyl (green triangles), and isobutyl methacrylate (blue circles) polymers exhibit an increased stiffness with increasing alkyl chain length (all using 3.4 kDa PEG, made in 90/10 benzene/toluene blend and concentrations of 16-20 w/v%).

C. 5 mol% isobutyl methacrylate (red triangles) and 5 and 10 mol% butyl methacrylate (blue circles and green squares, respectively) show an increased storage modulus with increasing PEG molecular weight from 2 kDa to 10 kDa. Isobutyl methacrylate polymers made using 95/5 benzene/toluene blend and concentration of 10 w/v%. Butyl methacrylate polymers made using 100% toluene at concentrations of 13-20 w/v%.

D. 5 mol% isobutyl methacrylate (red triangles) and 5 and 10 mol% butyl methacrylate (blue circles and green squares, respectively) show an increased storage modulus with increasing polymer molecular weight (PNASI-methacrylate polymer based

on PEO standards). All polymers using 3.4k PEG; isobutyl methacrylate and 5 mol% butyl methacrylate (smallest size) polymer concentrations of 16-20 w/v% and all others using concentrations of 6-8 w/v%.

E. 5 mol% methyl (red squares), ethyl (green triangles), and isobutyl methacrylate (blue circles) and 10 mol% butyl methacrylate (grey diamonds) polymers all exhibit the same trend of decreased storage modulus at 37°C with higher LCSTs most likely due to the higher solubility of the polymer in aqueous solutions affecting both properties when PEG concentration is increased. All methyl, ethyl, and isobutyl and small butyl methacrylates at concentrations of 16-20 w/v% 10 mol% butyl methacrylate made in 90/10 benzene/toluene ratio at concentrations of 6-8 w/v%. All polymers using 3.4 kDa PEG.

Effects of PEG length on hydrogel stiffness

Some hydrogels with LCSTs below room temperature did not immediately exhibit optimum mechanical properties at 37°C. Varying the molecular weight (MW) and concentration of the hydrophilic component (PEG) was anticipated to alter the spacing between the NIPAAm and the hydrophobic groups. This modification on the molecular structure is expected to translate into control over the bulk mechanical properties of the hydrogel. In order to understand the effect of PEG length on hydrogel stiffness, the molecular weight of the PEG was varied within the range of 0.6 kDa to 10 kDa. Polymers based on butyl methacrylate macrophase separated when containing PEG lengths of 0.6 kDa, 1 kDa, and 2 kDa (Figure A.4). However, varying the PEG length from 3.4kDa to 10kDa increased the storage modulus approximately an order of magnitude from ~10 Pa to ~100 Pa (Figure A.5c). A similar trend was elucidated for polymers based on isobutyl methacrylate. Therefore increasing the length of PEG used within the PNIPAAm-PEG-methacrylate polymers increased the stiffness at 37°C of the final hydrogel (Figure 2.2c).

Effects of polymer size on hydrogel properties

Most of the polymers optimized to have lower LCSTs and minimal phase separation had low molecular weights and were still not as stiff as desired. These polymers produced hydrogels which had some of the mechanical and rheological properties desired, however they were still too soft (approximately 100-400 Pa storage modulus for most gels) to successfully culture cells and last multiple days at 37°C (Figure A.5c,f). Increasing the PNASI-methacrylate polymer size was proposed to increase the stiffness of the resulting hydrogels due to an increase in the number of hydrophobic microdomain interactions occurring with each chain. Therefore, different overall PNASI-methacrylate polymer sizes were created by varying the ratio of benzene and toluene, as previous work showed that increasing the amount of benzene in the solvent for a similar synthesis increased the molecular weight most likely due to less chain transfer activity with benzene [35]. PNASI-methacrylate polymer size was quantified by gel permeation chromatography (GPC) with polyethylene oxide (PEO) standards. Polymers synthesized with 100% toluene produced the smallest molecular weight polymers (~6,000 g/mol based on PEO standards) while those synthesized with 100% benzene produced polymers with the largest molecular weight (~46,000 g/mol based on PEO standards) (Figure 2.2d,

Table 2.2). Polymers made with a majority of benzene produced hydrogels with the highest storage moduli at 37°C around 1-2kPa (Figure A.5e, f). However polymers made with 100% benzene would never reliquify after being held at 37°C no matter the concentration in solution, PEG concentration, or hydrophobic methacrylate group. Using a solvent ratio of 95/5 benzene/toluene with PNASI-isobutyl methacrylate generated hydrogels which could reliquify depending on the concentration in solution. Varying the solvent conditions during PNASI-methacrylate synthesis successfully produced polymers in a range of sizes from smallest to largest by using 100% toluene to 100% benzene and the polymers made with majority benzene produced the stiffest gels.

Table 2.2 PNASI-methacrylate polymer sizes based on PEO reference on DMF GPC for polymers made with different ratios of benzene and toluene.

Ratio of benzene/toluene	PEO reference size via GPC (g/mol)	Easily reliquifies?
0/100	6,000	Yes
50/50	10,000	Yes
60/40	11,000	Yes
80/20	17,000	Yes
90/10	23,000	Yes
95/5	35,000	At lower wt/vol%
100/0	46,000	No

Polymer size and hydrogel formation were not entirely predictable when comparing different hydrophobic groups in the polymer. PNIPAAm-PEG-butyl methacrylate polymers made with 50/50 benzene/toluene blend (~10 kDa based on PEO standards) made soft hydrogels, and polymers made with 100% toluene (~6 kDa) made the softest hydrogels for both 5 and 10 mol% butyl methacrylate (all less than ~500 Pa storage modulus) (Figure 2.2d, Figure A.5f, Figure A.6). These same polymers synthesized using 75/25 or 90/10 benzene/toluene blends (15 kDa or 25 kDa) would not reliquify after being warmed to 37°C. For PNIPAAm-PEG-butyl methacrylates with 10 mol% butyl methacrylate, the storage modulus at 37°C increased from around 300 Pa for polymers made with 75/25 benzene/toluene ratio to a range of ~600-900 Pa with 90/10 benzene/toluene ratio within the same PEG concentration range of ~25-33 wt% PEG (Figure A.5e). PNIPAAm-PEG-isobutyl methacrylates with 5 mol% isobutyl methacrylate saw a similarly large storage modulus increase with increase in polymer size from a range of ~100-900 Pa with 80/20 blends to a range of ~1-2 kPa with 90/10 blend for similar PEG contents. PNIPAAm-PEG-isobutyl methacrylate polymers made with 50/50 or 60/40 benzene/toluene blends (using 3.4k PEG) would not form hydrogels, but instead the polymer would precipitate from the solution. Polymers made with 80/20 or 90/10 benzene/toluene blends made hydrogels that were around 1 kPa storage modulus at 37°C (Figure 2.2d, Figure A.5e) and would easily reliquify, however they would immediately dissolve away upon addition of warm media or buffer using 3.4Da k PEG or become soft and dissolve after a couple days using 10 kDa PEG (Figure A.7). Polymers made with 95/5 benzene/toluene blend could make hydrogels that had a storage modulus around 1 kPa at 37°C (Figure 2.2c, d) and could reliquify after multiple days of cell culture using the appropriate concentration in solution. Batch to batch variability requires slight optimization for correct concentration to dissolve as hydrogels, remain stiff, and reliquify after multiple days

Cell culture within hydrogels

H1 and H9 human embryonic stem cells (hESCs) and TCTF induced pluripotent stem cells (iPSCs) were successfully cultured in synthesized PNIPAAm-PEG-methacrylate hydrogels using fully defined Essential 8 (E8) media. All current methods except for the previously published PNIPAAm-PEG system ([20]) require some amount of cell-cell contact for cells to survive between passages, minimizing the expansion potential and requiring stringent controls over aggregate size during passaging. Therefore we wanted to analyze whether the PNIPAAm-PEG-

methacrylate hydrogels could support the viability and growth of hPSCs from single cells as well as from small aggregates. H1 and H9 cells were successfully grown from small aggregates plated in as little as 24 hours from their single cell state and grew into large aggregates; these cell lines did not survive as single cells within these gels. TCTF cells did survive and grow from single cells and were cultured through four rounds of successive hydrogels as single cells (Figure 2.3). Multiple batches of PNIPAAm-PEG-isobutyl methacrylate polymers were used to culture TCTFs with an average of approximately 3-fold increase in expansion over the course of 4-5 days and a high of ~8-9 fold expansion after 4 days. Gels that had a storage modulus of approximately 1 kPa at 37°C were able to maintain their structure after multiple days and provide a stiff enough environment for cell growth. All of the hPSC lines grown in the gels stained positive for pluripotency markers Oct4 and Nanog (Figure 2.4) after one (H1 and H9 hESCs) to multiple passages within the gels.

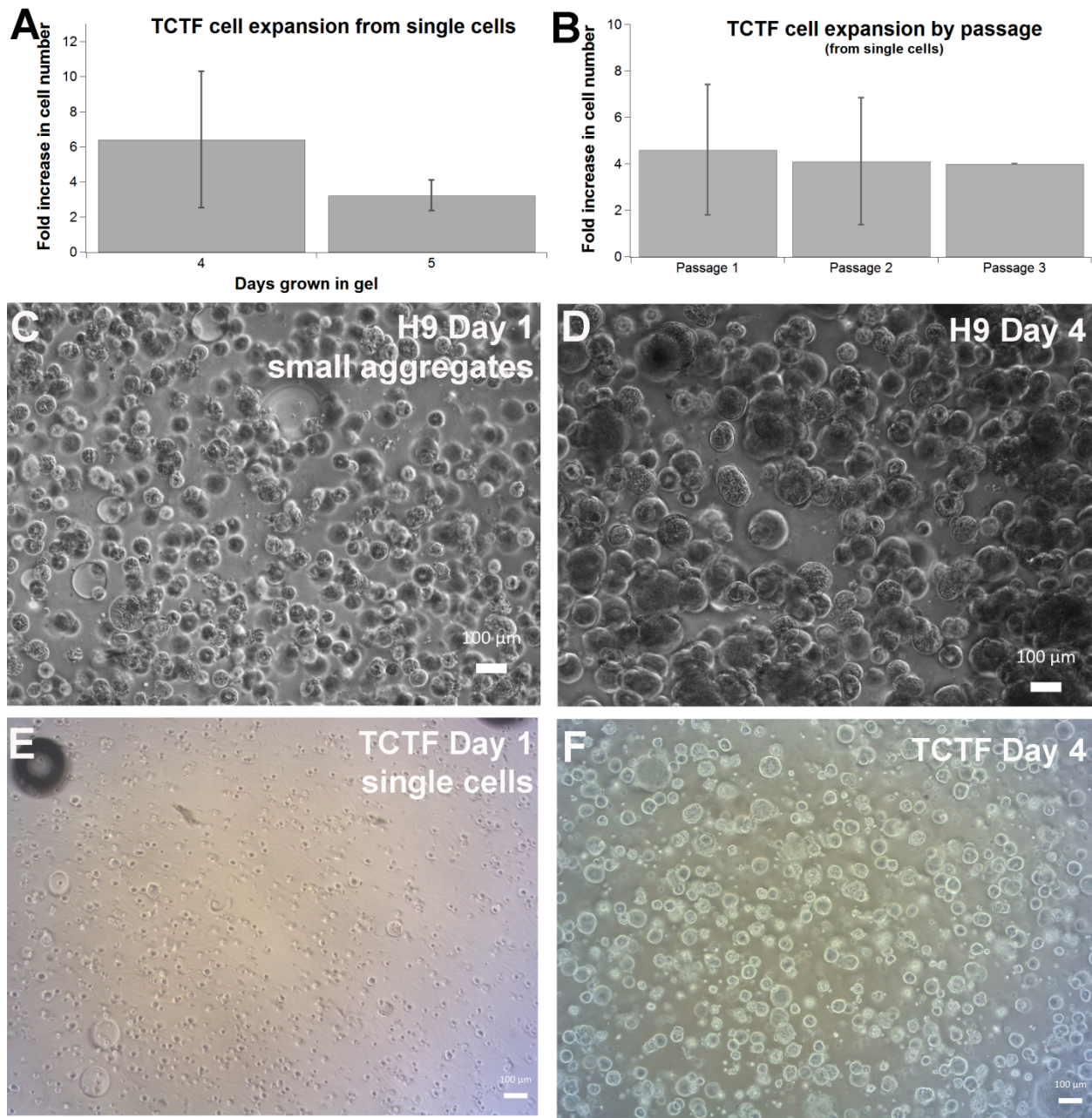


Figure 2.3. Stem cell expansion data and brightfield images depicting cell growth.

Cell expansion data for TCTF based on day (A) or passage number (B). In A, the black triangles correspond to cell count data without continuous counts each passage. C-F: Pictures of cells after different amounts of days in the gels. H9 hESC cell growth from day 1 as small aggregates (C) to larger aggregates after 4 days (D). TCTF iPSCs pictures after 1 day as single cells (E) and after 4 days (F). Scale bars are all 100 μ m.

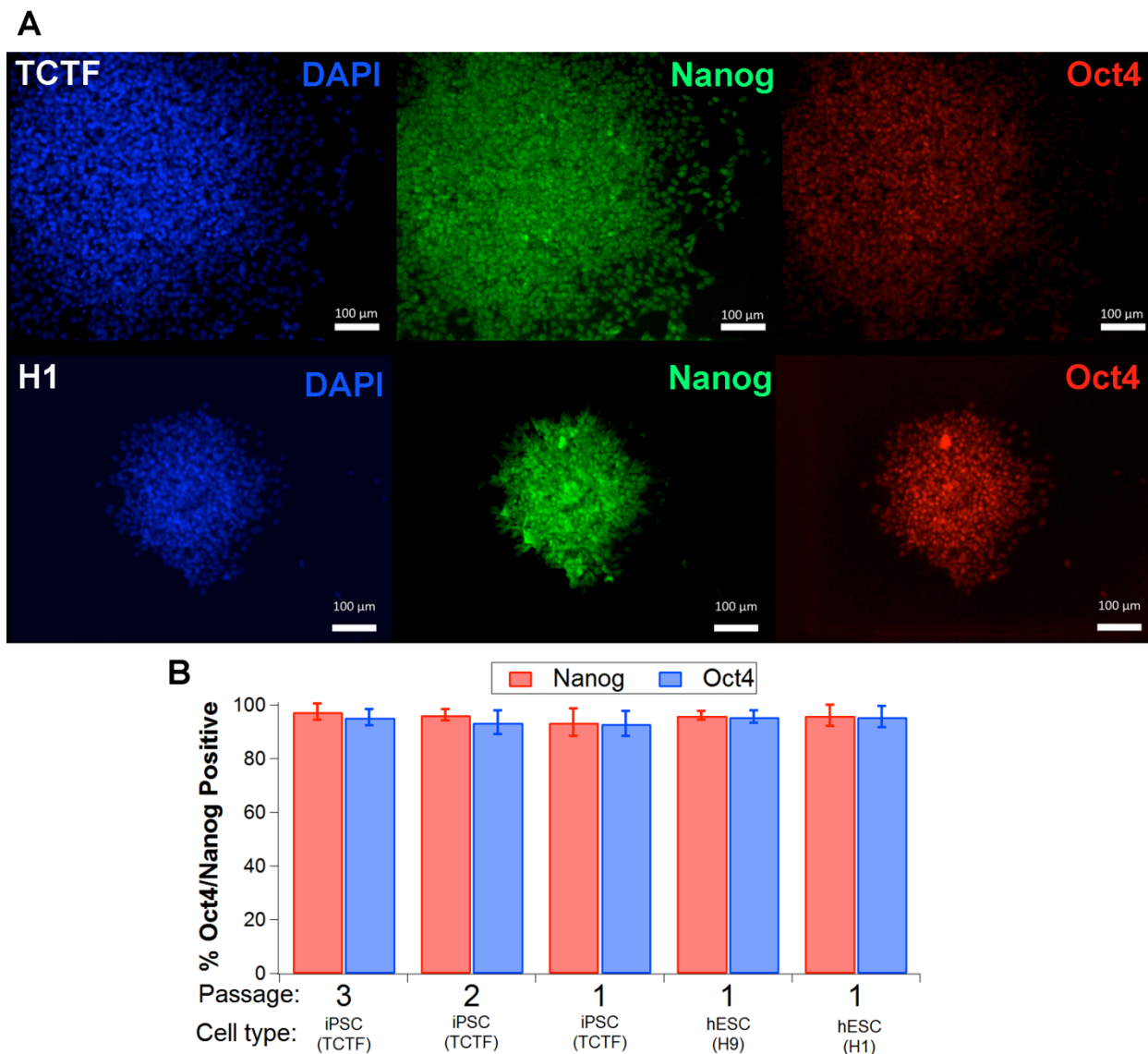


Figure 2.4. Pluripotency marker expression in hPSCs grown within hydrogels.

A. Example images of TCTF iPSCs and H1 hESCs staining positive for pluripotency markers Oct4 and Nanog, scale bars are 100 μ m. B. Statistics on cells grown for 1-3 passages within the gels are >95% Oct4 and Nanog positive.

Optimized hydrogel properties

Based on all of these characteristics and optimization protocols, the most effectively performing polymer is PNIPAAm-PEG-isobutyl methacrylate made using a 95/5 benzene/toluene blend with approximately 25 wt% of 10k PEG. This polymer had an LCST of 11-17°C and a storage modulus of approximately 1 kPa at 37°C (Figure 2.5). Gels made from this polymer easily reliquified after 4-5 days at 37°C. At 4°C using a shear rate of 100/sec, the viscosity of optimized PNIPAAm-PEG-isobutyl methacrylates were approximately 50-100 mPa·s (Figure 2.5b), lower than the commercially available Mebiol gel (~200 mPa·s) and other synthesized gels, which were an elastic string of polymer (~400 mPa·s) or completely entangled and unipettable (~1.3 Pa·s).

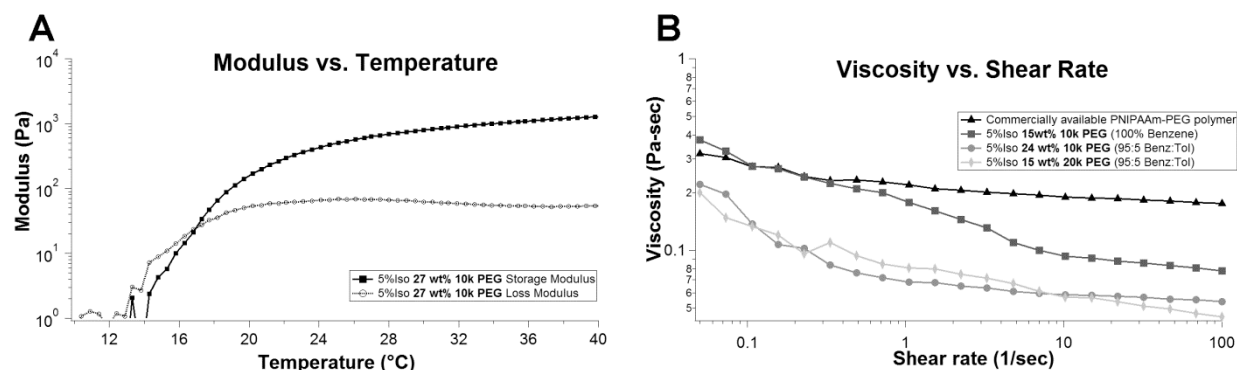


Figure 2.5. Optimized hydrogel temperature sweep and viscosity vs. shear rate.

A. Optimized PNIPAAm-PEG-Isobutyl methacrylate with desired mechanical characteristics was synthesized from 95/5 benzene/toluene ratio and 10k PEG. The LCST is well below room temperature, at approximately 17°C and the polymer stabilizes around 1kPa storage modulus above 25°C. B. Viscosity at 4°C of PNIPAAm-PEG-methacrylates of different makeup and consistencies compared to commercially available PNIPAAm-PEG polymer. Black triangles represent the commercially available PNIPAAm-PEG polymer, dark grey squares represent synthesized gels made in 100% benzene with 15wt% 10 kDa PEG, dark grey circles represent gels made in 95:5 benzene:toluene blend with 24 wt% 10 kDa PEG, and light grey diamonds represent gels made in 95:5 benzene:toluene blend with 15 wt% 20 kDa PEG.

Chemical functionalization for heparin incorporation and/or biochemical cue attachment

Soluble biochemical cues are typically utilized to regulate stem cell behavior, and such recombinant proteins are typically a highly resource intensive component of culture systems. It has been shown that immobilization of biochemical ligands to a material can result in a more potent cue, by increasing local concentration and reducing endocytosis-mediated ligand degradation [36-38]. In addition to direct chemical conjugation, protein factors that naturally bind heparan sulfate can be immobilized within a gel via the chemical incorporation of heparin into a material [39-43].

Incorporation of a thiol or methacrylate functionality along the polymer backbone allows simple conjugation of heparin that has been modified with a corresponding reactive group (Figure A.1, Figure A.8). Upon attachment of heparin to the hydrogel, the proteins will associate with heparin within the hydrogel and become much more accessible to the cells. Additional chemical functionality could be incorporated to attach multiple types of ligands within the gels. NMR data show the addition of methacrylate groups within the polymer structure after aminoethylmethacrylate is conjugated to the polymer backbone. NMR data show that after extensive dialysis with 300 kDa molecular weight cut-off heparin is still associated with the polymer (Figure A.9).

2.3 Discussion

There is a strong need for defined, scalable stem cell manufacturing processes, both cell expansion and differentiation, for cell replacement therapies for a range of degenerative diseases and even for high-throughput screening for pharmacology and toxicology screening. We have synthesized a novel thermoreversible hydrogel system that can be mechanically and rheologically tuned as well as readily incorporate biochemical cues. PNIPAAm-PEG is a

thermoreponsive material that has been explored for applications such as drug delivery and tissue engineering, due to its hydrophilic nature, LCST near body temperature, and mechanical properties [44-46]. We have recently utilized a PNIPAAm-PEG to establish the promise of thermoreversible, defined, and scalable hPSC culture systems [20]; however, this material had some rheological and biochemical limitations. We have therefore designed and synthesized a new family of thermoreversible gels to improve utility for stem cell culture and expansion. Specifically, we engineered gels with: an LCST below room temperature for ease of handling during media changes and imaging, tunable stiffness at 37°C to potentially optimize proliferation rates or aid in differentiation, lower viscosity at cold temperatures for ease of homogeneous mixing cells with polymer solution, and the capacity for chemical conjugation of biochemical cues within material.

Investigating any of the polymers with constant molecular weight (i.e. number of segments) and hydrophobic concentration, we controlled the hydrophilicity of the copolymers by altering the ratio of hydrophilic (PEG) components to hydrophobic monomer (acrylate or methacrylates) and observed a decrease in storage modulus and increase in LCST as PEG concentration increased. This finding suggests an improvement in solvent quality as the polymer-water interactions become more favorable, and is consistent with Flory-Huggins theory of polymer solutions where the critical temperature for phase separations is inversely proportional to the energy difference upon demixing at the monomer level. These copolymers are designed for use in aqueous conditions above the LCST (in the gel state) and therefore need to be designed to have tunable water content. Tuning the hydrophilicity of these copolymers altered the swelling ratio in the phase separated, physically crosslinked state. Based on phase equilibrium, if the temperature, pressure, and molecular weight of the copolymers are held constant, then the water content of the copolymers must change across the series due to differences in chemical potentials associated with composition.

Polymers were systematically optimized to yield hydrogels with several desired characteristics. The first priority was a 3D environment that suspends the cells and allows them to grow into small aggregates, accomplished by developing a temperature responsive hydrogel with PNIPAAm, a known biocompatible thermoreversible polymer. Conjugating PNIPAAm with PEG yielded materials that were liquids at cold temperatures below, and physically linked hydrogel networks above, the LCST. Therefore, the cells could be homogeneously mixed with the polymer solution at cold temperatures and then encapsulated upon heating to 37°C. An optimal range of ~15-35 wt% PEG generated hydrogels and within this range increased amount of PEG made softer and more transparent gels, though the higher PEG content polymers required a lower w/v% concentration in solution to dissolve. Multiple PNIPAAm-PEG polymers formed gels below 37°C however none of them transitioned below room temperature, proving problematic for cell culture. Modifications of future PNIPAAm-PEG polymers should aim to start with a range of 20-30 wt% PEG for gel formation.

The next design parameter necessity was to decrease the LCST of the hydrogels so that the liquid-gel transition temperature occurred between 4°C and room temperature, such that the material required some heating to gel and that small fluctuations around 37°C (e.g. during media changes or during routine culture monitoring by microscopy) did not cause cumulative gel dissolution and cell loss. The initial PNIPAAm-PEG polymers yielded gels with LCSTs above

room temperature. In order to lower the LCST below room temperature, hydrophobic groups were incorporated into the polymer to decrease the number of water molecules binding with the polymer at cold temperatures, thus decreasing the temperature at which the temperature-induced hydrophobic microphase separation occurs. The first hydrophobic monomer used, butyl acrylate, did not lower the LCST below room temperature; however, polymers using methyl/ethyl/butyl/isobutyl methacrylates did lower the transition temperature to $\sim 10^{\circ}\text{C}$ with as low as 5 mol% hydrophobic groups incorporated into the polymer backbone (Figure 2.2a). The polymers with higher LCSTs probably have more water inside the hydrogel in the phase separated state, allowing for polymer chains to move more easily and resulting in a softer gel.

Once we generated thermoreversible polymers with gelation temperatures below room temperature, we attempted to culture cells in these hydrogels; however, the initial polymers that were optimized for ideal LCST and thermoreversibility had low molecular weights and produced gels that were too soft to support cell culture over multiple days (data not shown). One possible means of increasing the stiffness of hydrogels was to change the spacing between the PNIPAAm and hydrophobic blocks, accomplished by varying the length of PEG attached to the polymer backbone. For the PNIPAAm-PEG-butyl methacrylate polymers of small molecular weight (made in 100% toluene), short PEG chains of 2k MW or less would cause polymer precipitation while PEG lengths of 3.4 kDa and 10 kDa produced gels of increasing stiffness (Figure 2.2c, Figure A.4, Figure A.5c,d). The effect of shorter PEG chains limiting the ability to gel is similar in behavior to having longer PEG chains but lower wt% within the polymers. This can be explained by the driving force of gelation in which the interactions of PNIPAAm acrylamide groups with each other or with hydrophobic moieties frees water molecules to hydrogen bond with each other or to hydrophilic PEG, which retains this water to prevent polymer precipitation and create a 3D network [21, 47, 48].

Results in literature suggest that PEG chains or “blocks” need to be long enough to disrupt hydrophobic domains and bind to the water released by PNIPAAm aggregating and that random equal arrangement of PNIPAAm and hydrophilic groups will not yield gels [21, 47, 49]. When analyzing the polymers with short PEG chains, there is a much higher mol% of PEG attached to the polymer backbone to get near the PEG wt% required for polymers with 3.4 kDa and 10 kDa PEGs to gel. These short PEG chains are interspersed between the PNIPAAm groups making the PNIPAAm “blocks” shorter but these PEG chains may not be long enough to disrupt the hydrophobic domains. Comparing polymers with the same wt% PEG but using 3.4 kDa or 10 kDa PEG, we noticed that the longer PEG chains make stiffer gels (Figure A.5b-c, Figure 2.2c). This increase could be due to the larger “blocks” of PNIPAAm with longer PEG chains (because there is less mol% PEG attached to the backbone) making the gel less porous and stiffer when the PNIPAAms aggregate in their hydrophobic domains and mobility is limited. This effect may also explain why higher wt% of PEG is needed to make gels when there is a higher mol% of hydrophobic group along the polymer backbone. Hydrophilic chain (PEG) length had a very large impact on hydrogel formation and stiffness and therefore should be taken into account when designing future types of similar polymers.

Another route pursued to increase hydrogel stiffness was to increase the overall hydrophobic polymer size. Polymers made using 100% toluene had smaller molecular weights (~ 6 kDa based on PEO standards), while polymers made with 100% benzene had the largest molecular weights

(~50 kDa), most likely by avoiding the chain transfer activity possible with toluene and some other solvents [35, 50, 51] (

Table 2.2). As anticipated, the largest molecular weight polymers made the stiffest gels for all of the PNIPAAm-PEG-methacrylates tested (Figure 2.2d, Figure A.5e,f); however, these would not reliquify when the temperature was reduced to 0°C. The PNIPAAm-PEG-Isobutyl methacrylate polymers made with 80/20 and 90/10 benzene/toluene ratios with slightly smaller molecular weights than 100% benzene would easily reliquify after the gel state, however the 3D network would dissolve or loosen upon addition of warm media (increasing time with increasing PEG length). This instability makes cell growth for multiple days difficult, therefore polymers with 95/5 benzene/toluene solvent ratios were also made. These polymers reliquify after multiple days in the gel state and maintain their stiffness and 3D network for cell growth. Higher molecular weight polymers were more viscous at cold temperatures than the low molecular weight polymers but were more pipettable and manageable than commercially-available system (Figure 2.5b).

Not only did incorporation of hydrophobic methacrylate groups lower the LCST of the polymers, they also made a large impact on hydrogel stiffness and aqueous solution solubility. There was a large difference in the ability of polymers to form hydrogels at low molecular weights between butyl methacrylate and methyl/ethyl/isobutyl methacrylate. The PNIPAAm-PEG-methacrylate polymers were only soluble at the highest concentration of ~20 w/v% for smaller polymer sizes and ~7.5-10 wt/v% for larger polymer sizes. All analyses of hydrogel storage and loss moduli were performed using the highest concentration the polymers were soluble, therefore increasing the concentration in solution was not feasible to increase the stiffness of softer gels. Butyl methacrylate polymers made gels when the polymers were as small as ~6 kg/mol based on PEO standards, however the methyl/ethyl/isobutyl methacrylate polymers would not make gels until the polymer was approximately 17 kg/mol based on PEO standards (Figure A.6, Figure A.7). In addition, isobutyl methacrylate polymers made stiffer hydrogels than ethyl methacrylate and methyl methacrylate. All of these properties could be attributed to the hydrophobicity of the resulting polymer and the amount of water it can uptake from aqueous solutions. As the polymer becomes more hydrophobic there are fewer interactions with water, as seen with a decreased LCST with lower PEG wt% incorporated and/or longer alkyl chain lengths on the methacrylate. Figure 2.2e shows storage moduli at 37°C increased as LCSTs decreased, suggesting that the more hydrophobic the polymer is (based on decreased LCST), the less the polymer interacts with the aqueous solvent which results in a higher storage modulus. Perhaps the butyl methacrylate polymers were more soluble than the methyl, ethyl, and isobutyl methacrylate polymers, giving rise to hydrogels while the other polymers precipitated (when comparing across the same molecular weight). In addition, the result of lower moduli for butyl methacrylate gels than isobutyl methacrylate gels could also stem from butyl methacrylate polymer solubility in water.

In order to help maintain a more relevant cell microenvironment *in vitro*, biochemical cue incorporation is very beneficial. Addition of proteins or peptides that are important for certain cell processes such as proliferation, cell adhesion, or differentiation could significantly impact cell growth rate or differentiation efficiency. The fully defined media Essential 8 (E8) used to culture hPSCs in this paper contains only 8 components, two of which are proteins vital for hPSC survival and proliferation: transforming growth factor beta (TGFβ) and fibroblast growth factor (FGF). Both of these proteins belong to a large subset of proteins that contain heparin-

binding domains, and thus incorporation of heparin into a material can enable non-covalent retention or presentation of such factors within a material [39]. Integrating these factors into the matrix increases potency, decreases potential of endocytosis, and increases local concentration [36-38]. Incorporating proteins within the matrix could also decrease the cost of media by minimizing the need for diffusion through the media and matrix. Attaching heparin and other biochemical cues to the matrix would help to support stem cell growth and other processes by presenting the cues in a more accessible and stable manner than when dissolved in media.

Therefore, the polymers were also designed in order to easily add chemical functionality for biochemical cue attachment. Based on the synthetic route, there are multiple ways to incorporate specific chemical functionality into the polymer. The NHS ester groups of NASI react with anything with a free, accessible amine group. PNIPAAm and PEG functionalities are incorporated through this route by reacting isopropylamine and diaminoPEG, respectively, with the NASI groups. We have also added in cysteamine and aminoethylmethacrylate to impart thiol and methacrylate functionality, respectively. Another option could be to use a heterobifunctional PEG with an amine on one end. These polymers can then be purified before biochemical cue attachment to eliminate potential for damage or degradation during polymer synthesis and purification.

Multiple types of cells survived and replicated while growing within these gels. This is only the second hydrogel system to successfully grow iPSCs from single cells for cell culture and expansion ([20]). hESCs (H9's and H1's) grew from small aggregates after at least 24 hours growing in Mebiol. The expansion rate for TCTF iPSCs was roughly 3-4 fold after 4-5 days within the gels. However, after the second passage the expansion rate was approximately 8-9-fold after 4 days, comparable to H9 or H1 hESC rates in Mebiol, and less than the rates for TCTFs in Mebiol. All cell lines tested stained positive for pluripotency markers Nanog and Oct4.

There was an obvious difference in cell survival in gels that had a storage modulus less than 1 kPa, either at the start or as the days progressed. When cells were plated into these gels as single cells, by 24 hours it was obvious that very few would survive and grow into aggregates, if any. If cells were plated as small aggregates they would appear to dissociate, perhaps due to minimal external force from the gel. If these aggregates dissociated, generally none of the cells would survive to grow into more aggregates. Some hydrogels were ~1 kPa when cells were seeded into the gels but would progressively become softer after multiple days, as seen with gel movement and grown aggregates dissociating. On the contrary, hydrogels that were stiffer than ~1 kPa and would not reliquify after multiple days seemed to have the highest growth rate. However, no analysis or cell counting was performed because cells could not be extracted. This suggests that there is a narrow range of stiffnesses or porosity that hPSCs require to maintain viability. Others have reported that with a PNIPAAm-PEG block copolymer system more PNIPAAm (larger PNIPAAm blocks) increased stiffness and increased cell viability of chondrocytes [46]. The large PNIPAAm blocks created larger voids during hydrophobic aggregation, forming stable gels with large pores for diffusion of oxygen and nutrients. Previous work has shown that 2D matrix stiffness affects stem cell fate by promoting differentiation to specific lineages when grown on substrates of similar stiffness to the respective native tissue, i.e. neurons on soft substrates, muscle cells on substrates of medium stiffness, and bone cells on hard substrates[52]. In

addition, hPSCs grown on soft substrates promoted differentiation towards the neuronal lineage (but not proliferation) while stiff substrates increased the colony spread area [53, 54]. These papers prove that mechanotransduction effects from 2D substrate stiffness can have strong impacts on stem cell fate, suggesting the stiffness of the 3D hydrogel environment could also alter stem cell processes like proliferation and differentiation. These novel hydrogels could be used as a model system to investigate cell behavior in 3D compared to the literature with 2D surfaces.

2.4 Conclusions:

Due to the large need for a defined, scalable cell culture system to generate stem cells for many therapeutic purposes, we designed and developed a novel, tunable hydrogel system. These hydrogels contain PNIPAAm, rendering them thermoreversible, along with PEG and hydrophobic methacrylates. Polymer LCSTs were decreased below room temperature by the addition of different methacrylate monomers to aid in handling and media changes. Hydrogels with a range of storage moduli were generated by varying the methacrylate group, amount and length of PEG blocks, and the overall polymer size. In general, a range of approximately 20-30 wt% PEG and PEG lengths of 3.4k or greater were necessary to generate hydrogels. In addition, the ability to reliquify and the viscosity at cold temperatures were also modulated, with polymers synthesized in 100% benzene unable to reliquify due to the large size and chain entanglement. Chemical functionalization with thiol or vinyl groups provided a route to attach biochemical cues directly or indirectly via heparin conjugation. Multiple hPSC lines remained viable, replicated, and stained positive for pluripotency markers after growing within these hydrogels; TCTF iPSCs were successfully passaged as single cells within these gels while hESCs were passaged from small aggregates. Hydrogel stiffness affected hydrogel durability as well as cell viability.

Transitioning from plating gels on tissue culture plates to forming microspheres in bioreactors would be necessary to scale up stem cell expansion in an industrial setting. However, these functionalizable and tunable hydrogels can be used for many types of purposes in addition to cell expansion. Beyond the current parameters and intended biochemical cues, a wide array of biochemical cues could be included to help increase rate of proliferation or guide cells to certain lineages, perhaps into neuronal or cardiac lineages. These hydrogels could also be used to understand the effect of stiffness in 3D systems for encapsulated cells to compare with current literature using 2D substrates and expand the potential of 3D cell culture and expansion.

2.5 Methods and Materials

Materials:

Monomer N-acryloxysuccinimide (NASI) was purchased from Fisher Scientific and all other monomers and aminoethylmethacrylate (90%) were purchased from Sigma Aldrich and purified by passing through a basic alumina column. Anhydrous solvents, azobisisobutyronitrile (AIBN), isopropylamine, cysteamine, and poly(ethylene glycol) diamines (PEGs) (MW 2k, 3.4, 10k) were purchased from Sigma Aldrich. PEGs with MW of 0.6k and 1k were purchased from Creative PEG works.

Synthetic steps:

The PEG-PNIPAAm-methacrylate polymers were made by first synthesizing a Poly(N-acryloxysuccinimide) (PNASI) polymer with or without a low molar equivalent of hydrophobic methacrylate monomer via reversible addition-fragmentation chain transfer (RAFT) synthesis using azoisobutyronitrile (AIBN) as the initiator (Figure A.1). The monomers, AIBN, and a blend of toluene and benzene were combined, sparged with Argon for 30 minutes, and then placed in a 60°C oil bath for 24 hours [55, 56]. The resulting polymer was precipitated into ethyl ether and dried in a vacuum oven overnight before further analysis or chemistry.

The PNASI-methacrylate polymer was then subjected to addition of chemicals with amine functionality to attach specific chemical groups via the NHS ester of PNASI (Figure A.1). Isopropylamine was added first to convert ~90% of PNASI to Poly(N-isopropylacrylamide) (PNIPAAm), followed by addition of a diamino-poly(ethylene glycol) (PEG) to attach a hydrophilic block [21]. At this point any additional chemicals with a free amine group could be added to react with the residual PNASI groups, providing added functionality. For instance, cysteamine could be added to generate a thiol-modified polymer, or allylamine could be added to incorporate a vinyl group. Once all additional chemical functionalization was completed, isopropylamine was added again to convert any remaining PNASI groups into PNIPAAm. The DMF solvent for all of these reactions was then removed via a rotary evaporator, and the polymers were redissolved in water followed by dialysis to remove unreacted chemical components.

The synthetic scheme could be altered for various polymer needs and properties. Hydrophobic methacrylate groups were included within the polymer to increase the tendency to microphase separate into hydrophobic domains, thereby reducing the LCST. Specifically, different methacrylate and acrylate groups were incorporated to tune this property. The various hydrophobic methacrylate groups also changed the storage modulus, or stiffness, at 37°C. Furthermore, the solvent utilized for PNASI synthesis – specifically benzene, toluene, or blends of these two – significantly changed the resulting polymer size. The hydrophilic chain included within the polymer could also be varied by changing the length of PEG used, chemical functionalities of PEG chain ends (i.e. carboxylic acid, maleimide, thiol, etc.), and/or the relative molar ratio of PEG attached to the PNIPAAm-methacrylate backbone compared to PNIPAAm and hydrophobic methacrylate groups.

Rheology analysis

Rheology analysis was performed using an AntonPaar Physica MCR 301 rheometer with a 7 mm flat plate. Temperature sweeps were performed at a rate of 1-2°C/minute at 1 Hz and 1% strain.

GPC

Analysis via gel permeation chromatography (GPC) was performed using a Viscotek GPCmax VE2001 solvent/sample module from Malvern with dimethylformamide (DMF) as the solvent and poly(ethylene oxide) (PEO) standards.

NMR

¹H NMR analysis was performed via Bruker 500 Ultrashield to determine mol% of hydrophobic methacrylate groups of PNASI-methacrylate polymers, ratios of PNIPAM and PEG peaks of final polymer, and confirmation of thiol, methacrylate, and heparin peaks.

Cell culture:

For culture within 3D gels, cells were either encapsulated after growing in 2D culture on Matrigel or after culture in Mebiol (CosmoBio) [20]. From 2D culture on Matrigel (Corning), Essential 8 (E8) (Life Technologies) media was aspirated followed by an Accutase (Life Technologies) incubation for 5 minutes at 37°C. Cell solution was collected, placed on ice, and manually pipetted to ensure cell dissociation. Cells and polymer solution were homogenously mixed on ice before pipetting onto tissue culture plates. To encapsulate cells after culture in Mebiol, ice cold PBS was added to wells containing hydrogel for liquefaction, and the cold hydrogel/cell solution was then collected and centrifuged at 3000 rpm for 3 minutes. Accutase was added to cell pellets, and a 10-12 minute incubation at 37°C enabled dissociation of cell aggregates into single cells for re-encapsulation. Cells and the hydrogel were homogenously mixed on ice before pipetting onto tissue culture plates. The plates were incubated at 37°C for 15 minutes before addition of E8 with 10 µM Rho kinase (ROCK) inhibitor (Y-27632, Selleckchem). When cells were seeded as small aggregates instead of single cells, the cells were grown in Mebiol for 1-2 days before using cold PBS to release from hydrogel; no accutase was used to break up the small aggregates. Cells were grown for 4-5 days until aggregates were ~100 µm in size. Recovering cells after growing from small aggregates follows the same procedure as recovering cells grown from single cells.

Antibody staining

To analyze pluripotency marker expression in cells cultured in the hydrogels, hPSC aggregates were recovered from the hydrogels by using cold PBS and centrifugation. They were plated onto Matrigel-coated plates and allowed to attach for 48 hours before fixing with 4 w/v% paraformaldehyde (PFA) at room temperature for 15 minutes, permeabilized with 0.25% Triton X-100 for 15 minutes and blocked with 5 v/v% serum plus primary antibodies at 4°C for 16 hours. After multiple washing steps, secondary antibodies in 2 w/v% BSA were added and incubated at 4°C for 4 hours. Cells were washed again multiple times before imaging.

Embryoid body differentiation:

To analyze the cells' abilities to differentiate into all three germ layers, hPSC aggregates were recovered from hydrogels and plated onto 0.1% gelatin-coated plates and grown in 20 v/v% fetal bovine serum (FBS) in DMEM/F12 media (Life Technologies) plus 10 µM β-mercaptoethanol, 1 v/v% Glutamax (Life Technologies), 1 v/v% minimum essential medium non-essential amino acids (Invitrogen), and 1 v/v% Penn/Strep. After 25-30 days, the cells were fixed and stained as above.

Antibodies: Oct4 (Santa Cruz Biotechnology; 1:200), Nanog (Abcam; 1:250), αSMA (Abcam; 1:200), FOXA2/HNF3β (Millipore; 1:500), and β3Tub (Covance 1:500).

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Chapter 3 - Thermoreversible and tunable hyaluronic acid-PNIPAAm hydrogel systems for 3D stem cell culture

3.1 Introduction

Human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have the abilities to replicate indefinitely and to differentiate to all cell types of the adult body [1-3]. Because of these benefits, hPSCs could be harnessed for biomedical therapies including cell replacement therapies (CRT), tissue engineering, or pharmacology/toxicology screening [4-9]. For CRT and tissue engineering applications, disease targets often involve large patient populations, large numbers of cells are typically needed [10-12], and cell survival after implantation into preclinical models is currently exceedingly low [10, 11]. As a result, scalable methods for hPSC expansion and differentiation are needed.

Most current systems rely on two-dimensional (2D) surfaces and involve animal or human tissue derived components [13]. For example, the standard protocol for culturing stem cells on 2D surfaces utilizes Matrigel, a gelatinous mixture of proteins secreted from mouse sarcoma cells, a process that has been utilized for clinical development but would be ideal to replace with reliable, defined systems due to lot-to-lot variability and the potential for pathogen or immunogen transfer to the human cells prior to implantation. Three-dimensional (3D) systems have also been developed to improve the yield over 2D culture and use animal-free materials; however shear stress and diffusion limitations exist for most of these methods [12]. hPSCs can be grown as aggregates floating in a suspension reactor, which better mimics *in vivo* growth but is difficult to culture homogenous aggregates and diffusion limitations can arise causing necrotic centers with cell death or differentiation and cells can experience harmful shear forces on the outside of the aggregates; microcarriers provide a scaffold for the cells to grow on but can also suffer from shear stress and diffusion limitations [12]. Encapsulation of cells within defined hydrogels is a promising solution to minimize the effects of shear stress and prevent large scale aggregation formation [14-16].

We have recently reported a defined, scalable 3D hPSC culture system, which is based on a thermoreversible hydrogel composed of poly(N-isopropylacrylamide) (PNIPAAm or PNIPAM) and poly(ethylene glycol) (PEG); this hydrogel system in combination with fully defined medium was able to maintain high cell viability and pluripotency as well as a high expansion rate of ~20 fold per 5 day passage, resulting in a 10^{72} fold expansion over a 280 day culture study [17]. This PNIPAAm-PEG hydrogel proved extremely useful for hPSC culture, however it was not amenable to attaching biochemical cues and was very viscous at cold temperatures, making cell encapsulation during scale up difficult. We wanted to develop another type of

thermoreversible hydrogel using hyaluronic acid (HA) as the major hydrophilic component instead of polyethylene glycol, which is protein and cell repellent. HA is a polysaccharide made up of disaccharide units found throughout the body in the extracellular matrix (ECM) and has both structural and biological roles in the ECM including cell differentiation, proliferation, and nutrient diffusion [18]. HA is biocompatible, biodegradable, and forms gels easily and has therefore been used in a range of biomedical applications including tissue engineering, drug delivery, and cell replacement therapies but never for *in vitro* scalable cell culture [19]. However, routine cell culture requires both ease of cell encapsulation and recovery, but HA hydrogels to date have typically involved gel formation via covalent crosslinking, such as for cell implantation applications where cell recovery or harvesting from the material is not relevant [20-24]. In one case, hESCs were cultured *in vitro* within crosslinked HA gels and maintained the ability to self-renew and form embryoid bodies [25], suggesting that HA gels could be beneficial for stem cell expansion. However, in general cell recovery from crosslinked HA gels involves enzymatic or chemical treatments which involve time and resources [25, 26].

Hydrogels are typically classified by their mode of gel formation; chemically crosslinked gels are the most common form and utilize chemical crosslinks between polymer chains to produce an irreversible network and physically linked gels utilize secondary interactions induced from an environmental stimulus (temperature or pH) including ionic or hydrophobic interactions [27, 28]. To obviate the need for covalently crosslinked gels, we have grafted a thermoreversible component (PNIPAAm based polymers) onto HA polymer chains and explored the potential of this family of polymers. We designed the system to have tunable mechanical properties for different cell culture applications, and the material could readily be functionalized to incorporate additional biochemical cues to modulate proliferation or differentiation. Subsequent tuning of the rheological and biochemical properties of this thermoreversible hydrogel system enabled successful culture of multiple hPSC lines with cells remaining viable and pluripotent after multiple rounds as well as successful neural stem cell culture through multiple rounds.

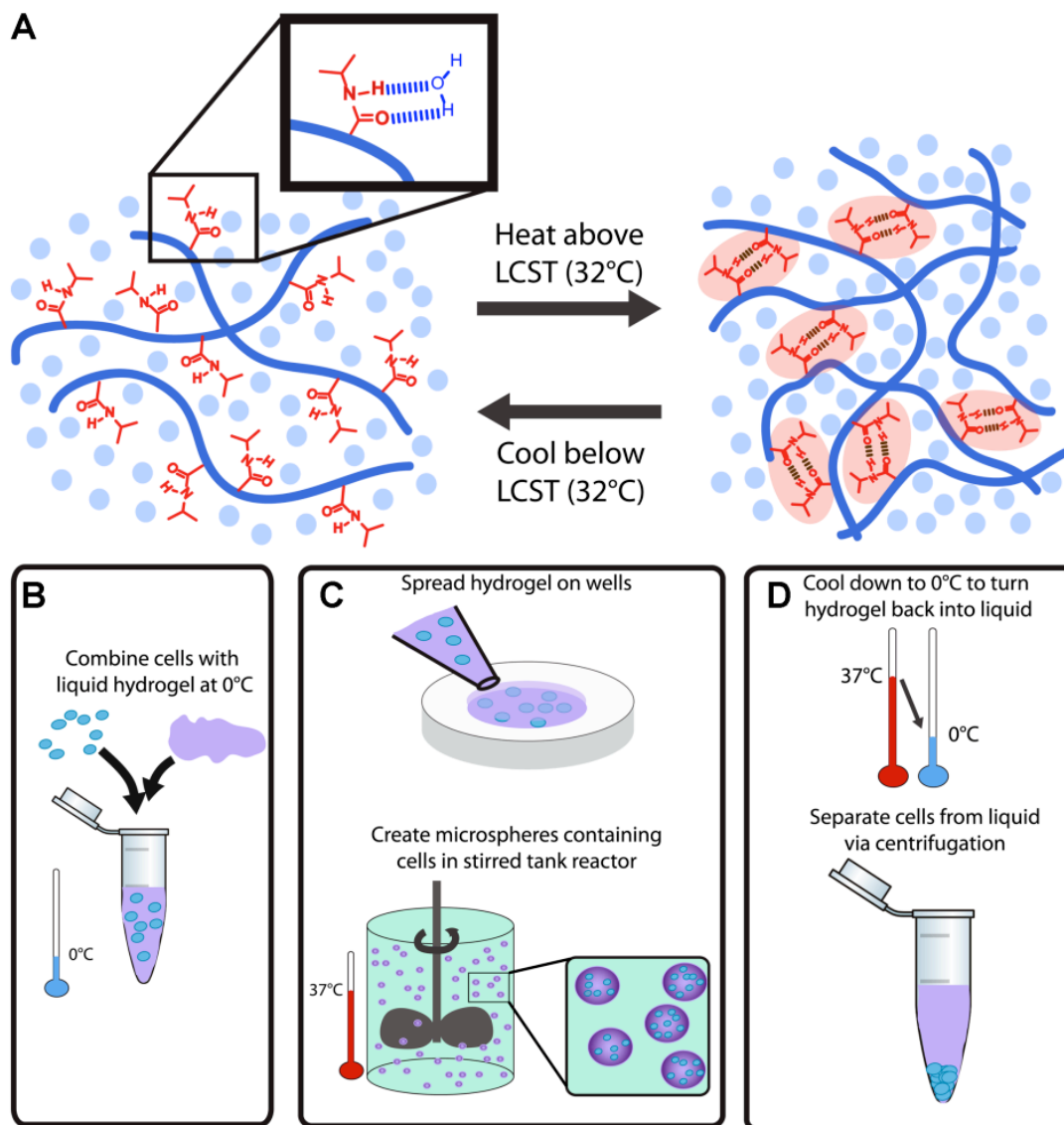


Figure 3.1 HA-PNIPAAm hydrogel gelling process and cell encapsulation procedure.

Schematic of HA-PNIPAAm showing PNIPAAm amide groups hydrogen bonding with water below the LCST and hydrogen bonding with each other above the LCST, creating hydrophobic microdomains (A). B-D illustrate the steps for cell encapsulation.

3.2 Results

Polymer synthesis results:

HA functionalization with divinyl sulfone (DVS) was varied to optimize PNIPAAm conjugation. HA is dissolved in NaOH to deprotonate a portion of the hydroxyl groups along the HA chains, providing functional chemical groups for the vinyl sulfones to react with. At 0.1M NaOH, there is a calculated concentration of approximately 0.1M deprotonated hydroxyl groups (4 OH's per disaccharide). A balance between number of vinyl sulfone groups and number of deprotonated hydroxyl groups is necessary to prevent crosslinking via divinyl sulfone groups reacting with two different HA chains or generating overfunctionalized HA which is no longer soluble in

aqueous solutions [29]. We achieved vinyl sulfone attachment to approximately 30%, 50% and 70% of the total number of disaccharides by using 1.5x, 2.5x, and 3.5x excess DVS to OH groups, respectively, for a range of HA molecular weights from 100 kDa to 1 MDa. Fresh 0.1M NaOH produced consistent results of how much the HA would be functionalized. Increasing amounts of vinyl sulfone groups from ~30% to ~70% attached to the HA disaccharides made the polymer more difficult to dissolve in aqueous buffers, most likely due to the more hydrophobic nature of the ethyl vinyl sulfone compared to the initial hydroxyl group reactant.

PNIPAAm-SH was synthesized via reversible addition-fragmentation chain transfer (RAFT) synthesis using azoisobutyronitrile (AIBN) as the initiator and 2-(Dodecylthiocarbonothioylthio)-2-methylpropionic acid (DMP) as the chain transfer agent (CTA) as shown in Figure B.1 [30, 31]. DMP and other similar thiocarbonylthio chain transfer agents are commonly used to synthesize polymers with the ultimate goal of cleaving these groups from the polymer chain ends to leave behind a thiol group for thiol-ene or disulfide reactions [32]. The CTA was reduced using NaBH₄ to generate thiols on the chain ends. PNIPAAm-CTA polymers were all approximately 20 kg/mol in size based on RI detection and PNIPAAm dn/dc values. Optimization of conditions for CTA reduction resulted in PNIPAAm-SH polymers with varying degrees of bulk thiol content potentially due to polymer precipitation during reduction causing different extents of polymer reduction (as measured via Ellman's assay).

Hydrogel synthesis results:

PNIPAAm is a thermoreversible polymer with a lower critical solution temperature (LCST) at ~32°C [33]. Below the LCST, the acrylamide groups hydrogen bond with water; however, above this key temperature it is more thermodynamically favorable for the hydrophobic acrylamide groups to interact with one another rather than water. When PNIPAAm is conjugated to a hydrophilic polymers such as hyaluronic acid, heating induces PNIPAAm microphase separation with accompanying physical links between polymer chains, leading to hydrogel formation [16].

To conjugate PNIPAAm to HA, PNIPAAm-SH was reacted with the vinyl sulfone groups available on the HA-VS disaccharides via thiol-ene chemistry. Initial ratios of 10:1 PNIPAAm-SH:HA-VS by mass were utilized to generate hydrogels. This corresponded to a thiol: vinyl sulfone molar ratio of ~0.63, 0.38, and 0.27 for 30%, 50%, and 70% functionalized disaccharides, respectively, which left residual functional groups for further attachment of biochemical cues or other components. Alternatively, the vinyl sulfone groups were quenched with an excess of cysteine. After extensive dialysis to remove unreacted PNIPAAm, ¹H NMR analysis was conducted and the relative ratios of HA peaks to PNIPAAm peaks were assessed (Figure B.2); after quenching with excess cysteine no peaks corresponding to vinyl groups appeared. Hydrogels were analyzed via amplitude sweeps, frequency sweeps and/or temperature sweeps from 4-50°C in order to determine their LCST, storage and loss moduli at 37°C and any unexpected rheology behavior such as shrinking/water excretion. Once PNIPAAm attachment was confirmed via NMR and rheology data showed the transition to hydrogel state, the hydrogels were utilized to encapsulate cells for cell culture. However, the initial hydrogels synthesized were either too soft or would dissolve away upon media addition. Therefore, the first optimization step for these polymers was to generate thermoreversible hydrogels that were stiffer

and maintained their gel-like consistency during cell culture, yet were still fluid enough at cold temperatures to pipette and easily mix with cells.

HA size affects mechanical and rheological properties:

To increase the stiffness of the thermoreversible HA-PNIPAAm hydrogels, we first probed whether the size of the HA would affect the mechanical properties of the resulting hydrogel. Generally increasing the molecular weight of a polymer will increase the stiffness for most systems. Using the same weight ratio of 10:1 PNIPAAm-SH:HA-VS with HA polymers that were all ~30% disaccharide functionalized and the same PNIPAAm-SH we saw a noticeable increase in stiffness with increasing HA molecular weight (Figure 3.2a). HA-PNIPAAm polymer solutions of 10 w/v% made with HA molecular weights of 100 kDa, 200 kDa, 700 kDa and 1 MDa had storage moduli at 37°C around 100 Pa, 600 Pa, 1 kPa, and 2 kPa, respectively. We also saw an increase in the LCST of these polymers with increasing HA molecular weight. HA-PNIPAAm polymer solutions of 10 w/v% made with HA molecular weights of 100 kDa, 200 kDa, 700 kDa and 1 MDa had LCSTs of approximately 33°C, 32°C, 30°C, and 29°C, though the 700 kDa HA hydrogel had a higher storage modulus than loss modulus before the temperature-induced stiffness change from a solution to a gel. Increasing the HA molecular weight increased the storage modulus of the thermoreversible hydrogels and decreased the gel transition temperature. The viscosity of the polymers made with the largest size hyaluronic acids were still at a low enough level to provide easy pipetting.

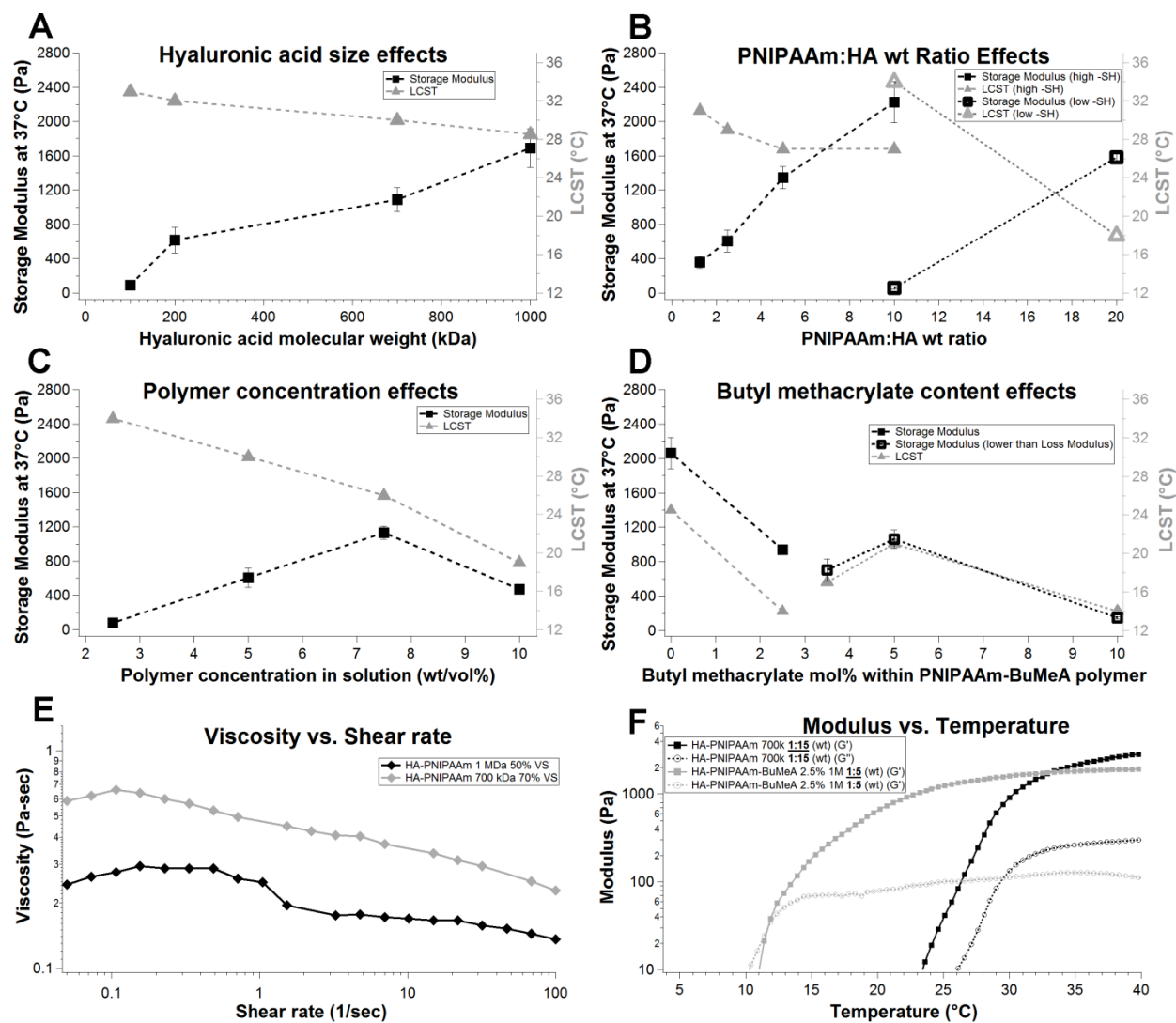


Figure 3.2 Rheology data for HA-PNIPAAm hydrogels.

A-D: Storage modulus at 37°C (black squares) and LCST (grey triangles) as a function of HA molecular weight (A), PNIPAAm:HA ratio (B), Polymer concentration in solution (C), Butyl methacrylate mol% within PNIPAAm-BuMeA polymer (D). All error bars represent average storage modulus between 35-39°C and concentrations are 10 w/v% unless otherwise noted. Panel A gels all were ~30% functionalized. Panel B shows two different conditions, one with high thiol content (solid squares and triangles), the other with low thiol content (open squares and triangles) as measured by Ellman's assay. Panel C shows the same 1 MDa 40% HA-PNIPAAm gel at different concentrations. Panel D shows the conditions with 3.5, 5, and 10 mol% butyl methacrylate with storage moduli less than loss moduli with open squares; gels with 0, 3.5 and 3 mol% butyl methacrylate all made from same 700 kDa HA-VS 40% batch and 5 and 10 mol% gels made from same 1 MDa HA-VS 25% batch. E. Viscosity of different HA-PNIPAAm hydrogels as a function of shear rate with 1 MDa HA (black) and 700 kDa HA (grey) at 10 w/v%. F. Storage modulus (G') and loss modulus (G'') as a function of temperature for a 700 kDa HA-PNIPAAm polymer at 7.5 w/v% (grey) and 1M HA-PNIPAAm-BuMeA with 2.5% butyl methacrylate at 10 w/v% (black).

Ratio of PNIPAAm-SH to HA affects mechanical and rheological properties

In addition to changing the molecular weight of HA to achieve stiffer and more durable hydrogels, we also altered the ratio of PNIPAAm-SH to HA to determine if this was another route to tune the mechanical properties of the hydrogels. Increasing the amount of PNIPAAm attached to a hydrophilic polymer was proposed to decrease the spacing between hydrophobic groups and ultimately form larger microphase-separated hydrophobic domains, thus generating

hydrogels with higher storage moduli. We varied the ratio of PNIPAAm-SH to HA using a constant 1 MDa HA that was 30% functionalized and the same PNIPAAm-SH polymer. Initial variations included 1:10, 1:5, 1:2.5 and 1:1.25 HA-VS: PNIPAAm-SH by weight, corresponding to a molar ratio of thiols to vinyl sulfones of 0.63, 0.32, 0.16, 0.08, respectively. As the weight ratio of PNIPAAm-SH increased from 1.25 to 10 we saw an increase in storage modulus; for weight ratio values of 1.25, 2.5, 5, and 10, the storage moduli values were approximately 300 Pa, 500 Pa, 1 kPa and 2 kPa, respectively (Figure 3.2b). The LCSTs also decreased from ~31°C to ~27°C from 1.25 to 10 weight ratio values. This suggests that as more PNIPAAm is attached to the functionalized HA there is an increase in hydrophobic phase separation upon heating, lowering the LCST and increasing the stiffness.

We also noticed for HA-PNIPAAm hydrogels made using PNIPAAm batches with lower bulk thiol content (based on Ellman's assays) a softer gel (>100 Pa at 37°C) was produced with a ratio of 1:10 HA-VS to PNIPAAm-SH. This was alleviated by utilizing a higher ratio of 1:20 HA-VS to PNIPAAm-SH with the same 700 kDa HA ~40% functionalized. The resulting HA-VS-PNIPAAm hydrogel had an increased stiffness of ~1 kPa, close to the range of ~1-2 kPa typically produced from PNIPAAm batches with higher bulk thiol contents. This proved that batch to batch variability with PNIPAAm-SH thiol content can be counteracted by the ratio of how much PNIPAAm reacts with the HA-VS. However, we have also found that incorporating too much PNIPAAm into the HA polymer can cause water excretion and hydrogel shrinking. This is seen visually but also by a decrease in the storage (and loss) modulus of the hydrogel as the temperature is increased above the LCST. If this mild shrinkage does not harm cells during the gelling process it adds an additional complication of lifting the gel off the bottom of the dish and this lifted gel could experience shear forces from moving around during media changes. Sometimes the water excretion and gel shrinkage could be delayed to a higher temperature by reducing the concentration of polymer in solution to make the hydrogel. A reduction in the concentration increases the LCST and makes the resulting hydrogel softer, as seen with Figure 3.2c and Figure B.6.

Incorporating hydrophobic butyl methacrylate lowers LCST

Once we generated HA-PNIPAAm polymers that produced hydrogels at an appropriate stiffness for cell culture and that did not dissolve or shrink a harmful amount for cell health we next focused on lowering the LCST below room temperature. Addition of hydrophobic groups such as butyl methacrylate has been used in synthesis of thermoreversible polymers to decrease the LCST [34, 35]. Similar to an increase in PNIPAAm attachment to the HA polymer, incorporation of hydrophobic groups into the polymer results in a decrease in the distance between hydrophobic groups. This in turn reduces the solubility of the polymer in water and lowers the temperature at which the microphase-separated hydrophobic domains cause the polymer to gel. To incorporate hydrophobic butyl methacrylate into the final polymer, it was synthesized with NIPAM monomer to make a random copolymer of PNIPAAm-butyl methacrylate-CTA (PNIPAAm-BuMeA-CTA). Initial molar ratios of 5 and 10 mol% butyl methacrylate were included within the polymer. These concentrations did significantly decrease the transition temperature at which the moduli increased (~14°C for 10 mol% and ~21°C for 5 mol%), however they did not show stable rheology with increased temperature (Figure 3.2d, Figure B.7). The 10 mol% BuMeA polymer had a storage modulus around 200 Pa at 37°C while the 5 mol% BuMeA polymer had a higher loss modulus than storage modulus, suggesting a more

fluid behavior than elastic, possibly from water excretion. Blending these HA-PNIPAAm-BuMeA polymers with HA-PNIPAAm polymers did not significantly improve the rheological or mechanical properties of the resulting hydrogels. Therefore a lower concentration of 3.5 mol% BuMeA was included with PNIPAAm next to determine if 3.5 mol% could alter the LCST and still make hydrogels with desired mechanical properties. This concentration of butyl methacrylate suffered the same rheological behavior as the 5 mol% BuMeA polymer with a higher loss modulus than storage modulus and also lowered the LCST below room temperature. A concentration of 2.5 mol% BuMeA was next incorporated with PNIPAAm and showed a decrease in LCST between 11-19°C depending on the ratio of HA-VS to PNIPAAm-SH and the size of the HA (between 700 kDa and 1 MDa) (Figure 3.2d and Figure B.7). The resulting HA-PNIPAAm-BuMeA hydrogels could attain storage moduli between 800 Pa and 2 kPa proving beneficial for cell culture. Incorporation of only 2.5 mol% hydrophobic butyl methacrylate into the PNIPAAm-SH used to make the HA-PNIPAAm polymers could successfully lower the LCST and maintain a high storage modulus at 37°C.

Hydrogel optimization

The HA hydrogels were optimized to have storage moduli around 1 kPa and maintain their gel-like consistency after multiple days in culture. This was accomplished by using HA sizes around 700 kDa – 1 MDa. This was also more likely achieved with HAs that were 50-70% functionalized with vinyl sulfone groups. Figure 3.2f shows two HA-PNIPAAm polymers, one with 2.5 mol% butyl methacrylate, which were optimized to reach ~1 kPa storage modulus at 37°C, did not shrink significantly upon gelling, and could continuously culture cells for at least 5 rounds as single cells (HA-PNIPAAm) or culture cells as aggregates (HA-PNIPAAm-BuMeA).

Stem cell culture and proliferation

Thermoreversible HA hydrogels were designed specifically to encapsulate cells for proliferation and expansion as well as potential future application for differentiation. The cells (single cells or small aggregates) were homogenously combined with the polymer solution at cold temperatures before pipetting onto tissue culture plates and placing in the incubator to solidify prior to media addition. The media were then changed daily until cells reached a desired density, at which point the hydrogels were cooled, and cells were harvested via centrifugation for additional culture.

hPSCs were encapsulated within the better performing thermoreversible hydrogels to investigate and quantify their expansion. H9 and H1 hESCs were able to successfully grow in these cells from small aggregates (after growing 24 hours in a PNIPAAm-PEG gel), and TCTF iPSCs were successfully grown from single cells continuously within these hydrogels. TCTFs were able to expand ~3-10 fold over the course of 4-5 days in these synthesized gels and aggregate size was consistent throughout the gels (Figure 3.3).

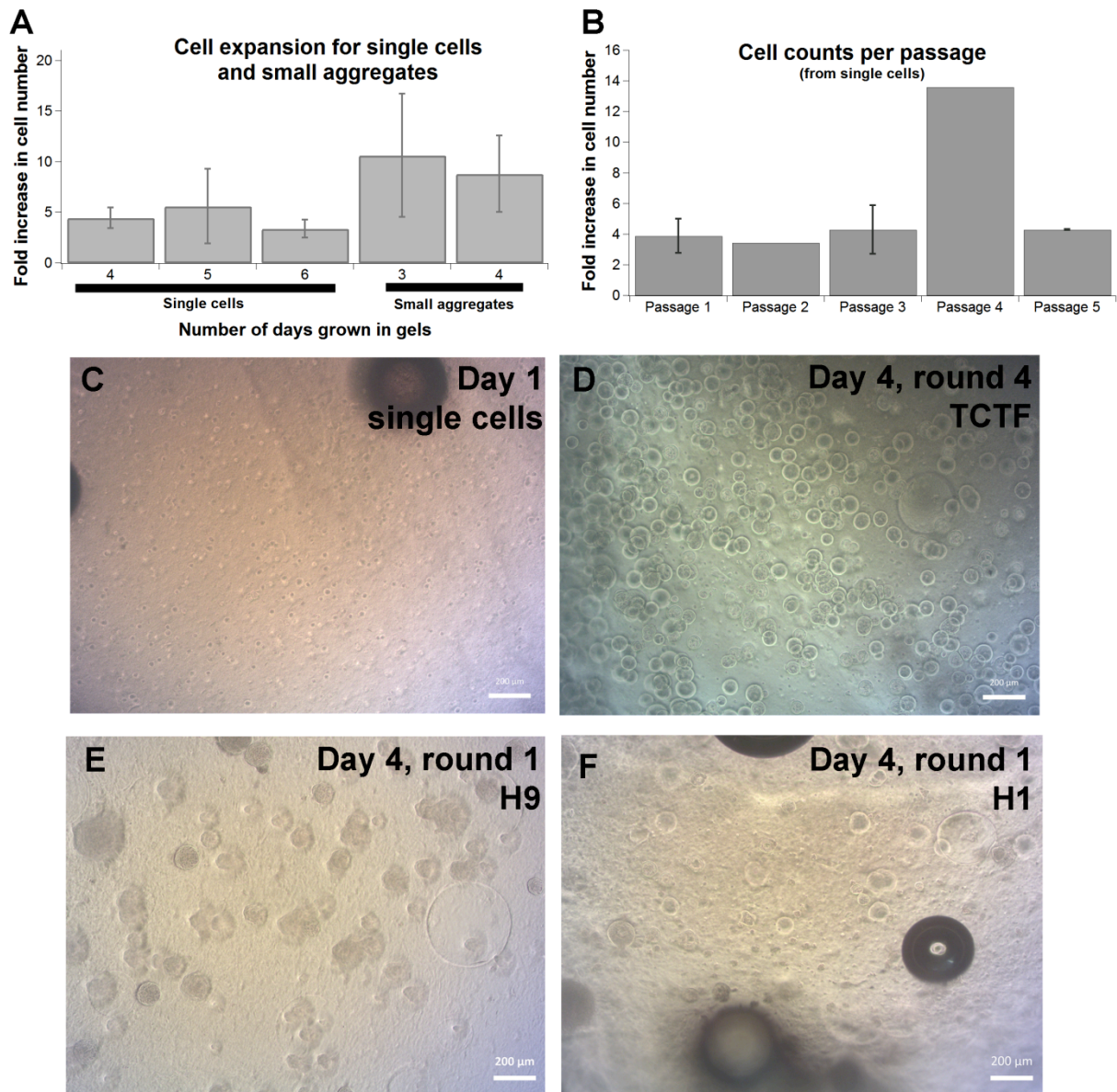


Figure 3.3 hPSC expansion data and brightfield images of cell aggregates.

A. TCTF cell expansion rates based on days grown from single cells for single cells and small aggregates plated after 2 days of growth from single cells. B. TCTF expansion rates based on passage number for multiple types of HA-PNIPAAm hydrogels: 700 kDa HA 50% functionalized round 1 (red), 700 kDa HA 50% functionalized round 2 (green), 700 kDa HA 70% functionalized round 1 (blue), multiple 700 kDa and 1 MDa gels individual data points (black open triangles). C-H. Pictures of cell growth for different cell lines. C. Day 1 TCTF single cells D. Day 4 in 4th round TCTF plated as single cells E. Day 4 H9 cells plated as single cells for 1 round F. Day 4 H1 cells plated as single cells for 1 round. Scale bars are all 200µm.

In order to determine whether the hydrogels could be used for multiple types of stem cells, rat neural progenitor cells (NPCs) were also encapsulated within the HA-PNIPAAm hydrogels at varying concentrations of polymer in solution to generate hydrogels with storage moduli ranging from ~100 Pa - 800 Pa (Figure 3.5a-b). Initial studies showed that the softer hydrogels supported NPC proliferation better than stiffer gels for the 100 Pa and 500 Pa gels, while the 800 Pa gel did not support NPC growth at all. NPCs within these gels had a 2-6 fold increase in cell number after 6 days of culture within these gels.

Stem cell pluripotency/multipotency

To test whether the hPSCs retained their pluripotency after 1-5 passages within the materials, we performed antibody staining for pluripotency markers Nanog and Oct4 as well as an embryoid body differentiation. Aggregates removed from the hydrogels were plated on Matrigel for 2 days to allow attachment before fixing and staining for pluripotency protein markers (Oct4 and Nanog). H9 and H1 hESCs and TCTF iPSCs maintained expression of pluripotency markers Oct4 and Nanog (after 5 passages for TCTF's) (Figure 3.4).

To functionally assess pluripotency, embryoid bodies (EB) were generated by allowing aggregates recovered from the hydrogels to attach to gelatin-coated plates (as opposed to growing on low-adhesion plates to form the EBs) with DMEM/F12 media supplemented with fetal bovine serum (FBS), beta mercaptoethanol, glutamine, non-essential amino acids, and gelatin. Cells were grown on the gelatin-coated plates for 25-30 days before fixing and staining for all three germ layers. The mesoderm layer lies in the middle of the embryo and ultimately forms muscle cells, bone cells, connective tissue, and blood. The endoderm layer is an internal layer that generates the gastrointestinal tract and lung. Finally, the ectoderm generates skin and the central nervous system. EB differentiation showed that cells stained positive for markers of all three lineages (actin for mesoderm, HNFb/FoxA2 for endoderm, and Tuj1 for ectoderm) after multiple successive rounds in the HA-PNIPAAm hydrogels.

In addition to assessing pluripotency of hPSCs, we also wanted to analyze NPC multipotency after growth within the gels. Therefore, NPCs grown in the hydrogels from single cells into aggregates were plated onto laminin-coated plates for attachment before antibody staining for Sox2, a marker of NSC multipotency. Figure 3.5c-d shows colocalization of DAPI nuclear stain and Sox2 NPC marker.

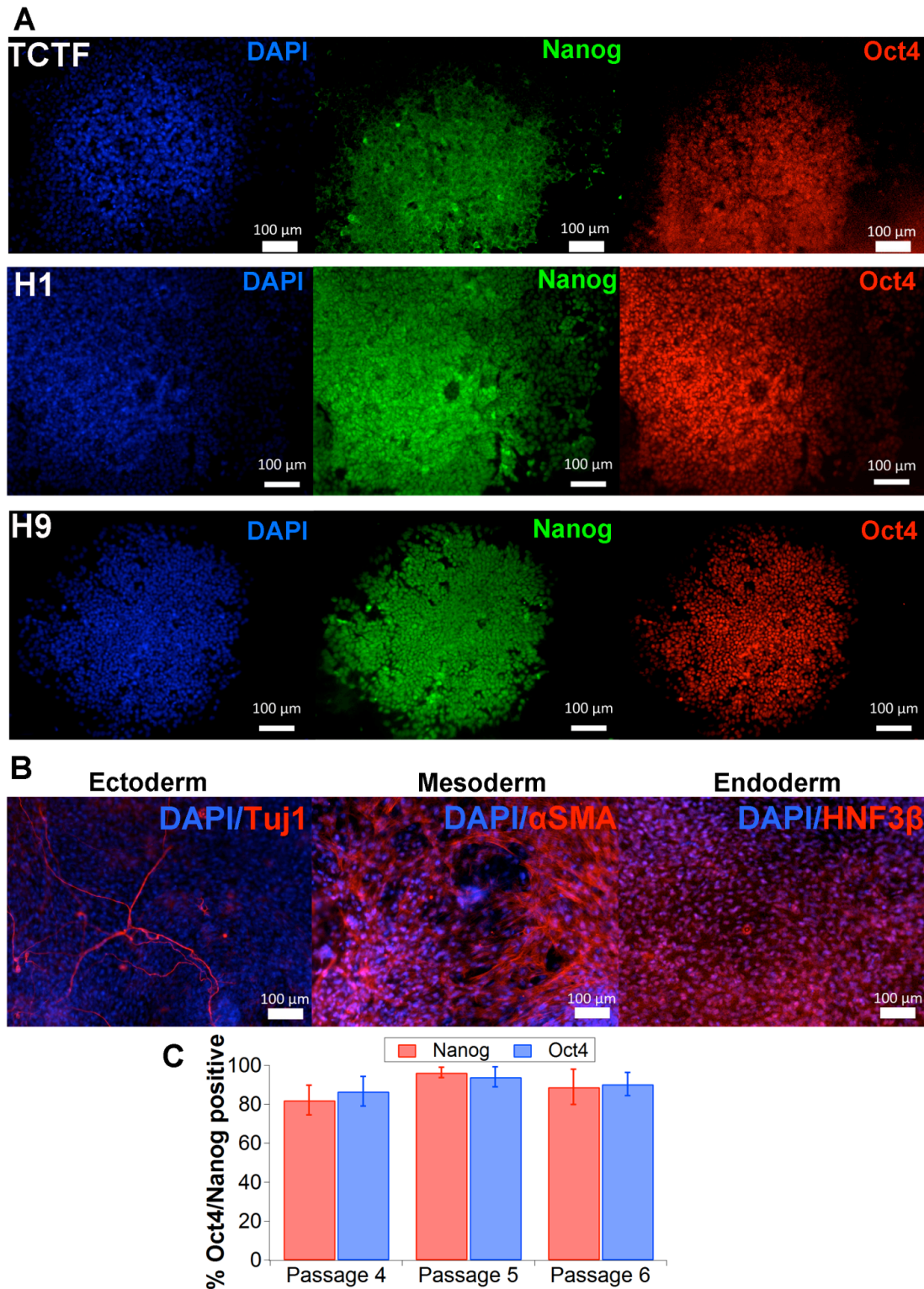


Figure 3.4 hPSCs express pluripotency markers and differentiate to all three germ layers after growth within the gels.

A. Antibody staining for pluripotency markers Nanog and Oct4 for TCTF iPSCs (after round 4), H1 hESC, and H9 hESCs (both hESC lines after round 1). B. TCTF iPSCs remain pluripotent after multiple rounds cultured in hydrogels. Aggregates were recovered from the hydrogels and plated on gelatin plates for ~25-30 days before performing antibody staining for all three germ layers (Tuj1 for ectoderm, α SMA for mesoderm, and HNF3 β /FOXA2 for endoderm). All scale bars are 100 μ m.

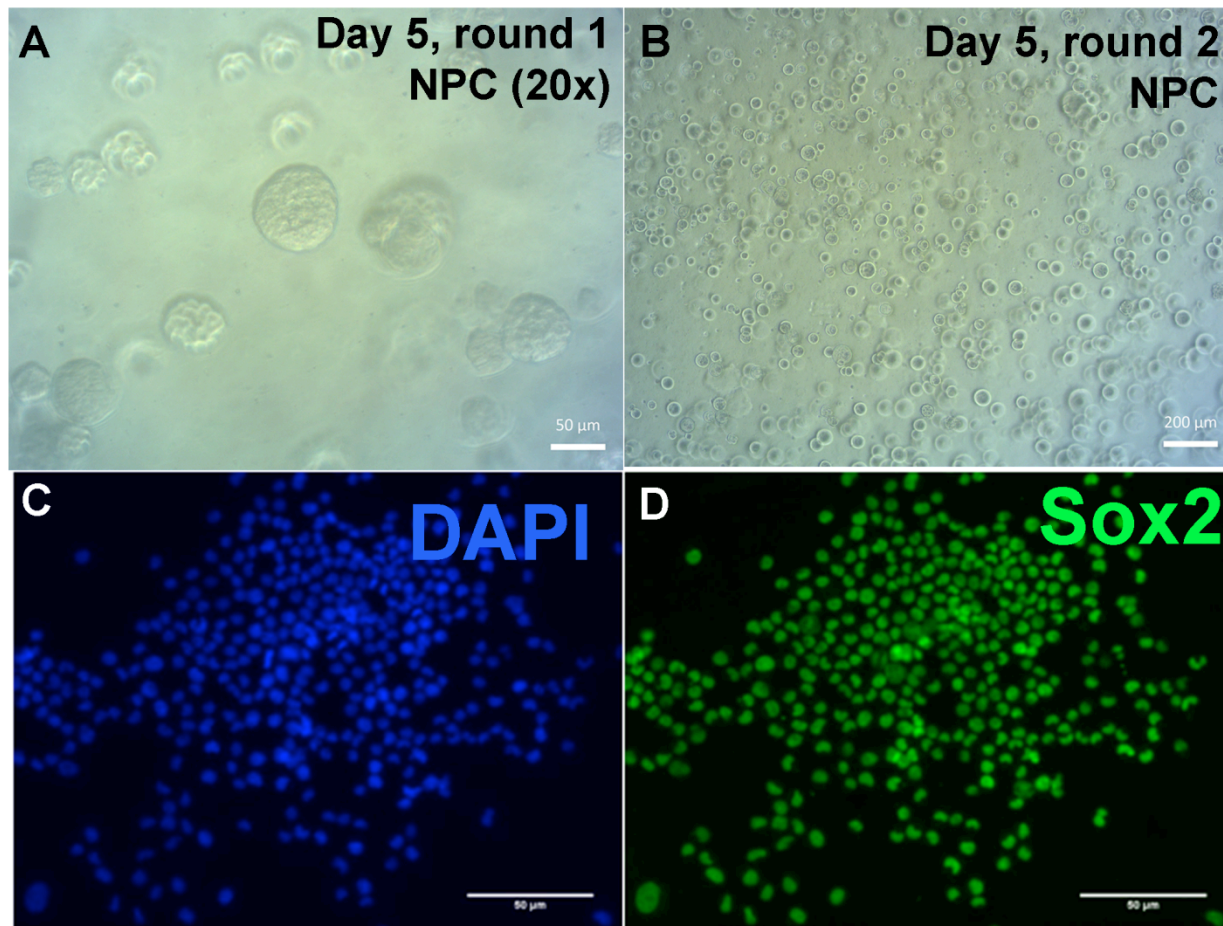


Figure 3.5. NPC growth and multipotency analysis.

Brightfield images showing how single cells grew into neurospheres by 5 days in the gels (A-B). C-D. Antibody staining for multipotency marker Sox2 and nuclear DAPI staining coincide for almost all NPCs.

Functionalization for heparin and biochemical cue incorporation

There are many biochemical cues that can be added to cell culture media to influence stem cell growth or differentiation; however these recombinant proteins can become extremely expensive in the quantities necessary to incorporate within the media. These biochemical components can be attached to materials in order to reduce the possibility of cell-mediated endocytosis as well as increase their local concentration and potency [36-38]. Some proteins contain heparin-binding domains which can noncovalently interact with heparin, therefore immobilizing the proteins wherever a heparin is conjugated to a material making it accessible to the cells without loss of functionality [39-43].

The hyaluronic acid hydrogels were designed with vinyl sulfone groups to attach polymers or biochemical cues via thiol-ene chemistry. There are sufficient amounts of vinyl sulfone groups along the hyaluronic acid chains so that both the PNIPAAm-SH and biochemical ligands or heparin can also be conjugated. Heparin functionalized with thiol groups could be conjugated to the polymer at a molar ratio of approximately 1 mol%. Heparin could then bind with two proteins contained in the E8 media (TGFβ and FGF) to potentially increase the growth rate of the stem cells.

3.3 Discussion

There is a major need for scalable stem cell manufacturing systems to support the clinical development of cell replacement therapies as well as for toxicology or pharmacology screening. Current culture methods typically involve limited scale 2D culture and/or undefined animal or human tissue derived components that raise challenges with reproducibility, scalability, pathogen transfer, and immunogenicity. We have developed a 3D thermoreversible system consisting of HA and PNIPAAm for scalable and defined culture, easy cell encapsulation and recovery, and tunable mechanical and biochemical properties to optimize cell proliferation or differentiation towards specific lineages.

The HA-PNIPAAm hydrogels were generated by the conjugation of synthesized PNIPAAm-SH to HA functionalized with vinyl sulfone groups. The PNIPAAm-SH was synthesized using RAFT polymerization with DMP as the chain transfer agent so that reduction of the thiocarbonylthio generated thiol chain ends. Hyaluronic acid was functionalized by reacting divinyl sulfone with the polysaccharide in basic conditions. Disaccharides were functionalized with vinyl sulfone groups to provide enough attachment sites for PNIPAAm-SH to conjugate and form a stable hydrogel at 37°C while still leaving some vinyl sulfone groups for further conjugation of other components such as peptides or heparin. HA chains with less than ~25% functionalized disaccharides never made stable hydrogels, most likely due to an insufficient amount of PNIPAAm attaching to create the necessary hydrophobic microdomains for physical linking of HA chains. HA chains that were highly functionalized with vinyl sulfone (~50-70% disaccharides) were increasingly more difficult to dissolve in aqueous buffers than those with lower vinyl sulfone functionalization, however conjugation of PNIPAAm-SH to the highly functionalized HA's resulted in a soluble liquid at cold temperatures that was less viscous than HA-VS by itself and a hydrogel at 37°C. Attachment of PNIPAAm-SH to vinyl sulfone-functionalized HA generated thermoreversible hydrogels.

This HA-PNIPAAm hydrogel system had many variables that were optimized for the characteristics and tunable properties desired for cell culture. We first designed the system to be temperature responsive to encapsulate cells at 37°C and maintain the gel-like consistency for multiple days without dissolving or shrinking; this was accomplished by attaching PNIPAAm to the HA-VS. In functionalizing the HA to attach PNIPAAm, we had remaining chemical functionalities that could be used to attach biochemical cues, another important requirement. Cell culture was attempted in initial hydrogels, however the hydrogels were too soft for cell culture or would dissolve upon media addition. Work specifically with hPSCs on 2D surfaces has shown that soft substrates (~100 Pa) promoted neuronal differentiation but not proliferation and stiff substrates (700 Pa and 75 kPa) increased the rate of proliferation [44, 45]. In addition, a comparison of crosslinked HA and agarose gels versus physically-linked alginate gels showed that encapsulated cells grew best in the physically-linked softer (~2 kPa) alginate gels compared to the ~3 kPa HA and agarose crosslinked gels [46]. Therefore we aimed to generate hydrogels with storage moduli ~1 kPa to promote hPSC survival and proliferation. In order to increase hydrogel stiffness, we varied the HA size, percent functionalization with vinyl sulfone groups, amount of PNIPAAm attached, and amount of hydrophobic group incorporated. During this optimization step we also produced many gels that shrank and excreted water during the gelation

and needed to ensure volume change would not affect cell health. Since the LCST of PNIPAAm is $\sim 32^{\circ}\text{C}$, most of the initial hydrogels had very high LCSTs very close to 37°C , leading to progressive gel reliquifying and loss even during rapid media changes and imaging. Therefore the LCST was decreased by incorporating small amounts of a hydrophobic methacrylate into the PNIPAAm polymer. In addition, we ensured the polymer solutions were still fluid enough to pipette at cold temperatures around $0-4^{\circ}\text{C}$. We were able to produce thermoreversible hydrogels with tunable mechanical and rheological properties with the ability to conjugate biochemical cues that supported hPSC culture and expansion.

In order to increase the stiffness of these HA-PNIPAAm hydrogels, we first varied the size of the HA polymer chains. HAs from 100 kDa- 1 MDa were functionalized with the same percent of vinyl sulfone and the same PNIPAAm-SH. As expected, the larger HA polymers generated hydrogels with higher storage moduli around 1-2 kPa and had slightly lower LCSTs than the hydrogels made with smaller HAs. By increasing the length of the HA chains while the percent of functionalized disaccharides remained constant, the polymer chains are more likely to become entangled and the number of interchain interactions for one polymer chain increases, forming a more stable and sturdier network.

We also varied the ratio of HA-VS to PNIPAAm-SH to generate hydrogels with a variety of stiffnesses and rheological properties. Increasing ratios of PNIPAAm-SH to HA-VS (and subsequently ratios of thiols to vinyl sulfone groups) produced hydrogels with increasing storage moduli. This result was from the smaller distance between PNIPAAm groups along the HA polymer chains, resulting in more inter- and intra-chain hydrophobic microdomains and ultimately generating a stiffer network. However, without the right balance of PNIPAAm groups and unfunctionalized HA disaccharides the PNIPAAm hydrophobic microdomains would become too dense, causing the hydrogel to shrink in volume and excrete water. Some hydrogels released only a minimal amount of water upon temperature elevation while others shrunk to thin films. This posed problems for cell culture within these gels because the result of volume change could exert harmful forces on the delicate cells encapsulated in the gel and the thin layer of water excreted would sometimes coat the underside of the hydrogel causing it to lift into solution upon media addition. Decreasing the concentration of polymer dissolved in solution sometimes alleviated this occurrence to a temperature above 37°C ; a more dilute solution increased the distance between polymer chains and decreased the ability for PNIPAAms to hydrogen bond with each other (and also generated softer gels). Increasing the ratio of PNIPAAm-SH to HA generally increased the stiffness of the hydrogels at 37°C but needed to be appropriately balanced to minimize hydrogel shrinking and water excretion.

Generating polymers which formed gels at a lower temperature (close or below room temperature) was the next design goal. Incorporating a hydrophobic group such as butyl methacrylate during the PNIPAAm-CTA synthesis produced random copolymers of PNIPAAm-BuMeA-CTA. Integration of a hydrophobic group with PNIPAAm will increase the hydrophobicity of the polymer and decrease the amount of interactions with water molecules, ultimately lowering the temperature at which the PNIPAAms form hydrophobic microdomains to physically link the polymer chains into a gel. Concentrations of 3.5-10 mol% butyl methacrylate within the PNIPAAm-BuMeA polymer did not create ideal hydrogels when conjugated to HA. These polymers provided too many hydrophobic groups and the resulting

hydrogels shrank, had unstable rheological profiles, or were more fluid than elastic; though the LCSTs were reduced compared to HA-PNIPAAm hydrogels, as predicted. A concentration of only 2.5 mol% butyl methacrylate within the PNIPAAm-BuMeA polymer conjugated to HA produced hydrogels with lower LCSTs than HA-PNIPAAm hydrogels and maintained stable rheological properties generating hydrogels with storage moduli around 1 kPa at 37°C.

hPSCs were encapsulated in the optimized hydrogels and grown for 4-5 days until they reached a desired cell density. We noticed that gels that had storage moduli of approximately 200-500 Pa or >2 kPa did not successfully support hPSC survival and growth. As mentioned previously, hPSCs grown on soft 2D substrates are primed for neuronal differentiation but not cell proliferation and those encapsulated in hydrogels had higher survival and proliferation rates in gels of ~2 kPa storage modulus compared to crosslinked gels around ~3 kPa storage modulus[44-46]. Therefore, an ideal stiffness for hPSC culture and proliferation in 3D may lay in the range of ~ 0.5-2 kPa in order to provide the appropriate mechanical properties for hPSC growth. In addition, previous work investigating stem cell responses to 2D substrate stiffness elucidated the differentiation of stem cells into neurons when grown on soft substrates, bone cells on stiff substrates, and muscle cells on substrates with medium stiffness, showing the stem cells differentiated to cells of particular cell lineages when grown on materials with storage moduli similar to the native environment [47]. This HA-PNIPAAm hydrogel system could be modified to generate a range of hydrogel stiffnesses in order to determine the optimal conditions for stem cell proliferation and also for promoting differentiation towards specific lineages.

H1 and H9 hESCs survived and grew from small aggregates after as little as 24 hours grown in a PNIPAAm-PEG gel. TCTF iPSCs were continuously grown for 5 passages, reaching a peak of approximately 10-fold increase after 5 days growth within the hydrogel. TCTFs grown from single cells through multiple passages maintained consistently-sized morphologically round aggregates each day. The HA- PNIPAAm hydrogels also provided an environment which did not cause the cells to spontaneously differentiate or lose their pluripotency. Cells recovered from the gels after multiple passages stained positive for pluripotency markers Nanog and Oct4 and turned into all 3 germ layers after an EB assay.

In addition, NPCs were grown within these hydrogels of varying stiffnesses from ~100-800 Pa to determine if multiple cell lines could be successfully cultured and potentially differentiated when encapsulated. Softer gels were chosen based on the previous work growing hPSCs on soft 2D surfaces to see if these softer gels would promote neuronal differentiation. Surprisingly, NPCs had a higher proliferation rate in gels around ~100 Pa than those ~500 Pa, while the NPCs encapsulated in gels ~800 Pa did not grow at all. This is the opposite effect of responses on 2D substrates seen previously [44].

3.4 Methods and Materials

Materials:

Hyaluronic acid (HA) was purchased from LifeCore Biomedical. Divinyl sulfone, N-isopropylamine (NIPAAm) monomer, butyl methacrylate monomer, azoisobutyronitrile (AIBN), 2-(Dodecylthiocarbonothioylthio)-2-methylpropionic acid (DMP), dioxane, sodium borohydride

(NaBH₄), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and ethylenediaminetetraacetic acid (EDTA) were all purchased from Sigma Aldrich. NIPAAm monomer was recrystallized in hexanes and butyl methacrylate was purified through an alumina column before use.

HA functionalization with vinyl sulfone groups:

HA (purchased) is first reacted with divinyl sulfone (DVS) to provide vinyl sulfone groups for the simple conjugation of components with free thiol groups. HA is dissolved in 0.1M NaOH followed by immediate addition of DVS of varying molar ratios [29]. The solutions are constantly mixed and allowed to react for 20 minutes before quenching with 1M HCl. The resulting solution is dialyzed against DI water for multiple days and freeze-dried. ¹H NMR spectrometry was utilized to determine the amount of vinyl sulfone groups attached to the HA polymer by comparing the peaks at ~2ppm to those above ~6ppm. Molar excess ratios of 1.5x, 2.5x, and 3.5x of DVS to deprotonated OH groups (4 OH groups per disaccharide) in solution (based on molarity) produced HA polymers that had ~30, 50, and 70% functionalized disaccharides.

PNIPAAm-SH synthesis:

PNIPAAm-SH was synthesized by reversible addition-fragmentation chain transfer (RAFT) synthesis using AIBN as the initiator and DMP as the chain transfer agent (CTA) (Figure B.1). NIPAAm monomer was dissolved in dioxane with AIBN and DMP and placed in a 60°C oil bath for 4-24 hours [30, 31]. Incorporation of butyl methacrylate groups to lower the LCST was achieved by adding in the butyl methacrylate monomer at the desired molar ratio during the synthesis of PNIPAAm-CTA. The resulting polymer was precipitated into hexanes, filtered, and dried in a vacuum oven overnight. A GPC with RI and UV detector using THF as the solvent was used to analyze the molecular weight of the PNIPAAm-CTA polymers. The CTA was cleaved to reveal a thiol group at the chain ends of the PNIPAAm polymer by reduction with NaBH₄ [32, 48]. The PNIPAAm-CTAs were dissolved in DI water in a 3-neck round bottom flask. NaBH₄ (30-50 molar excess to polymer chains) was dissolved in 5 mL DI water before quickly adding to the PNIPAAm-CTA solution. The vessel was hooked up to N₂ with an outlet valve to ensure gas buildup within the vessel did not occur and to maintain an oxygen-free atmosphere for the reaction. After 2 hours of reaction the PNIPAAm-CTA yellow solution was placed in dialysis tubing (MWCO 3400) and dialyzed against DI water with 0.1M NaCl for multiple days. 20mM TCEP and 10mM EDTA were then reacted with the PNIPAAm-SH solution for 2 hours to reduce any disulfide bonds that may have formed during purification. After disulfide reduction, the polymer solution was dialyzed against PBS with 1% Trifluoroacetic acid to remove any residual chemicals and to maintain free thiols. The PNIPAAm-SH solution was then centrifuged to separate any remaining solids or unremoved CTA and then lyophilized. ¹H NMR spectrometry and GPC were used to analyze resulting PNIPAAm-SH polymers.

Ultimately it was determined that a useful bulk thiol content was generated when the NaBH₄ molar excess to number of chains was approximately 30-50x. When even higher amounts of excess NaBH₄ were utilized, the polymer would ultimately end up precipitating to the top of the solution along with the cleaved CTA (as evidenced by a yellow solid floating on top of clear solution). This yellow solid was sometimes difficult to dissolve in water again for dialysis and further purification. Precipitation of the polymer out of solution caused an inconsistent amount

of thiocarbonylthio reduction and cleaved CTA removal based on bulk thiol content from Ellman's assay and faint yellow polymers.

HA-PNIPAAm polymer synthesis and hydrogel preparation

To attach the PNIPAAm-SH to HA-VS, first the HA-VS was dissolved in triethanolamine (TEOA) buffer pH 8.0 with 1mM EDTA. After fully dissolved, the PNIPAAm-SH was then added to the solution at weight ratios between 5:1 to 20:1 PNIPAAm: HA-VS. The solutions were constantly mixed at 4°C for 4-16 hours before adding 20mM TCEP and 10mM EDTA for ~1 hour to break any disulfide bonds and allow more PNIPAAm-SHs to attach. If heparin, peptides or other biochemical cues are desired within the hydrogel system, these thiol-functionalized components can be added at this point. 10mM cysteine is added last to react with any free vinyl sulfone groups that have not been reacted yet. These HA-PNIPAAm solutions were dialyzed against PBS using MWCO 100 kDa dialysis tubing for multiple days and then lyophilized. ¹H NMR spectrometry was used to analyze the relative ratios of HA peaks to PNIPAAm peaks of the final polymers.

Rheology analysis:

Hydrogels were made by dissolving the dry polymer in PBS or cell culture media at concentrations between 5-10 wt/vol%. An Anton-Paar MCR 301 rheometer was used to analyze the rheological properties of the polymers when dissolved in solution. Amplitude sweeps, frequency sweeps, and temperature sweeps from 4°C to 50°C (at a rate of 1-2°C/minute) were analyzed to determine which polymers had desired properties for cell culture and expansion.

Cell culture:

Encapsulating cells within these hydrogels from 2D culture on Matrigel (Corning) or continuously from hydrogel to hydrogel followed a previous protocol we developed [17]. Passaging cells from 2D culture on Matrigel was performed by first enzymatically treating the cells on Matrigel with Accutase (Life Technologies) for 5 minutes at 37°C to release the cells. The solution was rinsed over the plate multiple times to collect all the cells and then placed on ice. Mechanical disruption of any remaining aggregates using both a 1 mL pipette and a 200 µL pipette ensured single cells in the population. The solution was centrifuged for 3 minutes at 1.4 rpm and the cells were then combined at a density of 1E6 cells in 100 µL cold hydrogel solution and then pipetted onto tissue culture plates. The plates were placed in an incubator at 37°C for approximately 15 minutes before adding E8 media with 10 µM Rho kinase (ROCK) inhibitor (Y-27632, Selleckchem). After 4-5 days or when the cells reached a desired density, ice cold PBS was added to the wells to reliquify the hydrogel. The cell solution was centrifuged for 3 minutes at 1.0 rpm before Accutase was mixed with the cell pellet and placed in a water bath at 37°C for 10-12 minutes. Same protocol is followed at this point after the Accutase step from Matrigel. When cells were plated as small aggregates, the cells were grown in Mebiol (CosmoBio) for 1-2 days before using cold PBS and centrifugation to release from hydrogel; no accutase was used to break up the small aggregates.

Antibody staining

In order to investigate pluripotency marker expression in cells cultured in the HA-PNIPAAm hydrogels, hPSC aggregates were recovered from the hydrogels by using cold PBS and centrifugation. Aggregates were plated onto Matrigel-coated plates for 48 hours before fixing

with 4 w/v% paraformaldehyde (PFA) at room temperature for 15 minutes, permeabilized with 0.25% Triton X-100 for 15 minutes and blocked with 5 v/v% serum plus primary antibodies at 4°C for 16 hours. After multiple washing steps, secondary antibodies in 2 w/v% BSA were added and incubated at 4°C for 4 hours. Cells were washed again multiple times before imaging.

Embryoid body differentiation:

In order to analyze the differentiation capacity into all three germ layers, hPSC aggregates were recovered from hydrogels via cold PBS and centrifugation and plated onto 0.1% gelatin-coated plates and grown in 20 v/v% fetal bovine serum (FBS) in DMEM/F12 media (Life Technologies) plus 10 μ M β -mercaptoethanol, 1 v/v% Glutamax (Life Technologies), 1 v/v% minimum essential medium non-essential amino acids (Invitrogen), and 1 v/v% Penn/Strep. After 25-30 days, the cells were fixed and stained as above.

Antibodies: Oct4 (Santa Cruz Biotechnology; 1:200), Nanog (Abcam; 1:250), α SMA (Abcam; 1:200), FOXA2/HNF3 β (Millipore; 1:500), and β 3Tub (Covance 1:500).

3.5 Conclusions

Thermoreversible HA hydrogels were designed and developed to culture and recover hPSCs for cell culture expansion. The HA-PNIPAAms were tuned to produce hydrogels with storage moduli around 1 kPa by varying the hyaluronic acid chain length, amount of PNIPAAm attached to the HA, amount of vinyl sulfone functionalization, and amount of hydrophobic groups attached. The polymers were also tuned to attach biochemical cues such as peptides or heparin to provide biochemical signals for increased cell growth or differentiation. hPSCs were able to survive, grow and remain pluripotent within these hydrogels after multiple passages, suggesting a new route for large expansion of stem cells. NPCs encapsulated in these gels were also able to survive, grow, and remain multipotent in softer gels (~100 Pa) and did not grow well in stiffer gels (~800 Pa).

HA hydrogels which are thermoreversible and tunable could be utilized for a wide array of needs. The current polymer systems could be modified to determine the ideal conditions for enhanced hPSC proliferation to generate hPSCs at an even higher rate, potentially by modifying mechanical properties like stiffness or incorporating different biochemical cues. The mechanical properties of the hydrogels could be tailored to suit specific types of stem cells and the environments that support their cell growth, for instance potentially softer for neural stem cell culture or stiffer for mesenchymal stem cell culture. In addition they could be modified for advanced differentiation protocols to produce specific cell types desired for certain human therapeutics. There are also a multitude of biochemical cues which could be incorporated into this system to aid in differentiation or maintenance of cell lines. Ultimately it could be possible to expand cells within the HA-PNIPAAm hydrogels, incorporate biochemical cues for specific cell differentiation, and then inject into the body for cell implantation and therapies. Transitioning into production using bioreactors would greatly expand the potential of these hydrogels for scalable production of stem cells.

3.6 Acknowledgments

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3.7 References

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Chapter 4 - Spatial Organization of Cell-adhesive Ligands for Advanced Cell Culture

This chapter is adapted from a manuscript published as

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4.1 Introduction

The engineering of biomaterial surfaces and cell-adhesive ligands is an active and growing field for both basic biological investigation and biotechnological application. A major challenge to the development of artificial environments that can control cell function is our currently incomplete mechanistic knowledge of how natural extracellular matrix (ECM), as well as other solid phase components within a microenvironment, regulate a cell. Thus, advances in basic knowledge of how for example the spatial organization of ECM cues modulate cell behavior will both advance cell biology and enable the design of synthetic, biomimetic versions of *in vivo* environments to aid biomedical applications including tissue engineering.

As depicted in Figure 4.1a, biological processes on length scales ranging from nanometers to microns are important for understanding and engineering cell adhesion and downstream responses to ligand presentation. Integrins, a family of transmembrane heterodimeric adhesion receptors that interact with specific components of the ECM both biochemically and mechanically, connect the extracellular ECM with the intracellular cytoskeleton. The head diameter of an integrin is approximately 10 nm, which therefore sets the theoretical minimum scale at which these receptors can “resolve” adjacent ligands [1]. As receptors and ligands bind, cell-matrix interactions can be sorted into three additional, mechanistically-defined size scales. At the smallest extreme, individual ECM proteins (e.g. laminin) and fiber-forming proteins (e.g. collagen, fibronectin, and elastin) have dimensions ranging from 50-300 nm [2, 3]. At the next larger size scale, mature focal adhesions, which concentrate both biomechanical linkages to the substrate and also regulatory information to the cell’s interior, are roughly 1-5 μm in size [4]. Finally, individual cells and their processes extend to approximately 10-100 μm in size, with features on this scale regulating processes such as motility and cellular orientation.

To date, a variety of different patterning techniques have been developed to exogenously present biomechanical cues on different biomaterial substrates and at a range of such size scales to investigate biological consequences, pushing the envelope on the minimum feature size down to the nanometer scale [5-7]. In this review, we discuss these technological developments and how they can be harnessed to gain further insight into the biomechanical regulation of cells. Additionally, we will also discuss how biologically diverse cells could be affected by

presentation of a repertoire of cell-adhesive cues to aid our understanding cell-substrate interactions.

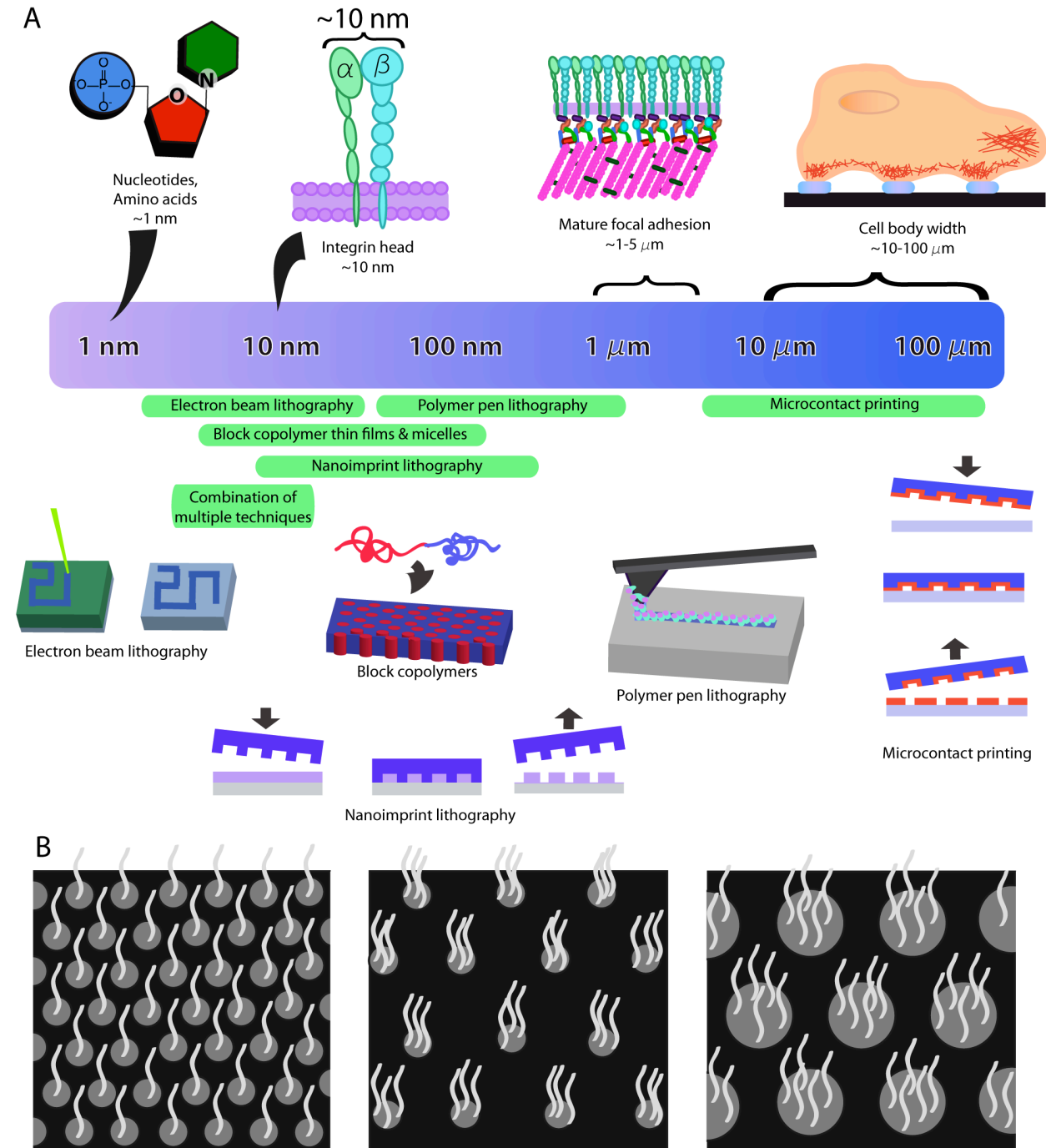


Figure 4.1 Schematics depicting length scales for cellular components and patterning techniques and difference between global and local concentration.

A. Length scales for cellular components and practical resolutions of micro/nanoscale patterning techniques. “Combinations of multiple techniques” refers to combination of electron beam lithography, nanoimprint lithography, metal evaporation and lift-off. B. Schematic illustrating difference between global and local ligand concentrations. Global concentration refers to the average concentration of ligands presented across a surface: μm - mm in size (held constant in all three patterns). In contrast, local concentration refers to the concentration heterogeneity on size scales comparable to adhesive complex components: tens to hundreds of nanometers (different for the three patterns depicted), or an adhesive complex: $\sim 1\text{-}5\ \mu\text{m}$. In many studies discussed

here, local concentration refers to the concentration of adhesive ligands within a cluster or isolated area on a patterned adhesive surface.

Numerous studies conducted with standard cell lines (such as fibroblasts, endothelial cells, and osteoblasts) [8, 9] have enabled a detailed understanding of focal adhesion structure and other downstream behaviors that result from cell regulation by the substrate [4, 10, 11]. These well-studied cells have very defined adhesive structures and are thus suitable for initial investigations of how cell processes and behaviors can be affected by the presentation of cell-adhesive ligands. However, much remains to be explored. For example, these approaches are increasingly being applied to other medically-relevant cell types – such as stem cells and cancer cells – with a focus on how cell-specific processes are regulated by the geometry of ligand presentation. Additional stem cell types, such as pluripotent stem cells, are likely to be studied in the future. In addition, while some integrin-ligand interactions have been well investigated (e.g. the integrin ligand Arg-Gly-Asp, RGD), future work is likely to encompass a broad range of cell-adhesive proteins and peptide sequences. Interfacing more complex cell types with more biomimetic surfaces may aid biotechnological applications such as tissue engineering.

4.2 Cell adhesion to ECM

Before proceeding to a discussion of material fabrication technologies, it is important to discuss cell interactions with natural surfaces in greater depth. Cell adhesion is a critical process that mediates cell communication with its surrounding physical environment via many complex extracellular and intracellular interactions. An in-depth discussion of the actions occurring at cell-ECM focal adhesions can be found in many review articles and are only briefly mentioned here [4, 10, 11]. When a cell initially encounters cell-adhesive ligands, adhesion receptors attach to key peptide or glycan ligands. These cell-adhesive ligands are often short polypeptide sequences within ECM proteins that bind directly to integrins and initially form nascent adhesions for cell attachment [12]. Upon forming complex linkages with the cellular cytoskeleton, integrins mechanically pull on the ligands, and this cell-generated force from actomyosin contraction is balanced by ECM resistance and surrounding cells [11, 13]. Next, cell spreading occurs as actin-rich lamellipodia (sheet-like protrusions) extend from the cell body to enable a larger adhered area and in some cases subsequent cell migration. Lamellipodia extend approximately 2-4 μm away from main cell body in the direction of spread (Figure 4.2) [4]. Additionally, filopodia are finger-like protrusions that – at a couple microns in length, a few hundred nanometers wide, and microns apart – extend even further from lamellipodium and search for cell-adhesive ligands in the environment [14].

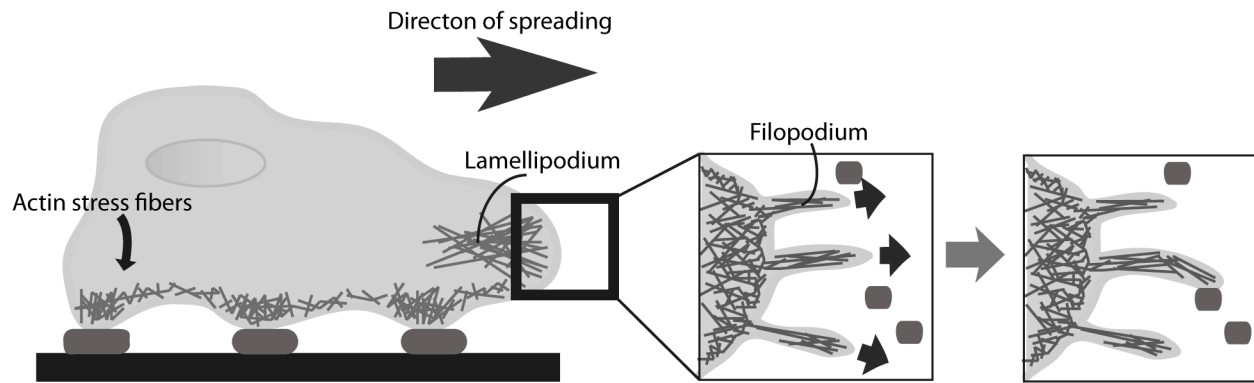


Figure 4.2 Schematic of cell spreading.

Cell spreading and searching for adhesive areas (dark ovals) using sheet-like lamellipodium and finger-like filopodia.

During these processes, focal adhesion maturation begins with integrin activation and clustering into larger complexes, which in turn recruit focal adhesion-associated proteins to connect the integrins to the cytoskeleton. Actin monomers are then recruited and polymerized to form actin microfilaments, which ultimately form the axial bundles termed stress fibers at the “bottom” of the cell, radially-oriented fibers found near the leading edge, or networks found throughout the cytosol [15-17]. Nascent adhesions are less than 250 nm in size and contain only the focal adhesion-associated proteins paxillin and talin. However, upon increased mechanical tension from the actin cytoskeleton to the integrin-ECM connection, these adhesions mature into focal complexes approximately 500 nm in size that contain additional proteins including focal adhesion kinase (FAK) and α -actinin [4]. The complex either proceed to mature focal adhesions that are 1-5 μ m in size and contain vinculin and zyxin if the integrin-ECM connection can provide sufficient resistance to the cytoskeleton-generated tension, or alternatively the adhesion complex can dissociate at any step and allow the filopodia to search for another adhesive site [4, 18].

Focal adhesion information can be relayed to the rest of the cell to modulate cell function via biochemical or mechanical signals, and these two processes are highly integrated. The biochemical response from focal adhesions starts from recruited signaling proteins such as focal FAK and Src, which can initiate signaling cascades that propagate throughout the cell. Downstream signaling effectors and processes can include the PI3K/Akt pathway, RhoGTPases, and target gene expression, which collectively contribute to regulating cell motility, morphology, polarity, size, adhesion, division, and differentiation [19]. Mechanotransduction refers to the mechanisms by which cells sense mechanical stimuli (such as integrin-ECM resistance to cell-generated forces) and convert that information into biochemical signals that can alter cell function and cellular or extracellular organization. As with biochemical signaling, there are clear mechanisms by which mechanical information can be transduced from cell surface receptors through the cytoskeleton and to the nucleus. For instance, some speculate that the cytoskeleton may pull on the nucleus which could change its shape, allowing different components to transfer in or out [20]. In reality, as discussed in greater detail in other reviews, the processes of mechanotransduction and biochemical signal transduction are highly integrated and at many junctures are effectively indistinguishable [20-24]. Finally, advances in technologies to control nanoscale surface patterning can affect cell function though both

signaling processes, which offers opportunities to gain basic biological knowledge and enhanced control over cell function for biotechnological application.

4.3 Types of presentation of cell-adhesive ligand

4.3.1 Micron-size ligand-covered islands

Microcontact printing:

As mentioned previously (Figure 4.1), mature focal adhesions are roughly 1-5 μm in size, and cell-body widths are roughly 10-100 μm . A system that can pattern ligands onto substrates in this range is thus ideal for creating patterns that can probe focal adhesion size and investigate effects of limiting the area on which cells can attach and spread. This ~5-100 μm range is possible with microcontact printing, a method that uses a soft polymeric stamp – typically poly(dimethyl siloxane) (PDMS) – replicated from a photolithography-made master mold to stamp self-assembled monolayers (SAMs) or biomolecules in specific patterns onto a substrate (see Figure 4.1) [6]. The areas in between the SAMs or biomolecules deposited by a stamp can be passivated to avoid cell or protein adhesion to these regions, and hydrophilic polymers such as polyethylene glycol (PEG) and poly(hydroxyethylmethacrylate) (PHEMA) as well as proteins such as bovine serum albumin (BSA) and casein are often used for this blocking [25-27]. The aspect ratio (height/length of features) of the stamp and surface spreading of the substance being transferred create a lower limit on pattern size of roughly a few hundred nanometers [28, 29]. Possible ways to extend this technology exist to print patterns with much smaller length scales (nanoimprint lithography); however, this very elaborate processes necessitates specialized coatings or rinse liquids to create a viable PDMS stamp without pattern collapse or other deformations [30, 31].

There has now been considerable cell biology work using microcontact printing, and multiple landmark studies will be mentioned here. Chen et al. utilized microcontact printing to understand the effect of cell morphology and size on the proliferation and death of endothelial cells [32]. Fibronectin was printed in various patch sizes, and as microscale adhesive island area increased, endothelial cell DNA synthesis increased, and apoptosis decreased [32]. In order to characterize whether this effect was due to ECM contact area or spread cell area, new patterns were created to allow cells spreading but offered much less actual ECM contact area within the patterns (Figure 4.3a). Results showed that a larger spread area increased growth and decreased apoptosis, suggesting endothelial cells are more affected by spread area than actual ECM contact area [32]. This discovery implies that cells do not require a continuous area of ECM in order to attach to a surface and instead need enough ECM contact area in order to resist cytoskeleton-generated forces and allow the cell to spread. A subsequent study found that fibronectin island size can also affect stem cell differentiation. Mesenchymal stem cells (MSCs) were cultured in medium that induces mixed lineage differentiation. Osteoblast (bone cell) differentiation increased, and adipocyte (fat cell) differentiation decreased as island size, therefore also cell spread area, increased [21]. Further analysis indicated that the GTPase RhoA and cytoskeletal tension underlay the effects of cell shape on lineage commitment [21]. These results showed that more biologically-diverse cells can sense and respond to differences in ECM patterns, and

therefore suggested that ECM presentation may impact many types of cellular behaviors in complex cells such as stem cells, cancer cells, etc. Based on these studies and others using microcontact printing, cell spread area, ECM contact area, and distances between focal adhesion should all be taken into account in investigating substrate effects on cell behavior [33, 34].

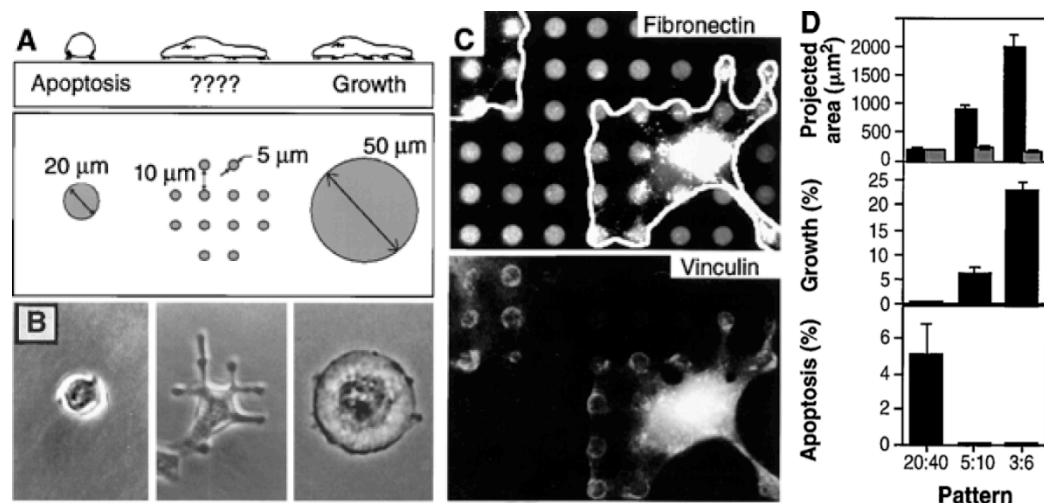


Figure 4.3 Microcontact printing of fibronectin (FN) islands to understand if cells respond more to ECM contact area or spread area [32].

A. Different substrates used to determine effect of ECM contact area vs. spread area. B. Phase-contrast micrographs of cells spreading on substrates illustrated in A. C. Immunofluorescence micrographs of cells on a micropatterned substrate stained for FN (top) and vinculin (bottom). White outline indicates cell borders; note circular rings of vinculin staining, which coincide precisely with edges of the FN-coated adhesive islands. D. Plots of projected cell area (black bars) and total ECM contact area (gray bars) per cell (top), growth index (middle), and apoptotic index (bottom) when cells were cultured on single 20-μm circles or on multiple circles 5 or 3 μm in diameter separated by 40, 10, and 6 μm, respectively.

4.3.2 Ligand clustering vs. random presentation

At a smaller size scale, an interesting question is whether cells can sense differences in the spatial organization of adhesive ligands. For example, a cell may interact with its surroundings by clustering adhesive ligands in the formation of a nascent adhesion [16]. For this reason, clustered cell-adhesive ligands may allow the integrins to bind in an already clustered format, which could affect the dynamics or extent of a mature focal adhesion formation and affect both cell force generation and biochemical signaling.

PEO stars:

In landmark studies investigating how cells respond to random vs. clustered presentation of ligand, polyethyleneoxide (PEO) star tethers were used [35, 36]. This system allowed for independent control over average surface ligand concentration (global concentration) and average number of ligands per cluster (ligand cluster size) with an inert PEO background that resisted cell adhesion and protein adsorption. Furthermore, the study used a minimal RGD-containing synthetic peptide to limit non-integrin receptor interactions with the substrate and avoid complexities such as unfolding of a substrate protein to reveal cryptic binding sites [36]. Cell speed increased as cluster size increased (from 1 to 9 RGDs/cluster) at similar global concentrations. Likewise, as global concentration increased so did the cell speed until saturation for each cluster size [36]. An analogous study showed that as global density increased, cell

adhesion strength also increased at constant cluster size [35]. Additionally, increasing cluster size (from ~1.7 to 5.4 RGDs/cluster) also increased the adhesion strength of the fibroblasts at constant global density [35]. These studies established that cells can sense and respond not only to overall, macroscopic surface ligand density but also local geometric features in the presentation of those ligands.

Alginate gels

Once ligand clusters were shown to have an effect on cell adhesion and motility, subsequent systems were developed to address additional questions about cell responses to ligand cluster density and global density. Alginate gels do not allow cell attachment in the absence of cell-adhesive ligands and are relatively biocompatible, and they therefore offer a tunable system in which cells attach only where cell-adhesive ligands are presented [37]. An alginate hydrogel system was designed to independently change overall ligand density (global concentration), number of ligands clustered in one location (local concentration), and idealized spacing between ligand clusters from roughly 40 – 170 nm. In this study, local concentration refers to the number of ligands clustered within the same area approximately tens of nanometers in size, spatially separated from other ligand clusters tens of nanometers away as depicted in Figure 4.1b.

The global density of RGD peptide could be held constant while the number of peptides per cluster, and therefore also the spacing between clusters, was increased (increasing local density). Monte Carlo models were used in parallel to infer average numbers of peptides per cluster and distances between clusters [37]. Trends showed that at a fixed global ligand density, increasing the spacing between RGD clusters and therefore increasing the number of RGDs per cluster (local concentration) decreased the growth rate and increased cell spread area [37]. This result does not follow an anticipated trend that increased spreading is correlated with increased proliferation, potentially due to the nature of ligand presentation in clusters from the alginate.

4.3.3 Receptor-sized ligand patterns

Although microcontact printing allows for an in-depth analysis of cell responses to ligand patterns roughly the size of focal adhesions, an understanding of how cells respond to ligands presented at smaller length scales could elucidate more specific interactions of integrins with hierarchically-structured proteins in the ECM. An integrin head diameter is roughly 10 nm, at least an order of magnitude smaller than the pattern size possible with microcontact printing, and therefore may interact with the surrounding ECM at a length scale of this order.

Block copolymer micelle lithography:

Block copolymers naturally assemble into micelles in solution on the appropriate length scale for investigating how ligand patterns on the order of integrin receptor sizes can affect cell adhesion. Block copolymers consist of two chemically dissimilar long molecules joined end to end, depicted as red and blue in Figure 4.4. In the most well studied block copolymer micelle lithography system, gold nanoparticles are coated with the block copolymer in solution to form micelles as shown in Figure 4.4a. Hard sphere repulsions between the micelles force them to generate roughly hexagonal networks when the solution is dip-coated on a substrate. The polymer can then be removed by heating, and the gold surfaces can be functionalized with

adhesive peptides via thiol chemistry [38]. In studies using this approach, the gold nanoparticles have a diameter of roughly 10 nm so that only one integrin should be able to bind per RGD-coated gold particle, with the inter-particle spacing roughly controlled via the dip-coating process. As a result, the actual number of ligands attached to each gold particle is irrelevant, as each particle can presumably bind to only one integrin. This system is ideal for patterning cell-adhesive ligand on the same scale as integrin receptor sizes, though it does not allow for the average overall ligand concentration (global concentration) to be varied independently of the number of ligands per local, adhesive complex sized feature (local concentration).

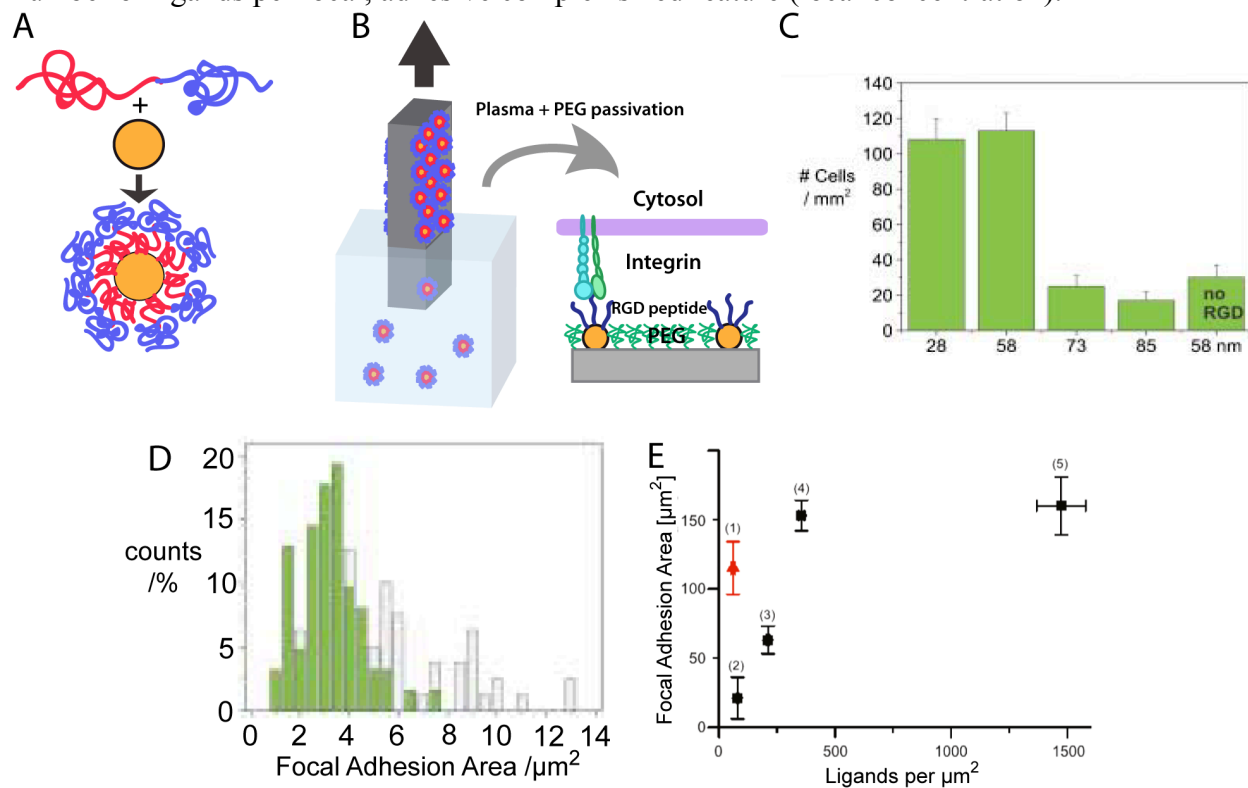


Figure 4.4 Block copolymer micelle lithography techniques.

A. Gold nanoparticle micelles forming from combination of block copolymer with gold nanodot. B. Dipcoating and processing techniques to create gold nanoparticle patterns passivated with PEG containing nanoparticles similar in size to integrin head diameter. C. Difference in adhesion between ligand spacings D. comparison of focal adhesion characteristics between extensive (continuous) 58 nm spacing (white) and micropatterns of 58 nm spacing (green) [38]. E. Focal adhesion area versus global ligand density of (1) micropatterns with 57 nm gold spacing (2) extensive 120 nm gold spacing (3) extensive 74 nm gold spacing (4) extensive 57 nm gold spacing (5) extensive 28 nm gold spacing (previous data) [41].

Spacings of individual ligands in a range roughly between 20-100 nm suggested that osteoblasts cannot adhere to ligands spaced on gold nanoparticles with global concentrations of 190 dots/μm² or less (73 nm or more apart) as shown in Figure 4.4c [38]. This system was also used to show that fibroblasts cannot form characteristic focal adhesions at the lower global concentrations and larger ligand spacings [39-41]. Processing by photolithography or E-beam lithography was applied to the gold nanopattern with a global concentration of 280 dots/μm² (58 nm spacing) to create micron-size patches (micropatches) of this nanopattern separated by micron-size unpatterned areas; these new micro-nanopatterned surfaces were created to determine if local ligand concentration within these micropatches was more important to cell attachment and spreading than ligand concentration across the entire surface (global

concentration) [38, 41]. Higher values of focal adhesion area and detachment force needed to remove a cell were found when the cells grew on surfaces with micropatches than when cells grew on surfaces with extensive (continuous, unpatched) nanopatterns even at a lower global concentration (larger spacing). These results suggest that the micropatches improved cell adhesion despite the lower global concentration [41]. That said, such “micro-nanopatterns” have only been made with a local concentration of the micropatches around 300 dots/ μm^2 , and therefore cell responses from micro-nanopatterns with higher and lower local concentrations have not yet been investigated.

Another study broaching the role of global and local concentrations, by comparing ordered and disordered ligand patterns at similar global concentrations, showed an increase in projected area and cell number with disordered patterns [42]. This increased adhesion was attributed to some ligands being arranged sufficiently close for integrins to cluster and form focal adhesions [42]. This system thus elucidated that there may be a minimum ligand concentration or maximum spacing between ligands to readily enable cell attachment. However, global and local concentration change in tandem when creating extensive nanopatterned surfaces, and pattern stability may be transient.

A modification of this system by the Ding group was recently used to investigate whether individual ligand spacing has an effect on MSC differentiation and morphology. The gold nanoparticle pattern was transferred to a PEG hydrogel by attaching an N,N-bis(acryloyl) cystamine linker to the gold nanoparticles for crosslinking to the hydrogel [43, 44]. Ligands on gold particles spaced 37-124 nm apart (global concentrations between ~ 60 -730 RGD/ μm^2) on the PEG hydrogels showed decreasing trends in cell number and cell spread area as RGD nanospacing increased and global concentration decreased. However, the dependence was continuous and did not exhibit a drastic drop in cell attachment below a ~ 200 RGD/ μm^2 global concentration (above the ~ 70 nm spacing) mark [44]. This effect could be due to differences in mechanical properties between the hard plastic and the hydrogel surface or from a difference in focal adhesion formation and focal adhesion-associated proteins required between MSCs and non-stem cell lines. This system also showed that osteoblast differentiation increased and adipocyte differentiation decreased as global and local concentrations decreased in tandem (ligand spacing increased) when MSCs were cultured in medium that induced mixed differentiation (osteogenic and adipogenic) [44]. That said, work by other groups shows an opposite trend in which osteoblast differentiation increased with strong adhesion on large ECM-patterned areas and with stiffer surfaces [21, 45].

These block copolymer micelle lithography studies have been useful in elucidating a range of ligand spacings and global concentrations that affect cell adhesion and focal adhesion formation in common cell lines. Before these studies were performed, it was unknown whether cells could even sense ligand patterns at this small of length scale. The work with fibroblasts and MSCs will encourage additional studies with more complex ligand presentation and additional cell classes.

Block copolymer thin films:

Block copolymer thin films provide the ability to independently change local and global concentration of ligand patterns with sizes and spacings on the order of ligand receptor dimensions (Figure 4.1b). Block copolymers consist of two or more different types of polymers

covalently attached to one another, and they can self-assemble into specific structures based on the competition between the free energy of mixing and entropy of the chains [46]. These specific morphologies include spheres, hexagonally close-packed cylinders, or lamellae depending on the volume fraction and molecular weights of each block (Figure 4.5a) [47]. Varying the volume fraction and/or molecular weight of one or both of the blocks can change the size and spacing of the morphology (i.e. larger cylinders spaced further apart). The range of feature size (i.e. cylinder diameter or lamellae thickness) available using this technique is roughly 5-200 nm. Some of the issues with this method are the synthesis time to create the block copolymers, modifying substrate surface energy to create the desired morphology [7, 48], and for standard polymer chemistries subsequent challenges with selective ligand attachment to one block but not the other.

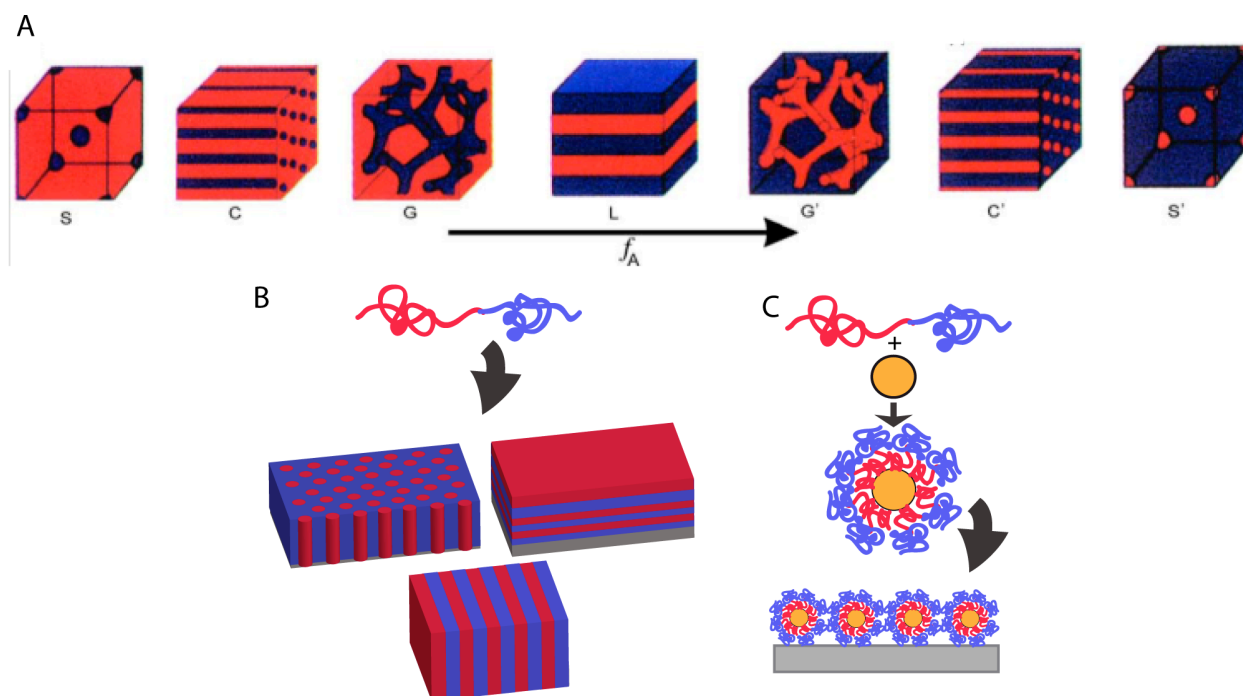


Figure 4.5 Block copolymer self assembly and resulting structures.

A. Block copolymer self assembly structures with increasing volume fraction of the blue block [47]. Morphology changes from blue spheres (S) to blue cylinders (C) to blue gyroid (G) to lamellae (L) then back to red gyroid (G') to red cylinders (C') to red spheres (S'). B. Self-assembly of block copolymer thin films. C. Self-assembly of block copolymer micelles with gold nanoparticles.

One of the first block copolymer thin film methods to pattern adhesive ligands for cell growth used a polystyrene-block-poly(ethylene oxide) (PS-PEO) copolymer with a maleimide (Ma) group attached to the PEO chain end for subsequent RGD peptide attachment [49]. PS-PEO is commonly used to obtain well-ordered upright cylinders in thin films, and this block copolymer system was self-assembled into hexagonally close-packed cylinders that were perpendicular to the top surface such that PEO domains were visible in a PS matrix. PS homopolymer was blended with the block copolymer to increase the center-to-center PEO-Ma spacing from roughly 34 to 62 nm (~ 860 to 260 RGD/ μm^2 if every PEO-Ma had only one RGD attached); however, the PEO domains become less uniform in size and spacing at around 50% block copolymer composition [49]. MSCs grown on these surfaces showed an increase in adipocyte

differentiation and decrease in osteoblast differentiation as average PEO center-to-center spacing increased from 34 to 62 nm, though the effect of spacing may be convoluted by the uniformity of PEO domains [50]. One challenge with this chemistry, however, is PEO swells in water and may thus cover parts of the underlying pattern in aqueous conditions necessary for cell culture.

Another recent system used PS-PEO with perfluorooctanethiol (PFOT) as well as allyl glycidyl ether (AGE) groups attached within the PEO domain, the latter for RGD peptide conjugation (Figure 4.6a) [51]. PFOT was included to modify interfacial interactions and thus enhance phase segregation (due to the fluorine groups not mixing with hydrophilic domains). It also maintained the ordered morphology in aqueous conditions by not allowing the PEO domain to swell (Figure 4.6b) [51]. AGE groups connected the peptide to the block copolymer, and therefore increasing the number of such groups increases the amount of peptide present on the surface. This system allows one to change the size and spacing of adhesive areas but requires different syntheses for different peptide surface concentrations. To date, this system has been used with fibroblasts, which were able to attach and remain viable on the ligand-patterned surfaces [51]. A different type of block copolymer has been used to pattern biomolecules but has not yet been used with cells. This polystyrene-block-poly(2-hydroxyethyl methacrylate) PS-PHEMA system utilized click chemistry to functionalize the PS domains with azide-tagged proteins in a protein-repellant PHEMA matrix [52].

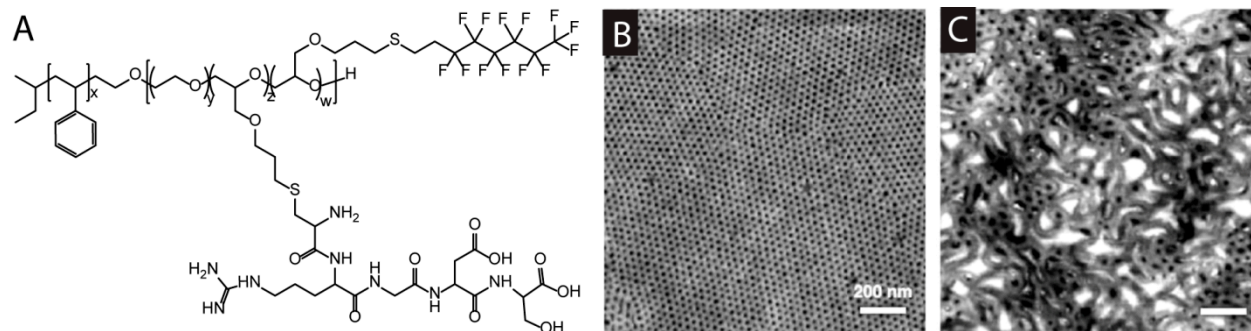


Figure 4.6 PS-P(EO-co-AGE) (with peptide) block copolymer self assembled thin films.

A. PS-P(EO-co-AGE) with peptide and PFOT groups covalently coupled. b) & c) Thin film containing RGD with PFOT (B) and without PFOT (C) in water [51].

These studies suggest a new ability to pattern ligand in an ordered manner that can allow independent changes in local and global concentration on the same length scales that were shown to modulate adhesion in studies using block copolymer micelle lithography (previous section). These studies may allow for more flexibility in the creation of patterns. Patterns that may define specifically where focal adhesions can start forming and limit how many integrins can bind could elucidate more about how cells respond to the specific size and locations of integrin-ECM contacts.

Electron beam lithography and nanoimprint lithography:

Production of ligand patterns that can probe length scales on the order of integrin head size and smaller could allow more in-depth analyses of integrin interactions with the surroundings. Electron beam lithography (E-beam) provides a very precise and controlled means to pattern surfaces in the nanometer range; however, patterning using an E-beam entails serially creating

one spot at a time. While E-beam technology has advanced significantly, this technique is thus more suited for complex patterning of small areas as opposed to large areas with imperfect periodicity created by self-assembly. That said, gold or other metals can be precisely deposited in nanometer-size patches with nanometer-sized distances between patches, allowing for exact production of patterns on a scale extremely difficult if not impossible to achieve with other techniques. Alkane-thiol chemistry can then be used to attach cell-adhesive ligand to the patterns. Therefore this technique can probe length scales on the order of integrin head size and integrin clustering (see Figure 4.1a). In an approach with related capabilities, nanoimprint lithography is a technique which uses a template created by lithography to imprint nanometer-sized features into a polymer resist [5].

E-beam lithography can be combined with nanoimprint lithography, along with some other nanofabrication techniques, to produce features that are sub-10 nm in size [53]. This combination has been used to explore whether fibroblast attachment requires a minimum number of RGD peptides per cluster (minimum local concentration) and whether spacing within and between clusters can also affect adhesion (Figure 4.7) [53]. One result with this system showed that fibroblasts spread when a minimum number of peptides are presented per cluster [53]. The same percentage of spread cells (i.e., cells that switched from round to flat with filopodia and maintained this spread form) was observed independent of local concentration once local concentration exceeded a minimum threshold of 4 dots/cluster (and with a constant global concentration of 50 RGD/ μm^2) [53]. This suggests that fibroblasts can be more sensitive to local concentration of ligand than global concentration at this very low global density. This result also may suggest that fibroblasts need a minimum number of integrins clustered together to start forming a focal adhesion. Another aspect of this experiment noted that varying global ligand density between ~ 50 RGD/ μm^2 and ~ 200 RGD/ μm^2 showed the same percent of spread cells [53] even though previous work using block copolymer micelle lithography showed a difference in cell adhesion in this range of global concentrations when changing from extensive (continuous) nanopatterns to micropatches of nanopatterns [38]. This may suggest that the lower limit for global or local concentration necessary for cell adhesion is reduced upon clustering of the ligands. Performing similar studies using different global concentrations could provide a more detailed understanding of how cells respond to these types of ligand patterns.

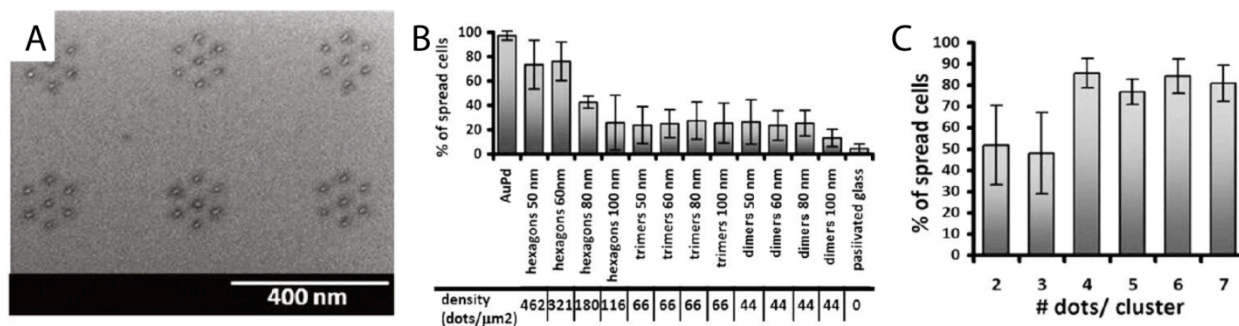


Figure 4.7 E-beam and nanoimprint lithography patterns and cell spreading area.

A. E-beam and nanoimprint lithography are used to create precise patterns of metal nanodots. B. Comparison of percent of spread cells on various nanopatterns. C. Comparison of percent of spread cells based on local concentration [53].

4.3.4 Ligand-patch arrays

Polymer pen lithography:

An array of ligand patterns or patch sizes/shapes could be used to screen cell responses to many variables simultaneously. Simple, continuous, and symmetric patterns can be easily fabricated via self-assembly techniques; however, they do not allow for multiple patterns on the same surface. Complex patterns can be created by microcontact printing or photolithography, yet require new masks or templates for any new patterns. In contrast to both of these options, dip pen nanolithography is a technique that can create arrays of different types of materials like alkanethiols or proteins in specific sizes and spacings with sub-50 nm resolution by taking advantage of the water meniscus formed when an atomic force microscopy (AFM) tip approaches a solid substrate, thereby depositing molecules in a controlled manner [54]. However, this technique does have similar limitations in throughput as E-beam lithography. To address this challenge, a variant technique called polymer pen lithography uses an array of soft polymer pens instead of an AFM tip and a scanning probe microscope to control the movement of the array [55]. Polymer pen lithography was recently used to create arrays of fibronectin patches of different sizes, based on the pressure and angle of the thousands of polymer pens depositing the fibronectin, to systematically screen MSC adhesion to different patch sizes [56]. This system showed that MSCs could differentiate to a greater extent into osteoblasts on the fibronectin patches with side lengths of 300 nm or 1 μm compared to nonpatterned surfaces, in the absence of differentiation-inducing media [56]. This type of system thus enables rapid screening of a wide range of conditions and sizes for numerous downstream applications.

4.3.5 Fluid surfaces with confinements

In addition to adhering to ligands presented from solid substrates, cells interface with the surfaces of neighboring cells. Ligands present in such fluid surfaces can undergo diffusion, and several synthetic systems have been developed to study how membrane fluidity can affect cell adhesion and function.

Lipid bilayers

Supported phospholipid bilayers, systems in which ligands are presented from a cell adhesion-resistant background surface that mimics the plasma membrane of a neighboring cell, have been used in many studies to mimic cell-cell interactions [57, 58]. For example, lipid bilayers can present ligands of many different kinds simply by attaching lipid tails to the ligands, which can thus easily be incorporated into the lipid bilayer. Surfaces can also be micropatterned with protein first, followed by lipid bilayer deposition around the protein [59, 60]. Lipid bilayers have recently been utilized to study stem cells, and in this study neural stem cells could adhere, proliferate, and differentiate on an RGD-presenting surface [29].

One interesting additional feature of these systems is that physical barriers microns in dimension can be added to restrict movement of ligands, for example to impede large scale receptor clustering and thereby gain insights into its role in cell function. Some methods to introduce barriers include manual scratching or blotting with a PDMS stamp, microcontact printing, or prefabricated barriers from photolithography [61]. One system included RGD peptide ligands in

a lipid bilayer along with physical barriers to limit the movement of these integrins in order to understand the role of RGD peptide clustering in cell adhesion and spreading [62]. Results tracked the focal adhesions formed with these ligands and showed movement forward for active protrusion and then rearward for retraction [62]. Supported lipid bilayers allow for more biologically-relevant responses such as clustering of ligands to occur because the presented ligands can freely diffuse.

4.4 Open questions

Over the last decade there have thus been many advances in the field of patterning cell-adhesive ligand to investigate how cell adhesion and other related behaviors are affected based on the presentation of ligand. We have learned that multiple responses – including proliferation, adhesion, viability, migration, and differentiation – of common or differentiated cell types are affected by spread cell area more than ECM contact area [21, 32]. This result suggests that as long as the focal adhesions can provide sufficient resistance to the cytoskeleton-generated tension, the actual area connecting to ECM may be less important for cell proliferation and viability. We have learned through several studies that ligand clustering improves adhesion and motility and that there may be a minimum number of ligands per cluster required for cell adhesion [35, 36, 53]. The minimum cluster size could result from a minimum number of integrins clustering together to recruit necessary focal adhesion-associated proteins and form a mature focal adhesion. Ligand clustering may also lower the minimum global concentration necessary for cell attachment and spreading. New surface engineering techniques have also shown that standard cell lines can detect differences in low global concentrations from ligands spaced apart on the order of receptor size [38]. Beforehand systems existed only to present ligands in a way that averaged out any differences in spacing between ligands. There may also be a difference between stem cell and fibroblast adhesion, as suggested with MSCs adhesion to surfaces at lower global concentrations than fibroblasts [44]. However, there are still many future questions that need to be explored.

Local concentration vs. global concentration

Several studies have investigated whether local concentration or global concentration of cell-adhesive ligand is more important for cell adhesion and spreading. This question is difficult to address since both local (i.e. concentration heterogeneity on size scales comparable to an adhesive complex or adhesive complex components) and global (i.e. average concentration across a surface) concentration change simultaneously when ligand spacing changes. Several results indicate that local concentration may impact cell adhesion and spreading more than global concentration, including investigations of differences between confluent and micron-sized patches of nanopatterned ligand [38, 41], comparisons of ordered and disordered ligand presentation [42], and exploration of minimum numbers of ligands required per cluster for cell adhesion [53]. However, underlying molecular size scales and mechanisms remain to be investigated. Tightly controlled studies that hold as many variables as possible constant while only changing one or two in order to pinpoint exactly which surface property affects cell adhesion and other behaviors will enable even more precise statements about integrin and focal adhesion interactions with ligands (e.g. Fig. 1b). Many integrin ligands that exist in the ECM are

multivalent and could therefore stimulate integrin clustering by themselves [1]. Local concentration patterning could thereby impact how many integrins can cluster together and how these clustered integrins recruit focal adhesion-associated proteins to start to form a mature focal adhesion. However, global concentration would affect the average number of adhesive bonds across the entire cell surface, also seemingly an important parameter. In addition, with the advent of fluid surfaces whose spatial patterns can be modulated, the roles of local and global adhesive patterning at cell-cell junctions can now be explored.

Cell sensing and responses to cell-adhesive ligand-patterned surfaces

In addition to trying to understand why local and/or global concentration may impact cell engagement with a surface, mechanisms by which ligand-receptor bonding regulates downstream cell behavior can be further explored. Considerable work has investigated how focal adhesion-associated proteins convey information from the ECM to the cytoskeleton [18]. In addition, increasing numbers of studies have established that these links can regulate complex downstream cellular responses to cell-adhesive ligand patterns on the micron scale, such as the work by McBeath et al. showing that RhoGTPase activity and actin-myosin-generated tension affect MSC differentiation [21]. However, an active area of research is how focal adhesions and the cytoskeleton convey mechanical information to the nucleus. Recently, Dupont et al. have shown that transcriptional coactivators YAP and TAZ act as sensors and relay mechanical signals from ECM proteins to alter cell behaviors [63]. Future work will likely establish causal connections between mechanical cues and such transcriptional regulators, as well as between these regulators and downstream cellular function.

Additional biological contexts

Most of the work with cell-adhesive ligands has been conducted with synthetic peptides containing RGD, a peptide sequence found in ECM proteins including fibronectin, laminin, and fibrinogen [12]. This canonical RGD sequence is clearly an important component of interactions between cell and ECM; however, the full range and richness of such interactions will extend beyond this motif. An increasing number of alternate cell-adhesive ligands have been used in other studies for their particular properties. For example, synthetic peptides were recently used to study the mechanisms of human embryonic stem cells (hESCs) binding to Matrigel surfaces as a step towards creating more defined culture substrates [64]. Others have used self-assembled monolayers as a platform to determine ideal peptide sequences for ESC growth and self-renewal [65]. In addition, bacterial peptide display was also used to screen many peptide sequences to determine which bind neural stem cells (NSCs) and support self-renewal and differentiation [66]. Using a different ligand could elicit recognition from other types of integrins or receptors, and different integrins may adopt different conformations upon connecting to alternate ECM motifs, which could change how cytoplasmic domains assemble and therefore affect how focal adhesion-associated proteins are recruited and how intracellular signals are relayed [1]. Therefore, different cell-adhesive ligands should increasingly be explored.

In addition to exploring more complex receptor-ligand interactions, additional cell types should be investigated. As mentioned previously, the majority of key research conducted with ligand-patterned surfaces uses model cell lines like fibroblasts, osteoblasts, endothelial cells, and others. These cell lines offer the advantages of being fairly straightforward to culture, having well-characterized adhesive structures and morphology, and exhibiting substrate-sensitive behavior

such as adhesion, spreading, and migration shortly after engaging with a substrate. Translating such investigations to less characterized cell types that exhibit complex behaviors on longer time scales, such as stem cells, will both advance basic knowledge and take steps towards biomedical application. Several studies for example have investigated the responses of stem cells to RGD peptide sequences spaced at varying distances, primarily with mesenchymal stem cells [44, 50, 67]. This work have benefitted from their similar adhesive behavior with fibroblasts, and investigating how more complex behaviors of MSCs and other stem cells – specifically their self-renewal and differentiation – will yield future advances and likely reveal further biological complexities. For example, based on the most recent work on individual RGD ligand spacing with MSCs, the ~ 200 RGD/ μm^2 (~ 70 nm spacing) limit found for osteoblasts, fibroblasts, and other common cell lines may not extend to other biologically-diverse types of cells [44].

Additional complex behaviors may also be investigated in the future. Behaviors such as proliferation or apoptosis on ligand-patterned surfaces were investigated in foundational work by Chen et al [32], and this work can be extended with different cells and patterns. Additionally, investigating cell motility, polarization, contractility, cell-cell signaling and organization, and other behaviors on ligand-patterned surfaces may provide useful information for designing biomaterials for implants.

4.5 Conclusions

Though the past decade has brought many advances in understanding how cells sense and respond to cell-adhesive ligand patterns, many important studies are still needed to elucidate many questions. A deeper understanding of how cells respond to the presentation of cell-adhesive ligands can aid in creating biomaterials that mimic characteristics of the *in vivo* environment more precisely. This will benefit basic biological studies of how biochemical and biophysical engagement with the ECM regulate cell function, as well as facilitate more controlled biological studies in general. Furthermore, this information could aid *in vitro* stem cell differentiation into specific lineages, which would benefit applications ranging from drug screening to scalable cell culture systems. Finally, regenerative medicine and tissue engineering efforts are likely to benefit from a deeper understanding of how cells respond to cell-adhesive cue presentation.

4.6 References

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Appendix A: Supplementary Material for Chapter 2

A.1 Supplementary Figures

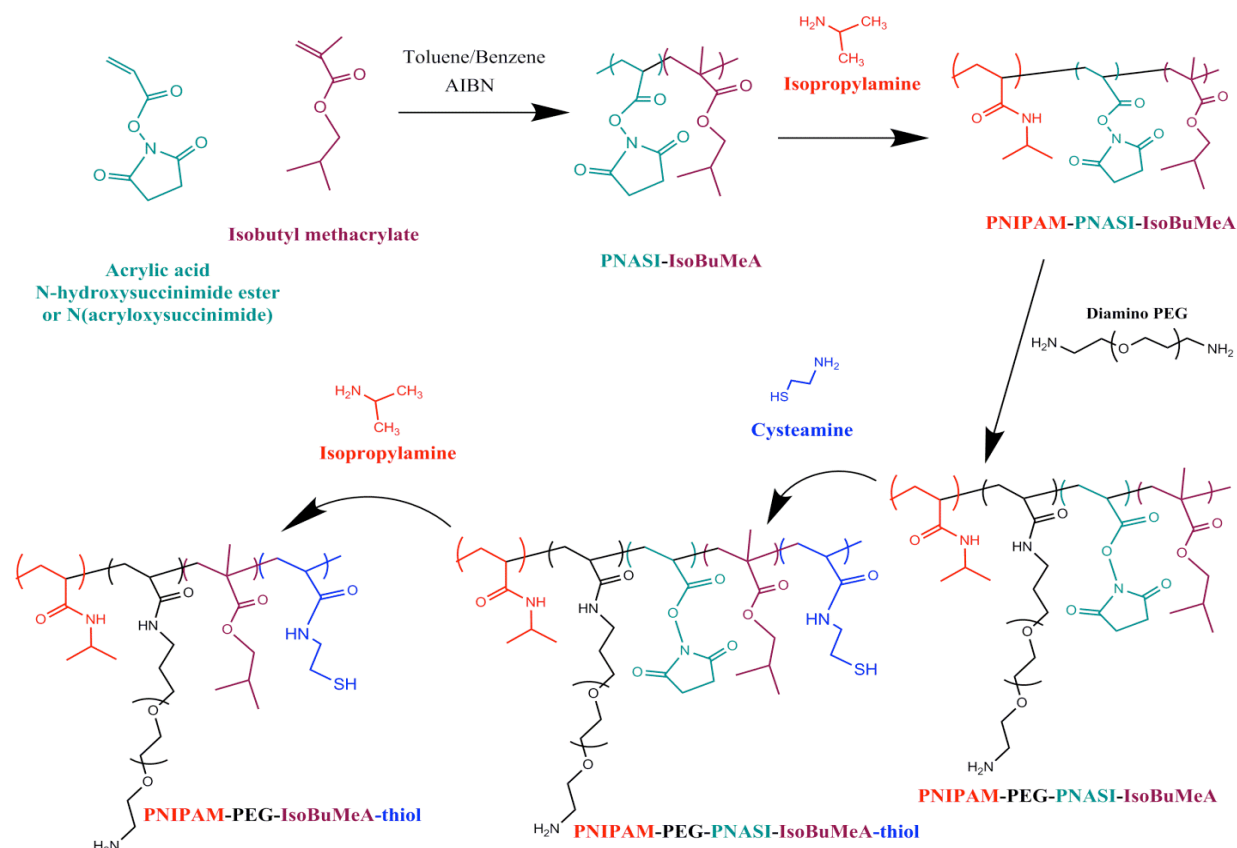


Figure A.1 PNIPAAm-PEG-Isobutyl methacrylate-thiol synthesis.

N-(Acryloxysuccinimide) and Isobutyl methacrylate (or other hydrophobic methacrylate) monomers are combined with AIBN and a mixture of toluene and benzene and reacted at 60C for 24 hours before precipitating into ethyl ether. The PNASI-methacrylate polymer is reacted first with isopropylamine to turn PNASI groups into PNIPAAm followed by addition of diaminoPEG to attach PEG. Cysteamine can be added at this point if thiol functionalization is desired. Excess isopropylamine is added at the end of synthesis to convert any remaining PNASI groups into PNIPAAm.

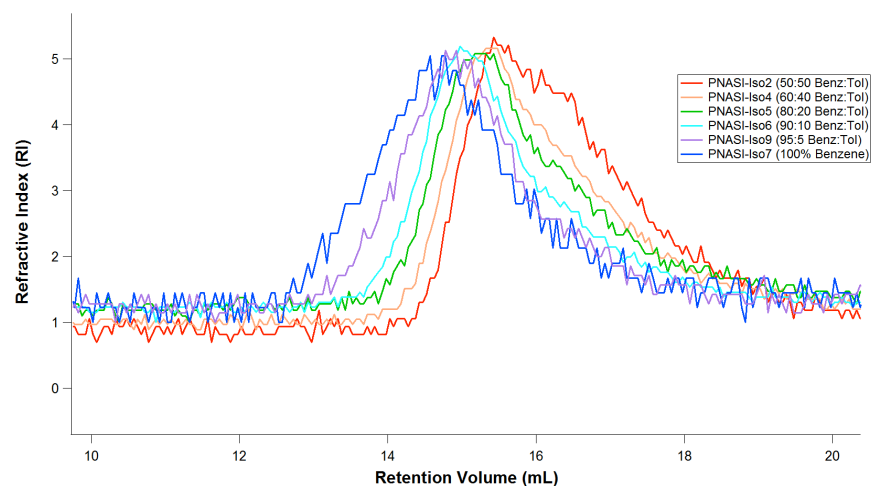


Figure A.2 GPC traces of PNASI-Isobutyl methacrylate polymers synthesized with different ratios of benzene and toluene.

As benzene content is increased polymer size increases as seen by the shift towards lower retention volumes. Ratios of benzene to toluene are: 50/50 (red), 60/40 (orange), 80/20 (green), 90/10 (teal), 95/5 (purple), and 100/0 (blue).

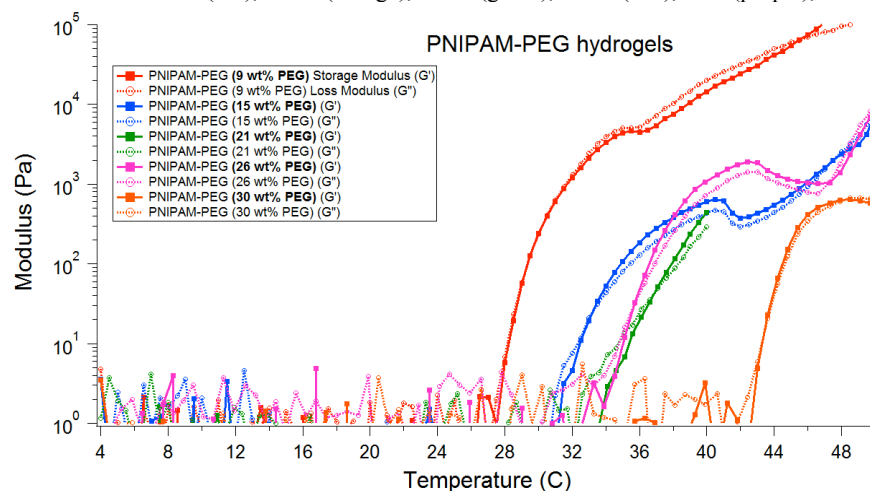


Figure A.3 PNIPAAm-PEG hydrogel rheology as a function of temperature.

All polymer concentrations are 20 w/v% in PBS. Curves are based on wt% 3.4k PEG: 9 wt% (red), 15 wt% (blue), 21 wt% (green), 26 wt% (pink), and 30 wt% (orange).

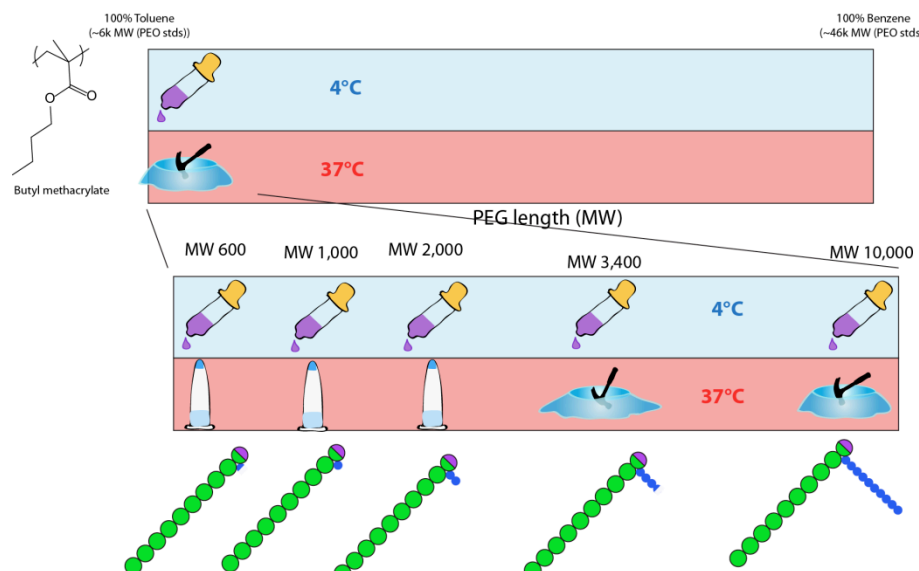


Figure A.4 Illustration of PNIPAAm-PEG-butyl methacrylate polymers with differing PEG lengths.

PNIPAAm-PEG-butyl methacrylate polymers made with 100% Toluene showed varied hydrogel properties with different PEG lengths. With PEG lengths of 600, 1k, and 2k the polymers precipitated or formed macroscopically distinct solids. Polymers made with 3.4k PEG made very soft hydrogels while polymers made with 10k PEG made slightly stiffer hydrogels. All polymer solutions were pipettable at cold temperatures below the LCST.

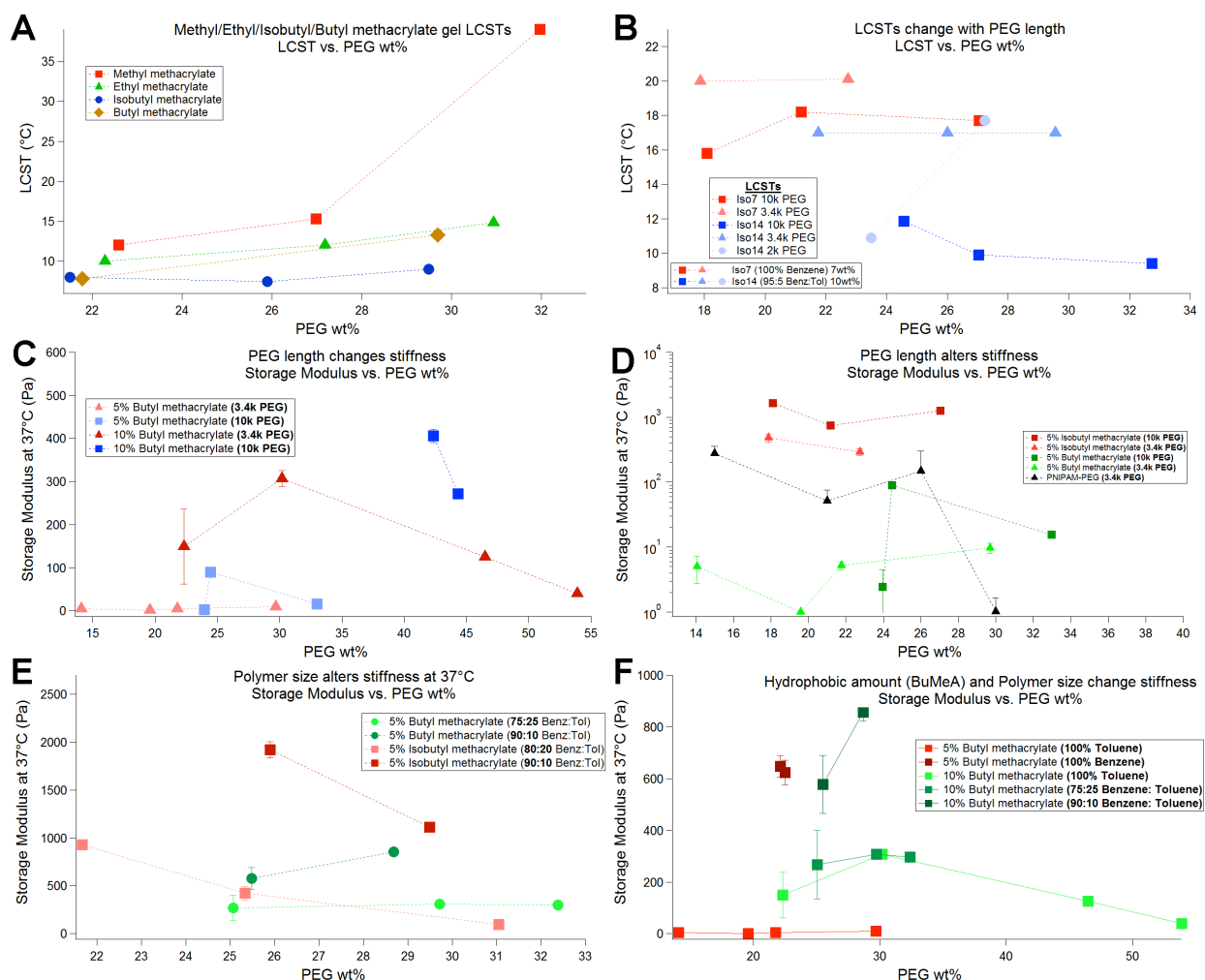


Figure A.5 LCST and storage modulus at 37°C versus PEG wt% for many polymer variations.

A. Simplified plot of LCST versus PEG wt% using only 5 mol% methyl, ethyl, isobutyl, and butyl methacrylates. All polymers were made with 3.4k PEG and all polymer concentrations between 16-20 wt/vol%. PNIPAAm-PEG, butyl acrylate and butyl methacrylate polymers shown here made in 100% toluene; methyl, ethyl, and isobutyl methacrylates made in 90/10 benzene/toluene blend. B. LCST for multiple PNIPAAm-PEG-Isobutyl methacrylates with PEG lengths of 2k (circle), 3.4k (triangle) and 10k (square) for polymers made in 100% benzene (red) or 95/5 benzene/toluene ratio (blue). C. 5 and 10 mol% butyl methacrylate polymers made in 100% toluene with 3.4k (triangles) or 10k (squares) PEG show an increased stiffness with increased PEG length for each percent hydrophobic groups. D. 5 mol% isobutyl methacrylate (95/5 benzene/toluene ratio) (red) and 5 mol% butyl methacrylate (100% toluene) (green) polymers both have increased storage modulus with increased PEG length from 3.4k (triangle) to 10k (square) PEG. E. Increased polymer size made with a higher ratio of benzene to toluene during synthesis produces stiffer hydrogels for both butyl methacrylate (green) and isobutyl methacrylate (red) polymers; all polymers made with 3.4k PEG. F. An increased amount of butyl methacrylate from 5 mol% (red) to 10 mol% (green) increases stiffness. Increased polymer size (as conveyed by higher benzene ratios) increased hydrogel stiffness as well. All polymers made with 3.4k PEG.

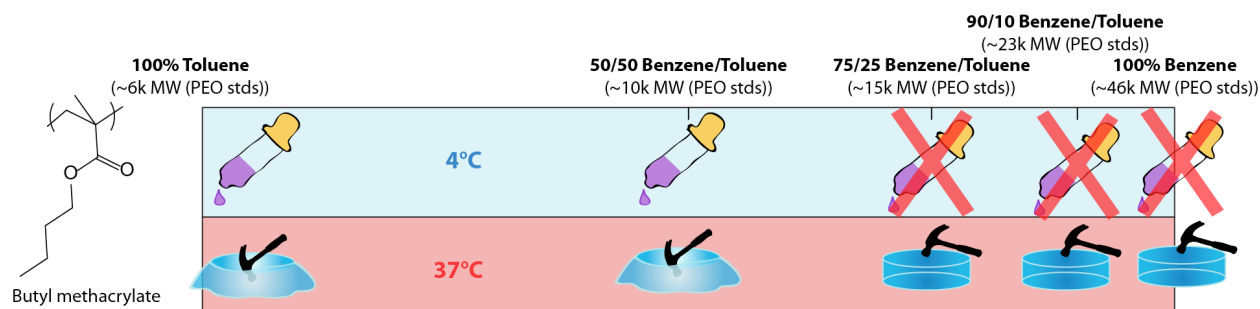


Figure A.6 Illustration of PNIPAAm-PEG-butyl methacrylate solution properties for polymers of different sizes. PNIPAAm-PEG-butyl methacrylate hydrogel properties spanning different molecular weights (from different benzene/toluene ratios) using 3.4k PEG. Polymers made with 100% toluene and 50/50 benzene/toluene ratio generate soft hydrogels while polymers made with 75/25, 90/10 benzene/toluene and 100% benzene generate stiff hydrogels that do not reliquify after 37°C.

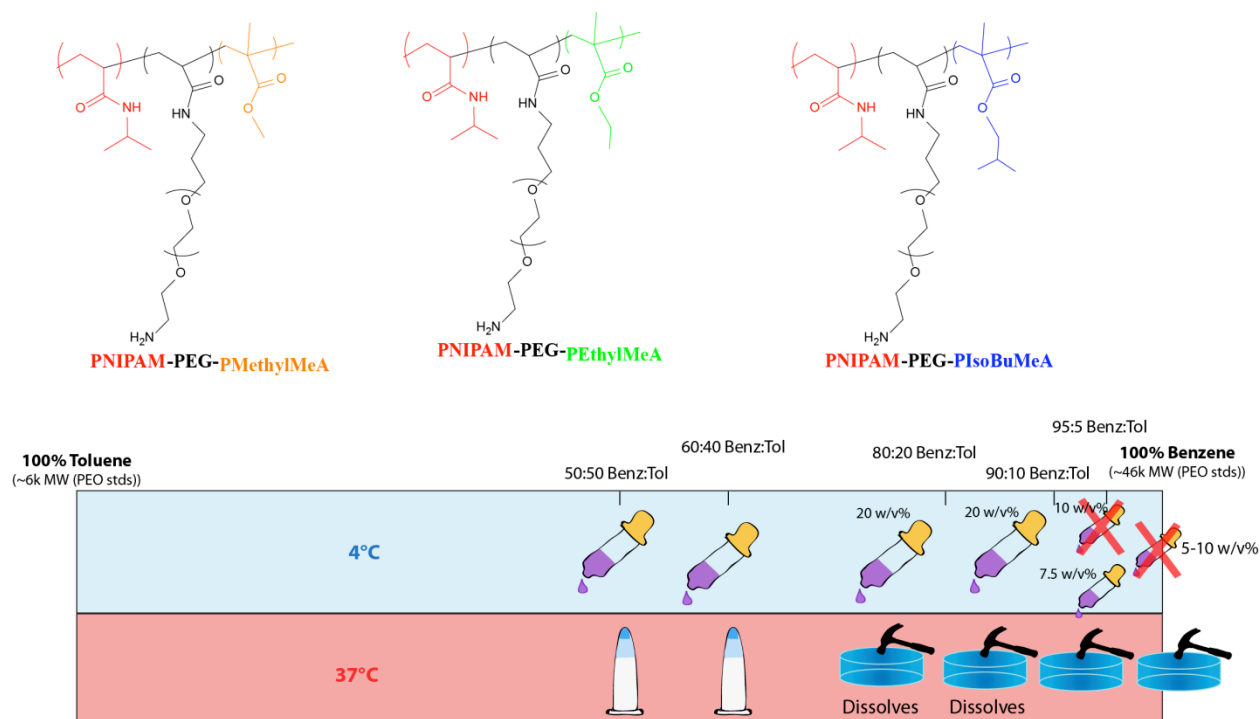


Figure A.7 Illustration of PNIPAAm-PEG-methyl/ethyl/isobutyl methacrylate hydrogel properties spanning a range of molecular weights (from benzene/toluene ratios during synthesis).

Polymers made with 50/50 and 60/40 benzene/toluene ratios do not make hydrogels but instead phase separate from the water. Polymers made with 80/20 or 90/10 benzene/toluene ratios make stiff hydrogels that reliquify, however these dissolve in media at 37°C. Polymers made with 100% benzene do not reliquify as well as polymers made with 95/5 benzene/toluene ratio at high concentrations.

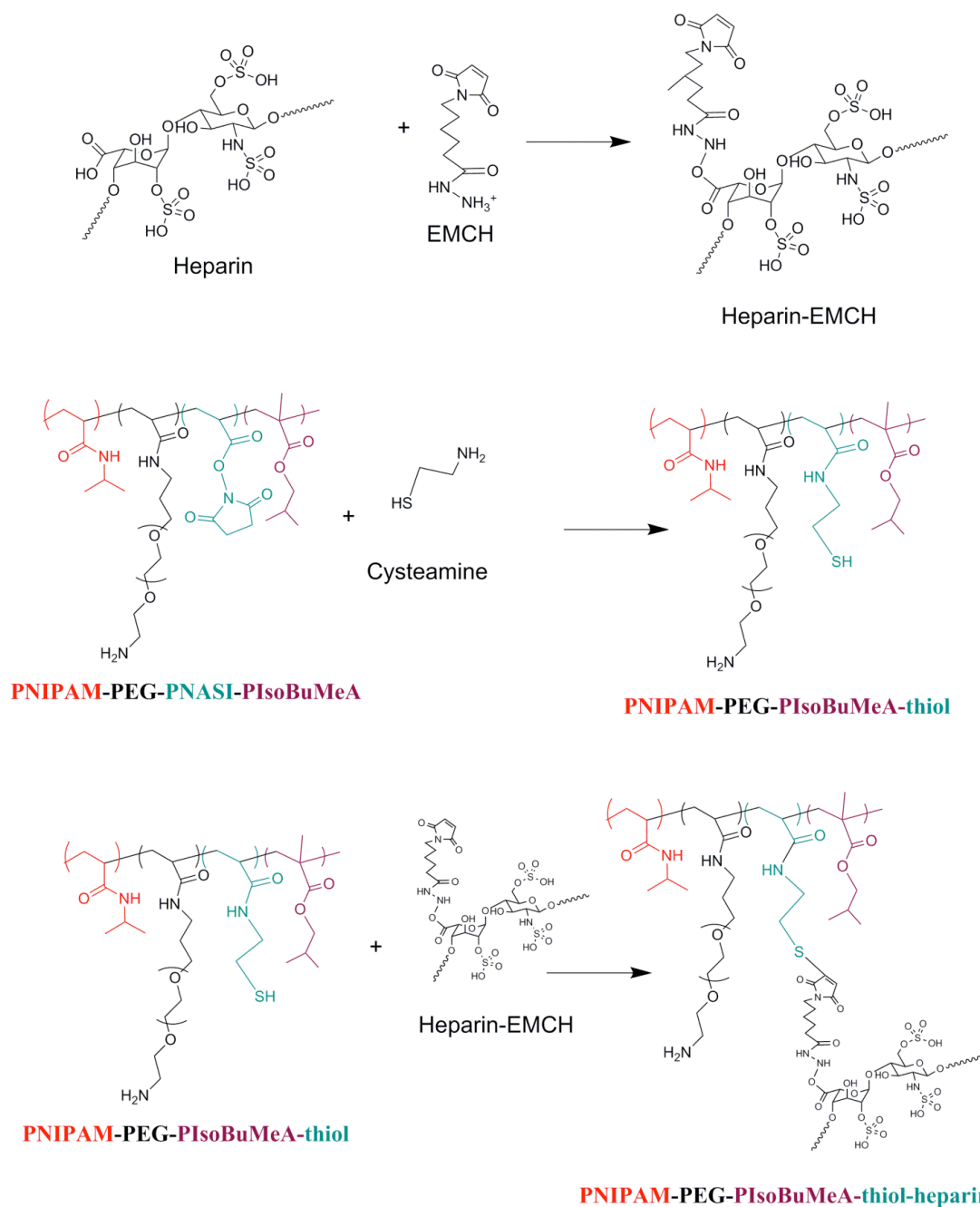


Figure A.8 Schematic of heparin functionalization and conjugation to PNIPAAm-PEG-isobutyl methacrylate-thiol
Heparin conjugation to PNIPAAm-PEG-Isobutyl methacrylate polymers occurs after thiol functionalization via cysteamine. Once the heparin is functionalized with EMCH, the maleimide group can react with the thiols within the polymer.

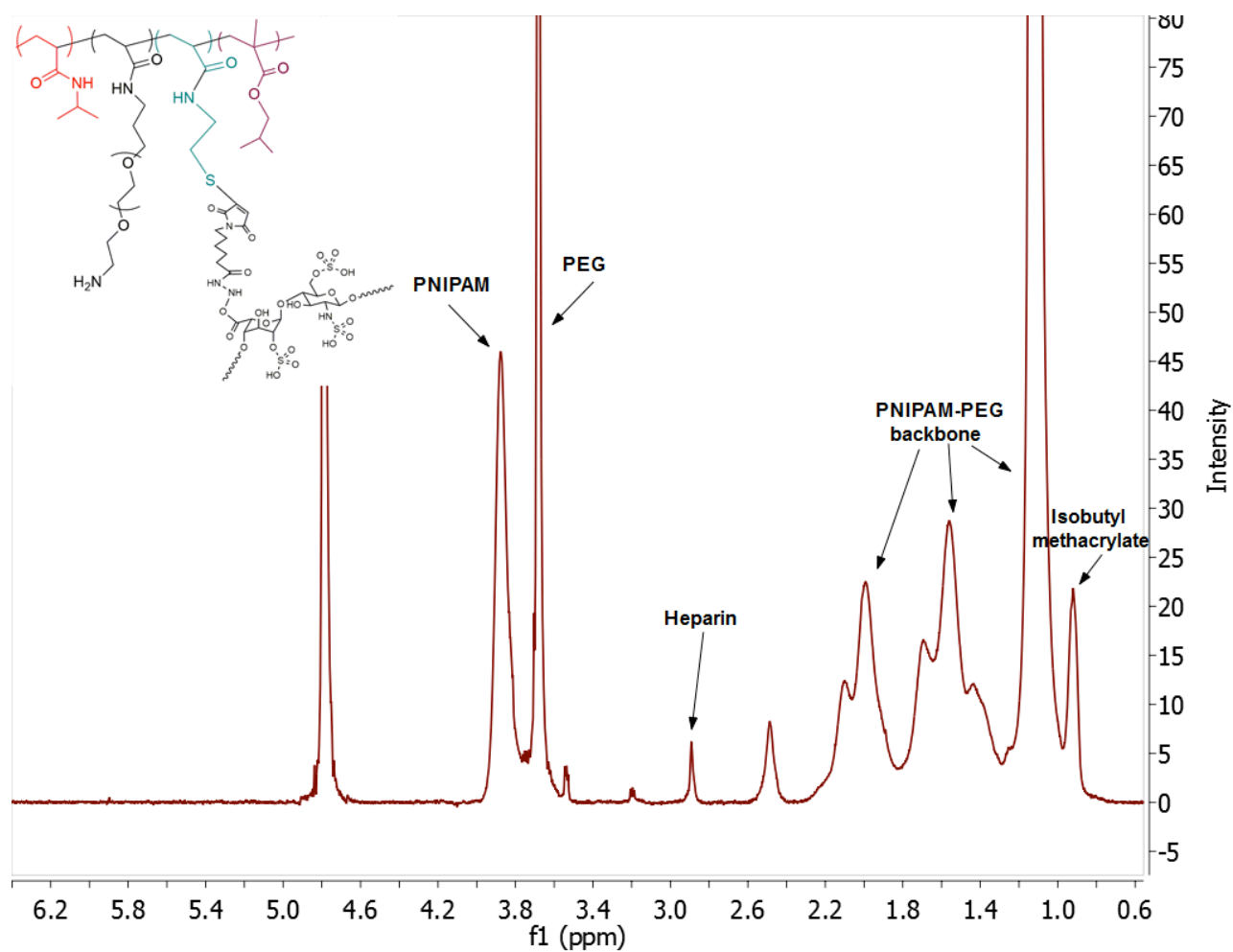


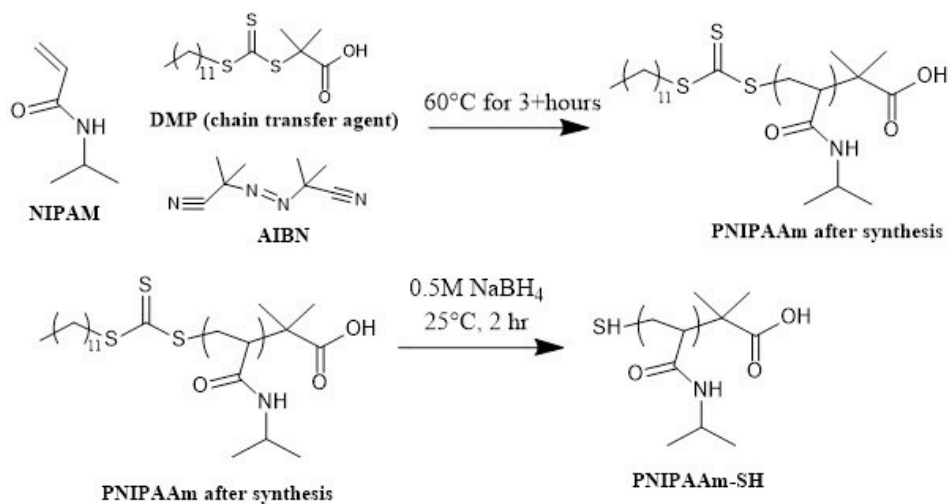
Figure A.9 ^1H NMR of PNIPAAm-PEG-isobutyl methacrylate with heparin conjugated.

^1H NMR spectrum shows heparin peaks present within the PNIPAAm-PEG-Isobutyl methacrylate polymer after exhaustive dialysis using 300 kDa MWCO dialysis tubing.

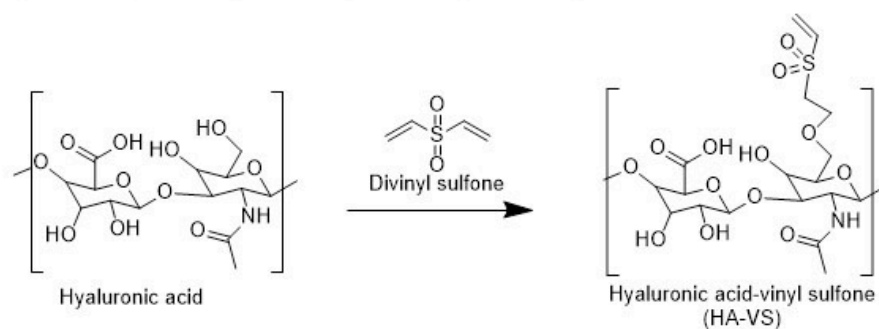
Appendix B: Supplementary Material for Chapter 3

B.1 Supplementary Figures

A



B



C

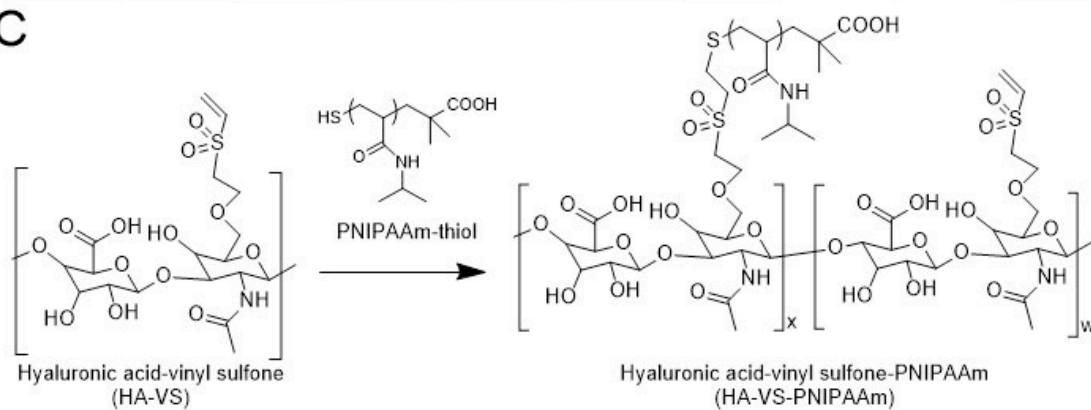


Figure B.1 Polymer synthesis schemes for PNIPAAm, HA-VS, and HA-PNIPAAm.

A. PNIPAAm synthesis via RAFT polymerization followed by thiocarbonythio reduction with NaBH₄ to produce thiols on chain ends. B. Synthesis scheme of functionalizing HA with divinyl sulfone C. Conjugation of PNIPAAm-SH to vinyl sulfone groups on the HA.

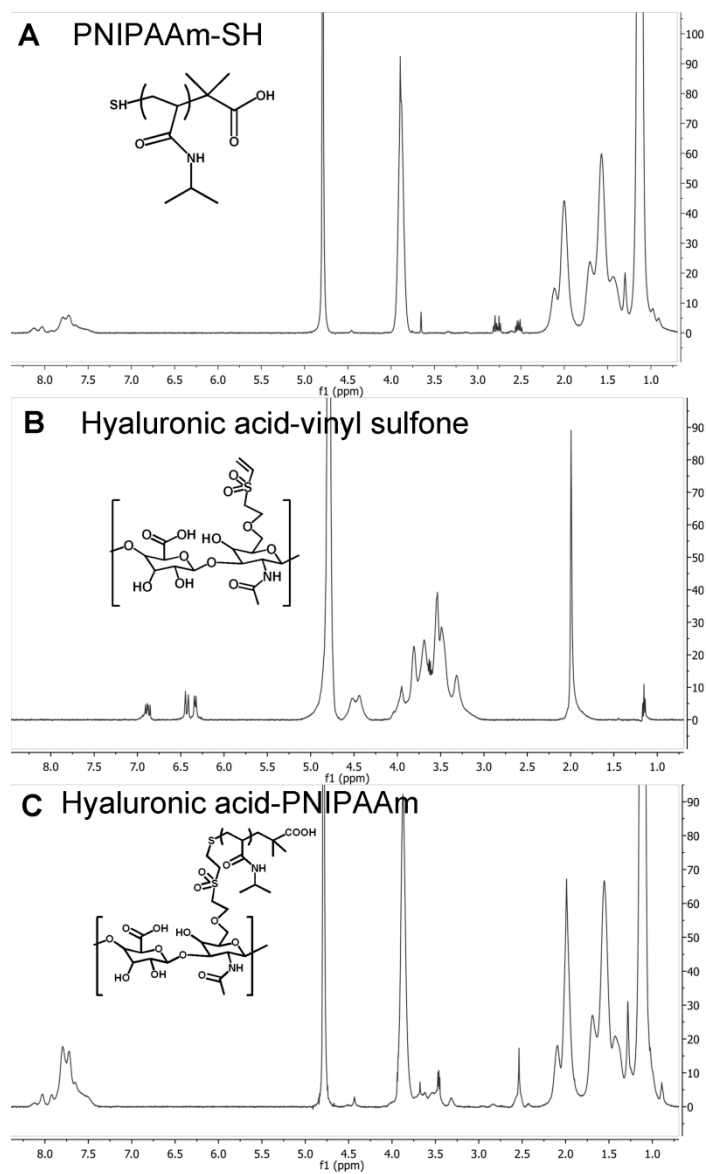


Figure B.2. ¹H NMR spectra of HA-PNIPAAm components

¹H NMR spectra of PNIPAAm-SH (A), Hyaluronic acid-vinyl sulfone (B), and Hyaluronic acid-PNIPAAm (C)

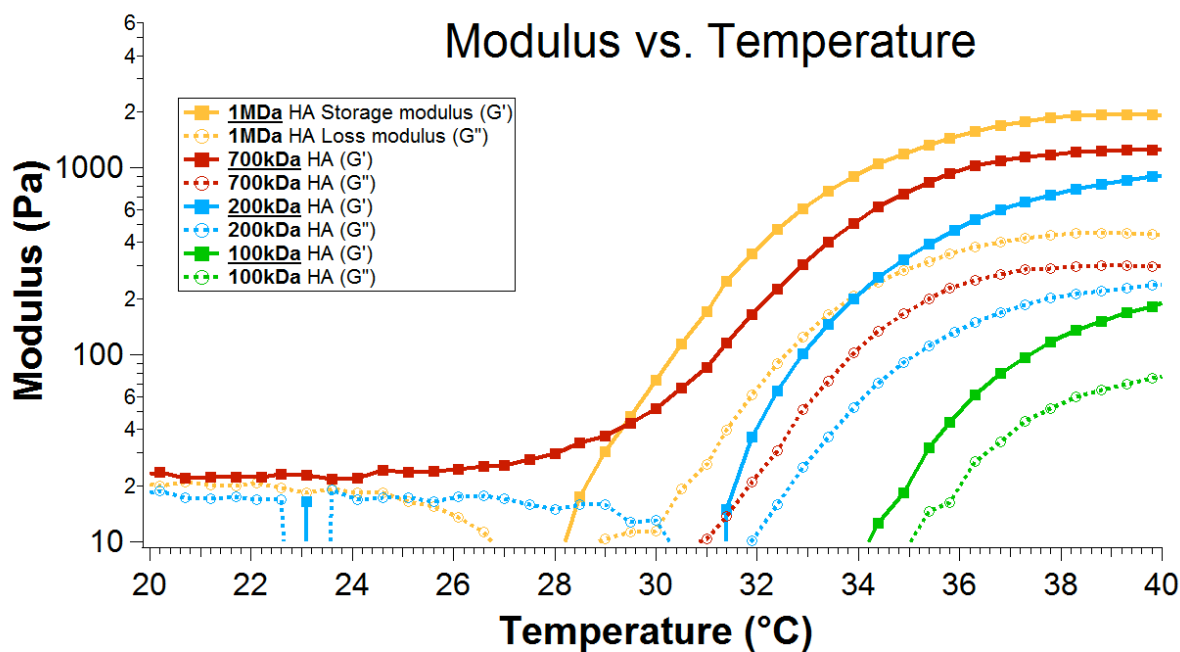


Figure B.3 Modulus as a function of temperature with varying HA sizes.

Rheological data showing modulus as a function of temperature for HA-PNIPAAms at 10 w/v% with different HA molecular weights: 1 MDa (yellow), 700 kDa (red), 200 kDa (blue), 100 kDa (green).

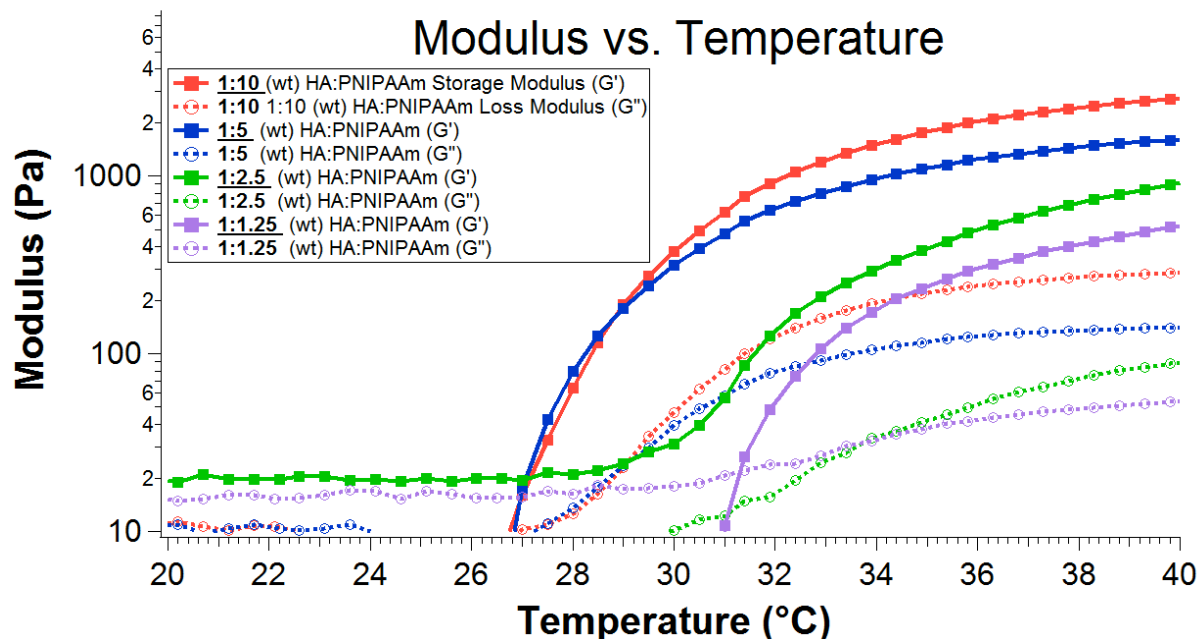


Figure B.4 Modulus as a function of temperature with varying HA:PNIPAAm ratios.

HA:PNIPAAm ratios of 1:10 at a concentration of 7 w/v% (red), 1:5 at a concentration of 5.5. w/v% (blue), 1:2.5 at a concentration of 4.5 w/v% (green), 1:1.25 at a concentration of 4.5 w/v% (purple).

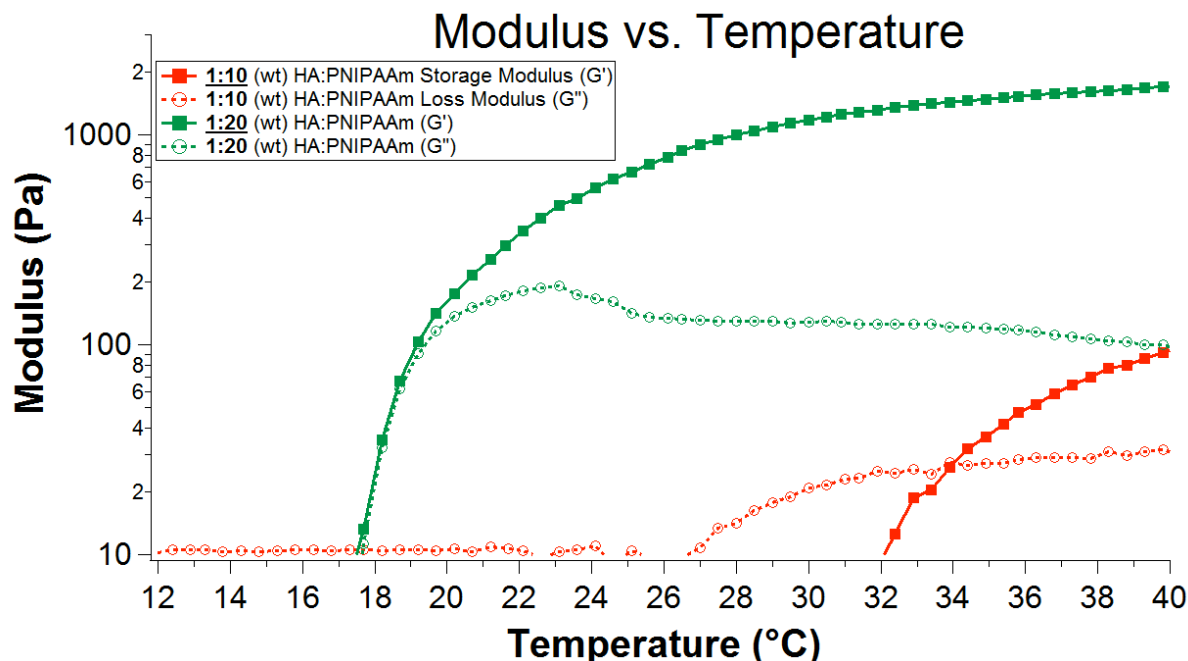


Figure B.5 Modulus as a function of temperature for HA-PNIPAAm gels with low bulk thiol contents using different HA:PNIPAAm ratios.

HA-PNIPAAms gels were at a concentration of 10 w/v% using 700 kDa HA 40% functionalized and a PNIPAAm-SH batch with low bulk thiol content with weight ratios for HA: PNIPAAm-SH of 1:10 (red) and 1:20 (green). Increasing the amount of PNIPAAm attached to the HA increases the storage modulus and decreases the LCST. Stiff hydrogels can still be made with PNIPAAm-SH's with low bulk thiol contents by increasing the ratio of PNIPAAm to hyaluronic acid.

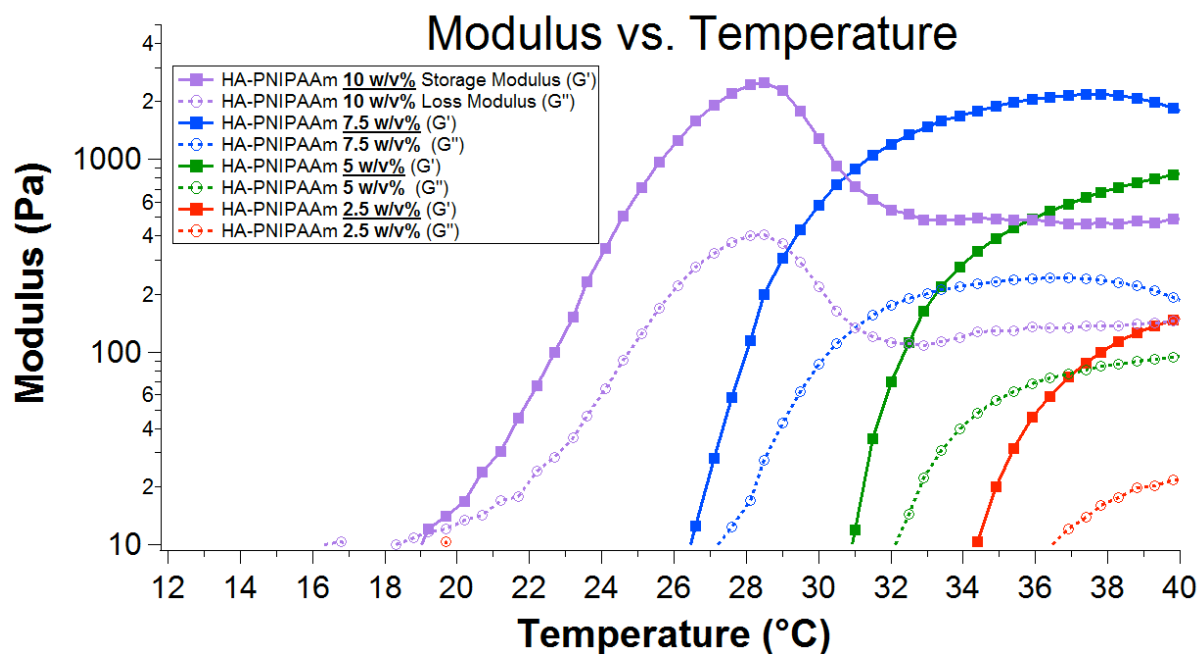


Figure B.6 Modulus as a function of temperature for HA-PNIPAAm polymer at varying concentrations.

HA-PNIPAAm polymers were made with the same 1 MDa 30% functionalized HA and PNIPAAm-SH. Concentrations of polymers in solution were 10 w/v% (purple), 7.5 w/v% (blue), 5 w/v% (green), and 2.5 w/v% (red). As concentration decreased the storage modulus decreased and the LCST increased. The shrinking/water excretion change (as seen by the decrease in storage and loss modulus after a peak) was shifted to higher temperatures at lower polymer concentrations.

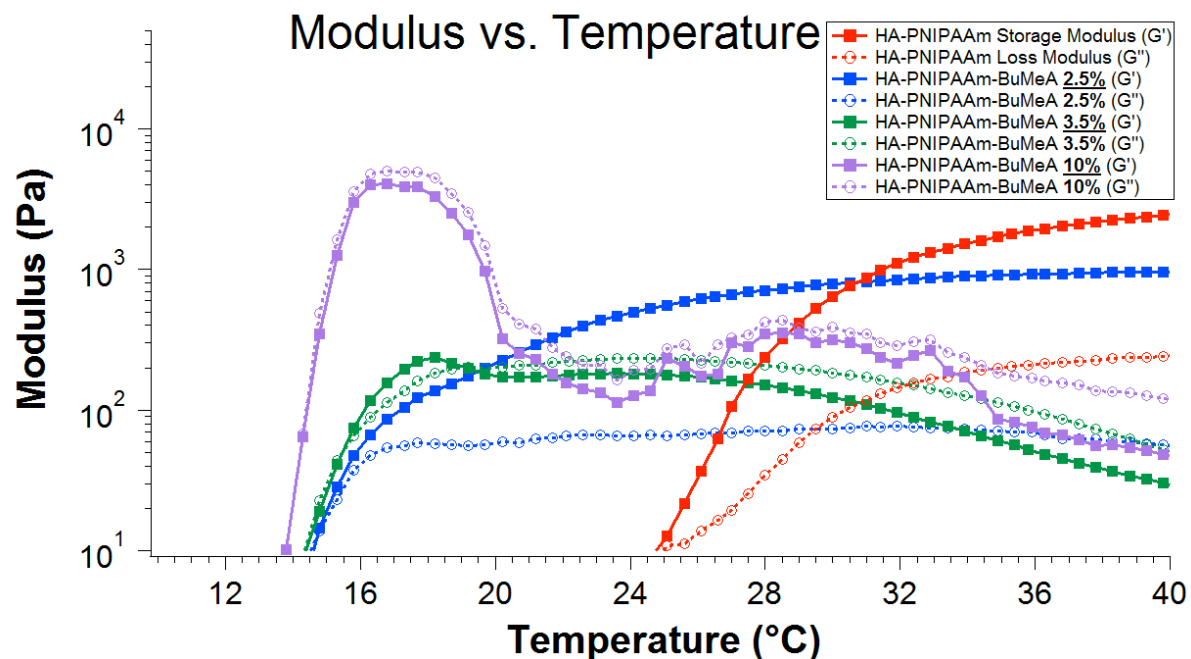


Figure B.7 Modulus as a function of temperature for HA-PNIPAAm hydrogels with different amounts of butyl methacrylate attached to the PNIPAAm

Butyl methacrylate amounts: 0% (red), 2.5 mol% (blue), 3.5 mol% (green), and 10 mol% (purple). All gels shown are 10 w/v% made with 700 kDa HA that was 40% functionalized and reacted at a weight ratio of 1:10 HA:PNIPAAm-SH except for 10 mol% BuMeA which was made with 1 MDa HA.