Poly(ethylene glycol) based biomaterial platforms for guiding cell behavior through control of presentation and release of bioactive, therapeutic proteins.

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Poly(ethylene glycol) based biomaterial platforms for guiding cell behavior through control of presentation and release of bioactive, therapeutic proteins.

by

Joshua Daniel McCall

B.S., North Carolina State University, 2003

A thesis submitted to the
Faculty of the Graduate School of the University of Colorado in partial fulfillment of the requirement for the degree of Doctor of Philosophy

Department of Chemical and Biological Engineering

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Poly(ethylene glycol) based biomaterial platforms for guiding cell behavior through control of
presentation and release of bioactive, therapeutic proteins

written by Joshua D. McCall

has been approved for the Department of Chemical and Biological Engineering

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Christopher N. Bowman, Ph.D.

The final copy of this thesis has been examined by the signatories, and we
find that both the content and the form meet acceptable presentation standards
of scholarly work in the above mentioned discipline.
Abstract

McCall, Joshua D (Ph.D. Chemical Engineering)
Department of Chemical and Biological Engineering, University of Colorado at Boulder.

Poly(ethylene glycol) based biomaterial platforms for guiding cell behavior through control of presentation and release of bioactive, therapeutic proteins

Thesis directed by Dr. Kristi S. Anseth

Poly(ethylene glycol) (PEG) based biomaterials offer a number of advantages for applications in biomedical technology, including drug delivery and tissue engineering. PEG is notable for both its hydrophilicity and bioinert properties, and is widely used to create hydrogels used for 3D cell culture. There is growing interest in strategies to introduce biological functionality into PEG-based materials, in order to develop platforms to study the complex biochemical and biomechanical cues that govern cell behavior in physiologically relevant context. This thesis explores photopolymerization conditions for hydrogel synthesis that maintain a high degree of bioactivity for proteins in-situ. Such reactions are then utilized to create hydrogels capable of directing cell fate and behavior.

Photopolymerization conditions for the formation of hydrogels were characterized, as these reactions are widely used for protein encapsulation. First, the role of affinity peptides was studied during photoencapsulation of the cytokine transforming growth factor β (TGFβ). When peptides with affinity for TGFβ were included in monomer solutions, they increased the amount of soluble, bioactive protein released from PEG diacrylate hydrogels formed via a chain-growth polymerization. We then studied protein protection during photopolymerization of step-growth networks using a thiol-norbornene reaction. Thiol-ene reactions were shown to be milder than
acrylate chain-growth, as they fully maintained the bioactivity of TGFβ and lysozyme. Protein deactivation was correlated to total photoinitiated radical concentrations in order to more fully characterize reaction conditions that maximized protein protection.

We then applied this knowledge to create biomaterials that incorporated covalently linked, bioactive proteins capable of directing complex cellular functions. TGFβ was thiolated via reaction with 2-iminothiolane, a modification that had no impact on bioactivity. This thiol-functionalized protein could then be readily linked into hydrogels using either thiol-acrylate or thiol-ene polymerizations. When human mesenchymal stem cells were encapsulated into tethered TGFβ hydrogels, the growth factor induced chondrogenic differentiation at levels similar to or exceeding that of soluble delivery. Further, tethered TGFβ hydrogels were used to culture valvular interstitial cells in order to study the combined roles of substrate elasticity and immobilized TGFβ play in activation of myofibroblast phenotype. The photopolymerization conditions and resulting functionalized hydrogels developed in this thesis demonstrate the use of PEG hydrogels to investigate complex cell-material interactions of interest in tissue engineering applications.
To my brothers Adam and Michael,

who have always believed in me
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Chapter 1.

Introduction

1.1 Overview

Hydrogels hold great promise for utilization in the field of regenerative medicine, especially as cell and drug delivery vehicles. These versatile, highly swollen polymer networks are characteristically formed with a high water content, via a variety of cytocompatible, aqueous chemistries\textsuperscript{1-6}. By tuning polymeric properties like elasticity and diffusivity, hydrogels provide a facile method for creating three-dimensional cell-laden scaffolds with properties similar to many soft tissues. Unlike traditional cellular biology approaches, where cells are cultured on rigid, two-dimensional surfaces (e.g., petri dishes, tissue culture flasks, etc), hydrogels provide a powerful tool to study cellular behavior in three-dimensional systems that more closely resemble the native extracellular matrix (ECM).

Poly(ethylene glycol) (PEG)-based hydrogels are one of the most widely studied biomaterial platforms, as PEG offers a number of desirable properties as a scaffold choice, including tunable mechanics\textsuperscript{2, 7, 8}, low protein adsorption\textsuperscript{9-11}, and high viability of encapsulated cells\textsuperscript{4, 12-14}. Further, PEG hydrogels can be tailored to provide a sustained and local presentation of proteins to cells\textsuperscript{15-20}. Many such biological macromolecules, including hormones, growth factors, and others, function \textit{in vivo} to guide a host of cellular functions, making them a promising tool for tissue engineering. However, protein signaling is a complicated process and
often necessitates delivery platforms capable of both spatial and temporal control of their presentation. This thesis is broadly focused on photopolymerization schemes of PEG hydrogels that maintain a high degree of bioactivity for proteins encapsulated in situ, with the ultimate goal of developing materials capable of presenting bioactive protein signals to study and direct cell behavior.

1.2 Poly(ethylene glycol) based biomaterials

Poly(ethylene glycol) (PEG) is an attractive choice for biomaterials, as PEGs have been utilized in a variety of FDA-approved applications including food additives, emulsifiers, anti-thrombotic coatings, and in PEGylation of therapeutic proteins. The carbon-carbon-oxygen mer (Figure 1.1) is imminently hydrophilic; in aqueous environments, the ethylene glycol repeat unit binds water molecules, thereby limiting sites available for adsorption of other species

Edward Merrill is often credited for pioneering the use of PEG in biomedical applications. While studying the viscosity of blood in the early 1960s, he observed rapid clot formation upon contact with most membrane materials in use at the time. In contrast, he found PEG was impressively bioinert and had clear application as an anti-thrombotic material, a discovery that facilitated the development of early biomedical technologies like kidney dialysis and blood oxygenators. Some sixty years later, PEG-based polymers are ubiquitously used in biomedical applications.
PEG chains can be functionalized with reactive groups and subsequently used to create a crosslinked material, as shown in Figure 1.1 B. Many reactive groups have been used to form crosslinked PEG hydrogels, including amines, carboxylic acids, thiols, (meth)acrylates, maleimides, vinyl sulphones, and others. This vast array of functionalized PEGs provides a similarly diverse range of mechanical properties in resulting hydrogels. Hydrogel crosslinking density can be tuned by choice of PEG molecular weight, e.g. diacrylated PEG of molecular weight 2,000 Da forms a hydrogel with a higher crosslinking density than does 10,000 Da PEG diacrylate. Crosslinking density can also be tuned through differences in monomer functionality and number of PEG arms. As an example, a PEG hydrogel formed with 4-arm PEG (M$\text{\textsubscript{n}}$=10,000) norbornene can be crosslinked with a linear bis(thiol) (M$\text{\textsubscript{n}}$=2,000) via a thiol-ene polymerization; crosslinking density can be tuned by changing the molecular weight of the bis(thiol) crosslink, the molecular weight of the 4-arm PEG ene, or by increasing the number of arms on either species, as shown in Figure 1.1. The utility of PEG hydrogels is enhanced through such facile control of crosslinking density, as this in turn controls important mechanical properties, including elasticity, diffusivity, and degradation$^{7,24-30}$. 

Figure 1.1. A) linear PEG structure, and B) multi-arm PEG structure
1.3 Photopolymerization approaches for forming PEG hydrogels

While hydrogels can be synthesized from a variety of chemistries, photopolymerization offers a number of distinct advantages. Photoinitiated reactions can proceed under mild conditions, notably in aqueous systems at physiological temperature and pH. These approaches are widely used for polymerization in the presence of sensitive encapsulants, such as cells or proteins due to the high cytocompatibility afforded by photoinitiation\textsuperscript{1, 4, 6}. Of further interest, photoinitiated polymerizations provide precise spatial and temporal control, extending the versatility of materials formed from such reaction schemes. For radically mediated photopolymerizations, radicals are generated from an initiator species according to Equation 1.1:

\[
R_i = \frac{2f\Phi\epsilon\lambda I}{N_A h\nu}[C_i]
\]  

(1.1)

where \( R_i \) is the rate of primary radical generation, as a function of \( C_i \), the concentration of unreacted initiator. The initiator efficiency, \( f \), and quantum yield, \( \Phi \), are characteristic for each initiator. Molar absorbptivity, \( \epsilon \), describes the amount of light absorbed by an initiator at a given wavelength, \( \lambda \), while the intensity of incident light, \( I \), is a measure of the amount of radiation delivered to the system for initiation. For biocompatibility, light wavelengths and intensities are critical parameters: wavelengths above 365 nm are generally considered cytocompatible, and initiators with high absorbptivity at these longer wavelengths allow for reduction in the dose of radiation, thereby increasing cell viability\textsuperscript{1, 4, 31}. 


1.3.1 (Meth)Acrylate chain-growth polymerization

![Schematic of an acrylate chain-growth reaction and resulting network structure](image)

One of the most common photochemical approaches to forming PEG hydrogels is the radically-mediated chain-growth reaction of (meth)acrylated PEG species. Photoinitiation of acryl chain-growth is widely used, via a number of water-soluble light-cleavable initiator species\textsuperscript{1, 4}. In the presence of a radical source, both acrylate and methacrylate functional groups can undergo chain-growth polymerization. Following radical transfer to the vinyl carbon of the carbon-carbon double bond, the radical propagates until termination produces a dead polymer chain\textsuperscript{32}. For tetrafunctional monomers, e.g. PEG di(meth)acrylate, the reaction results in a crosslinked polymer material. Although susceptible to oxygen inhibition\textsuperscript{33}, the (meth)acrylate chain-growth reaction is considered a robust method for synthesizing hydrogels at dilute functional group concentration in aqueous environments, while maintaining viability of \textit{in-situ} encapsulated cells and proteins\textsuperscript{34}. When mesenchymal stem cells were encapsulated in PEG hydrogels formed through photopolymerization of acrylated-PEG monomer, Nuttelman \textit{et al.} reported >95% viability of encapsulated cells\textsuperscript{13}. 
1.3.2. Thiol-ene step-growth polymerization

In contrast to (meth)acrylate homo-polymerization, thiol-ene “click” reactions proceed via a step-growth mechanism and require two unique monomers. The reaction occurs through two steps: upon cleavage of an initiator species, radicals transfer to a thiol in solution, creating a thiyl radical. Propagation occurs with the addition of the thiyl radical across a carbon-carbon double bond, the ene. If the ene is selected properly, the carbon radical cannot homopolymerize, but instead, can only chain transfer back to another thiol, regenerating a reactive thiyl radical. This unique reaction allows step growth of PEG networks via a radical-mediated process\(^2\). As an example, PEG hydrogels have been photopolymerized using this reaction scheme with four-arm PEG-norbornene and bis(thiol) crosslinkers, with high maintenance of cell viability and function\(^{14}\). The resulting ideal polymer networks possess macroscale homogenous mechanical properties, making them an attractive choice for encapsulation platforms for biologics. Further, the thiol-ene reaction is not oxygen inhibited and can be used to rapidly form hydrogels using low initiator concentrations and/or light doses\(^2, 35\), and this approach has been utilized to synthesize hydrogels for encapsulation of radically sensitive payloads, including cells\(^{14, 36-38}\) and bioactive proteins\(^{39}\).
1.3.3 Thiol-acrylate mixed-mode polymerization

As the name suggests, the thiol-acrylate photopolymerization is a hybrid scheme combining aspects of chain- and step-growth. While the reaction can propagate via a chain-growth mechanism, thiol present in the monomer solution can abstract a radical to produce a thiyl species. This thiyl can then chain-transfer to an unreacted acrylate, creating an acryl radical that can further propagate. The chain-transfer constant for this mixed mode has been reported on the order of $1.5 - 2^{40-42}$ and has been utilized to incorporate numerous thiol-functionalized molecules into PEG hydrogels.

Both the thiol-ene and mixed-mode thiol-acrylate photopolymerization approaches involve covalent reactions of thiol-containing species. One mutual benefit these synthetic routes is the facile incorporation of thiol-containing biomolecules into hydrogel materials.

Figure 1.4 – The thiol-acrylate polymerization scheme and resulting polymer network
1.4 Introduction of biologically active moieties into synthetic polymer materials

There is growing interest in ways to incorporate biological cues into synthetic cell scaffold biomaterials, in order to study more complex cell-material interactions in a physiologically relevant three-dimensional model. *In-vivo*, a host of signals from the cellular microenvironment influence cell behavior, including cell-cell, cell-matrix, and cell-signaling protein interactions. Hydrogels synthesized from bioinert materials like PEG represent a blank slate for cell culture, one that can be functionalized via chemical modification to introduce biological cues. In this way, it is possible to isolate and characterize individual cues, like adhesion ligands or substrate elasticity, and the role that they play in guiding cell function. PEG hydrogels formed via acrylate chain-growth or thiol-ene step-growth polymerization are particularly attractive for such studies, as thiol-functionalized biomolecules can be covalently incorporated into the polymer itself.

1.4.1 Peptide-functionalized hydrogels

Peptides are short polymers comprised of amino acids linked by amide bonds. Peptides can be synthesized at yield and quantity similar to that of small molecules, and in many cases can provide functions similar to that of a full-length protein. This property makes peptide functionalization an attractive choice for introducing biological cues into synthetic hydrogels.

Perhaps the most extensively studied example of peptide functionalization is that of the integrin-binding peptide sequence Arg-Gly-Asp-Ser (RGDS), isolated from the ECM protein fibronectin. This peptide has been identified as an adhesion site used by many adherent cells, and binds with a number of integrins expressed by a multitude of cell types. The inclusion of
RGDS peptides in PEG hydrogels provides an adhesion site and mimics the native cellular microenvironment, where ECM proteins facilitate integrin binding.

The native ECM is a dynamic structure, and cells are capable of remodeling their pericellular microenvironment through enzymatic degradation. Matrix-metalloproteinases (MMPs) are cell-secreted enzymes capable of degrading specific peptide sequences of ECM proteins like collagen, fibronectin, laminin and others. Numerous peptide sequences susceptible to MMP-cleavage have been identified, including MMP-1 cleavable peptides Val-Arg-Asn (VRN) and Ala-Pro-Gly-Leu (APGL), among others. Including these MMP-degradable peptide sequences as crosslinkers in synthetic PEG presents a facile method to allow cell-mediated degradation of a synthetic matrix.\(^45\)

Alternatively, peptides have been exploited for use as protein affinity ligands. Peptide sequences with binding affinity for proteins of interest can be isolated via a variety of techniques, including but not limited to bacteria phage-display screening or isolation of short sequences from longer proteins known to bind a target. Such affinity peptides bind reversibly with a target protein, and the interactions are governed through kinetic rates depicted in Equation 1.2:

\[
\text{Protein} + \text{Ligand} \rightleftharpoons k_{\text{forw}} \text{Protein-Ligand} \\
K_D = \frac{k_{\text{rev}}}{k_{\text{forw}}} 
\]

Incorporation of such affinity ligands has been used to functionalize PEG hydrogels used for protein delivery and inhibition. The peptide sequence Lys-Arg-Thr-Gly-Gln-Tyr-Lys-Leu (KRTGQYKL) has binding affinity for the growth factor bFGF. When this peptide was covalently incorporated in PEG hydrogels, the resulting polymer matrix was effective in
controlling the release of photoencapsulated growth factor over time scales ranging from 1 to 4 weeks\textsuperscript{19}.

Peptide mimics of full proteins provide another application for peptide-functionalized hydrogels. Numerous peptide sequences have been reported as passable analogs of full-length proteins and can be produced at a fraction of recombinant expression approaches required for proteins. Peptide mimics have been reported for the cytokines BMP-2 and BMP-7,\textsuperscript{46,47} both of which are implicated in the osteogenic differentiation of human mesenchymal stem cells. Such discoveries and reports regarding new peptide sequences open opportunities for the creation of innovative peptide functionalized matrices to direct critical cellular functions.

\textit{1.4.2 Protein-functionalized hydrogels}

While peptide-functionalized hydrogels have been effective tools for introducing biochemical cues into synthetic hydrogels, methods to covalently link full, bioactive proteins into hydrogel matrices are of interest, as they can potentially provide a more physiologically relevant signal to encapsulated cells. Such an approach provides a versatile method to precisely control protein concentrations, while limiting protein diffusion out of the target site, potentially minimizing undesirable collateral interactions with surrounding tissues.

A number of proteins have been successfully tethered into hydrogel materials for tissue engineering applications\textsuperscript{15,16}. Valvular endothelial growth factor (VEGF) has been acrylated and covalently immobilized in PEG diacrylate hydrogels, in order to promote endothelial cell tubulogenesis\textsuperscript{48}, and platelet-derived growth factor (PDGF) has been similarly incorporated into PEGDA hydrogels with results demonstrating promotion of angiogenesis and tubulogenesis\textsuperscript{49}. 
TGFβ is an attractive target for tissue engineering application, as it is known to regulate a diverse number of cellular responses in a wide range of biological systems. Mann et al. acrylated TGFβ using acryl-PEG-NHS chemistry and subsequently crosslinked this growth factor into PEGDA hydrogels. When smooth muscle cells were encapsulated in PEG hydrogels with tethered TGFβ, the cells produced ECM components indicative of TGFβ stimulation, at levels higher than that of a control system, namely TGFβ that was dosed solubly. More recently, affinity peptides binding to TGFβ receptors were immobilized on glass substrates, in an effort to spatially concentrate receptors to, in effect, spatially pattern TGFβ. This approach sensitized mouse mammary gland cells, as surfaces patterned with the peptides promoted αSMA upregulation at levels similar to that of solubly delivered TGFβ. Finally, TGFβ and interleukin 1β were thiolated by Hume et al. and covalently tethered into PEG diacrylate hydrogels to form material surfaces capable of down regulating dendritic cell response.

1.5 The cytokine transforming growth factor beta (TGFβ)

The elucidation of in-vivo protein signaling between organs and systems is a relatively recent achievement of modern biology. The name Pavlov will always be associated with conditional responses of the nervous system (as well as slobbering dogs); he was himself a stringent opponent to the idea of non-nervous system signaling, as were most biologists of the time. In their seminal experiment in 1902, Ernest Starling and William Bayliss removed the nerves from the intestine of a canine, then simulated digestion and observed the production of pancreatic juice. Despite the lack of a nervous system connection between the two organs, there was no decrease in pancreatic function. This was the first demonstration of the role played by proteins in controlling cell and organ function. The ramifications of this discovery reverberate
more than a century later, as medical technology continues to pursue and exploit such complex signaling for the treatment of disease.

Many classes of signaling proteins have since been characterized, including hormones, antibodies, and growth factors. The term ‘growth factor’ is vestigial, as these proteins and factors were originally known for their ability to illicit changes, such as an increase in the rate of cellular proliferation\textsuperscript{54, 55}. More appropriately, this class of cell-secreted proteins is now more commonly referred to as ‘cytokines,’ from the Greek words for ‘cell’ and ‘movement.’ Cytokines can regulate a variety of complex cell behaviors and play an important role in both homeostasis and pathologies. While they are potent, with functional concentrations in the nano- to picomolar range, they are unstable in circulation, often with serum half-lives on the order of minutes\textsuperscript{18, 56}. Another limitation is that receptors for many cytokines are widely conserved among multiple cell types\textsuperscript{17, 18, 57, 58}, restricting the utility of systemic dosage. However, as these proteins are designed to incite cellular responses, they are increasingly targeted for applications in regenerative medicine and tissue engineering\textsuperscript{16, 17, 19, 56, 57, 59-62}.

Figure 1.6 TGF\(\beta\) signaling pathway (adapted from Sigma Aldrich)
Transforming growth factor beta (TGFβ) was first isolated in 1983 and has since been widely studied, as this 25 kDa protein and other members of the TGF super-family play an important role in a vast array of cellular processes. TGFβ is found in three different isoforms, typically referred to TGFβ-1, TGFβ-2, and TGFβ-3. While TGFβ-2 only functions in conjunction with TGFβ1/3, isoforms 1 and 3 are almost identical in bioactivity and sequence, and TGFβ-1 is used exclusively in this work. TGFβ is clearly implicated in regulation of proliferation, differentiation, motility, adhesion, and apoptosis. Receptors for TGFβ are highly conserved across systems and species, as quoted from Massague’s 1998 review: “Expressed in complex temporal and tissue-specific patterns, TGFβ and related factors play a prominent role in the development, homeostasis, and repair of virtually all tissues in organisms, from fruitfly to human.” TGFβ is also implicated in both pathology and wound response, including fibrosis, activation of immune response, blood vessel development, ECM secretion and remodeling, and myocardial homeostasis.

Of specific interest in this thesis, TGFβ can induce differentiation of hMSCs, promoting a chondrocyte-like phenotype. Chondrocytes are the primary cell type found in articular cartilage tissue. This tissue, composed of polysaccharides known as glycosaminoglycans (GAGs) and collagen type II, is avascular, a factor that leads to the limited healing capacity observed for damaged cartilage. hMSCs cultured in media augmented with soluble TGFβ undergo chondrogenic differentiation, resulting in neochondrocytes capable of secreting GAGs and collagen-II. As hMSCs can be readily isolated from patient bone marrow, this approach has potential as a tissue-engineering solution for cartilage repair.

TGFβ is also known to promote activation of valvular interstitial cells (VICs), where the cells undergo a transition from a quiescent, fibroblast-like state into a myofibroblast phenotype.
This myofibroblast population helps repair damaged heart valve tissues following injury, secreting ECM components to replace and reorganize the matrix. However, sustained activation of these cells can itself lead to valve failure, as the leaflets stiffen and can cease to properly regulate blood flow through the aorta\textsuperscript{66, 69, 70}. VIC activation can be initiated by both substrate elasticity and TGFβ dosage\textsuperscript{50, 71-75}, and indeed, TGFβ is immobilized in calcific nodules that form in response to prolonged activation\textsuperscript{72}. Less understood is the crosstalk between these two factors, as elucidation of the roles played by substrate stiffness and TGFβ concentration requires a model with independent control of each.

1.6 Approach of this thesis

In this thesis, we broadly investigate photopolymerization methods and conditions for synthesis of PEG hydrogels that maintain the bioactivity of in-situ proteins. We use these reaction parameters to demonstrate the utility of PEG hydrogels for both encapsulation and covalent tethering of TGFβ and its application in directing cell function. The specific objectives and experimental plan of this thesis are discussed in greater detail in Chapter 2.

In Chapter 3, we focus on the use of affinity peptides and their ability to increase the recovery and release of active TGFβ that is photoencapsulated in PEG hydrogels formed via a chain-polymerization. Two peptides with affinity for TGFβ are identified and characterized. When included in monomer formulations, we study how affinity peptide sequences increase the amount of soluble, bioactive TGFβ released from the hydrogel following photoencapsulation.

In Chapter 4, we further characterize photoencapsulation reaction conditions that preserve the bioactivity of in-situ proteins. PEG hydrogels are formed using either a chain-growth (acrylate) or step-growth (thiol-ene) mechanism. Loss of protein bioactivity from
photogenerated primary radicals is characterized and compared to destruction in the presence of a competing acryl chain-growth or thiol-ene step-growth polymerization.

After studying photopolymerization conditions that maximize in-situ protein bioactivity, we then apply this knowledge by covalently crosslinking bioactive proteins into PEG hydrogels. In Chapter 5, we show the utility of this approach by developing a biomaterial platform capable of promoting chondrogenic differentiation of encapsulated hMSCs. Thiolated TGFβ is incorporated into PEGDA hydrogels using a thiol-acrylate polymerization. A reporter cell line is first utilized to demonstrate the bioactivity of tethered TGFβ in 3D culture. Then, hMSCs are encapsulated in these tethered TGFβ hydrogels to study how tethered growth factors promote their chondrogenic differentiation, as evaluated via immunohistological analysis of secreted proteins and stem-cell markers. Chondrogenesis of hMSCs encapsulated in tethered TGFβ gels is compared to that of cells encapsulated in blank PEG gels and dosed with higher amounts of soluble TGFβ. In Chapter 6, we use a similar approach in PEG hydrogels created using a thiol-ene polymerization. PEG hydrogels are formed with excess [ene] relative to [SH], allowing a subsequent reaction to couple thiolated TGFβ. Bioactive proteins are then spatially patterning through the use of photolithographic techniques. We demonstrate the utility of this approach by studying the fibroblast to myofibroblast activation of valvular interstitial cells.

Chapter 7 then concludes with a brief summary of the full scope of the work included herein, as well as preliminary results indicating the feasibility of future applications for this knowledge. Collectively, the results presented in this thesis combine to demonstrate facile methods for photoencapsulation of bioactive proteins capable of directing multiple cell functions.
1.7 References


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Chapter 2

Thesis Objectives

Broadly, this thesis aims to develop hydrogel platforms capable of presenting bioactive proteins to cells in a physiologically relevant context. To this end, we investigate photopolymerizations as a facile method to either encapsulate or covalently tether signaling proteins in PEG hydrogel scaffolds, with the goal of minimizing the loss of protein bioactivity during such reactions. Synthetic polymeric materials are ubiquitously utilized for both cell encapsulation and drug delivery, as they can be easily fabricated with a wide array of biomechanical properties via mild synthesis conditions. Further, synthetic materials like PEG function as a “blank slate”, one that provides minimal biological cues to encapsulated or proximal cells, and a unique system from which to introduce protein signals and ask basic questions about their influence on specific cellular functions.

*In-vivo*, the cellular microenvironment presents a complex milieu of external cues that can guide behavior and influence cell fate. These signals can be from the ECM itself, e.g. integrin-adhesion site binding or tissue elasticity; cell-cell contact can also play a role in cell function, through cell-adhesions and other mechanisms. Of specific interest to this thesis, signaling proteins like cytokines and chemokines are also powerful regulators of cell fate. One such signaling protein, the cytokine transforming growth factor β (TGFβ), plays a role in directing numerous cellular processes, including proliferation, homeostasis, and differentiation. Signaling proteins interact with both cells and the ECM in native tissues, where the ECM can
sequester growth factors via electrostatic affinity interactions, preserving protein bioactivity and stability.  

There is much interest, then, in methods to recapitulate such biological signaling capacity in PEG scaffold platforms, to improve the basic understanding of biological processes, as well as to accelerate and improve healing. Towards that goal, this thesis pursues two general aims: i) the characterization of bioactive protein recovery following exposure to chain- and step-growth photopolymerizations and ii) the use of such reactions to covalently tether bioactive proteins into polymeric biomaterial platforms and study their influence on cell function.

Related to the first of these aims, we investigate the role that radically mediated polymerization reactions play in protein deactivation and loss of function, and characterize methods to ameliorate this damage. These polymerization reactions enjoy wide use as a method for protein encapsulation in polymeric matrices. By forming an encapsulating matrix in-situ, through the inclusion of protein in monomer solutions, the total protein payload can be precisely controlled. One drawback of this approach, however, is the potential for loss of protein function, due to side reactions between monomer species and protein.

We hypothesize that this protein damage can be ameliorated in two ways. First, since affinity ligands bind their target proteins to form a ligand-protein complex, this binding event may shield the protein from some radical-mediated damage during polymerization. Secondly, we systematically study the role that the radical mechanism of polymerization itself can play in loss of protein function, by comparing relative bioactivity changes after exposure to chain- and step-growth reactions. The following specific aims are proposed to test this two-fold hypothesis:
Specific Aim 1: Examine the protective effect of affinity peptides on TGFβ bioactivity during photoencapsulation in poly(ethylene glycol) diacrylate hydrogels.

We investigate the role that affinity peptides can play in the recovery of the cytokine transforming growth factor β (TGFβ) encapsulated in PEG diacrylate (PEGDA) hydrogels. Profiles of TGFβ release are generated from hydrogels composed of various molecular weights of end-functionalized linear PEG acrylate species. The photoinitiated chain-growth reaction used to form these hydrogels can be modeled using solution polymerizations of monofunctionalized PEG acrylate species. Using this approach, TGFβ recovery is characterized and related to polymerization conditions using both ELISA and bioactivity assays. Peptides with reported affinity for TGFβ are synthesized, and their binding affinity confirmed with surface plasmon resonance. Affinity peptides are then included in monomer solutions at varying peptide to protein ratios, and shown to increase the amount of bioactive TGFβ recovered when the cytokine is photoencapsulated in PEG hydrogels.

Specific Aim 2: Characterize and compare the recovery of bioactive protein from photoinitiated chain- and step-growth radical photopolymerization reactions.

Since we hypothesize that protein damage will correlate with radical concentrations during hydrogel evolution and protein entrapment, the effects of the radical polymerization mechanism on protein bioactivity was studied using a model protein, lysozyme. First, photopolymerization conditions for hydrogel formation via acrylate chain-growth and thiol-ene step-growth are characterized. Next, the effect of photogenerated radicals on lysozyme bioactivity is detailed, using the initiator lithium acylphosphinate (LAP). Protein damage is quantified for multiple initiator concentrations and light intensities, and then plotted as a function
of total primary radical generation. Non-crosslinking solution polymerizations are then used to measure the relative bioactivity loss of enzyme exposed to chain- and step-growth radical reactions, and protein damage is correlated to total photogenerated primary radicals. This approach is then used to characterize recovery of bioactive lysozyme and TGFβ following exposure to chain- and step-growth reactions, using polymerization conditions determined for hydrogel formation. Protein loss-of-function is further characterized for the thiol-ene reaction, for various initiator concentrations and light intensities, and protein destruction is correlated to total number of photogenerated primary radicals. Lastly, we demonstrate the translation and utility of this approach by studying the recovery of bioactive TGFβ from cross-linked thiol-ene hydrogels.

Related to the second general aim of this thesis, we sought to create PEG hydrogels capable of presenting sustained protein signaling to cells, through the incorporation of a covalently tethered protein hydrogels. Rather than a vehicle for soluble protein delivery, these platforms present a non-diffusing, conjugated protein molecule, detectable by cells seeded onto the gel surface and/or encapsulated in a three-dimensional culture model. In-vivo, the extracellular matrix functions as a storage depot for a variety of growth factors, which are typically bound via electrostatic interaction with structural ECM proteins. Cytokines typically interact with receptors on a cell’s surface, rather than being internalized, and we hypothesize that covalently tethering proteins into a PEG hydrogel could not only recapitulate a physiologically similar growth factor presentation, but also contribute a sustained, persistent signal to encapsulated cells.
Many recent studies have demonstrated the feasibility of tethering bioactive proteins onto a variety of substrates\(^3\)-\(^5\). The reagent 2-iminothiolane can be used convert a primary amine, such at that present on a protein’s N-terminus, into a thiol. Such thiolated proteins may then be incorporated into PEG based hydrogels, through the use of either a thiol-acrylate or thiol-ene reaction, to form a protein-functionalized biomaterial platform. The resulting material provides not only a physical scaffold, but also one capable of presenting complex biological cues to cells in contact with the hydrogel. Further, the use of covalently tethered proteins allows for experiments to test the effects of presentation of a more persistent growth factor signal. To demonstrate the utility of tethered-protein hydrogel platforms to guide cell function, we propose to study the effect of tethered TGF\(\beta\) on 1) promoting the chondrogenic differentiation of mesenchymal stem cells encapsulated in PEG hydrogels and 2) myofibroblastic activation of valvular interstitial cells seeded on PEG hydrogel substrates. To test these hypotheses, the following specific aims are proposed:

**Specific Aim 3:** Investigate the use of tethered TGF\(\beta\) in promoting chondrogenic differentiation of human mesenchymal stem cells encapsulated in PEG diacrylate hydrogels.

TGF\(\beta\) is a potent cytokine, capable of directing numerous cell functions, including the differentiation of human mesenchymal stem cells (hMSCs) down a chondrogenic pathway. The wide cross-reactivity of TGF\(\beta\), coupled with its short half-live \textit{in-vivo}, makes it an ideal model to demonstrate proof of concept for the utility of a tethered protein hydrogel platform. TGF\(\beta\) is thiolated using 2-iminothiolane, and bioactivity of thiolated TGF\(\beta\) is compared to native TGF\(\beta\), using the PE.25 reporter cell line. Thiolated TGF\(\beta\) is then crosslinked into PEGDA hydrogels
using a thiol-acrylate mixed-mode reaction scheme. The resulting hydrogels are assayed for detectable TGFβ using a modified three-dimensional gel ELISA approach, and bioactivity of tethered TGFβ is confirmed via encapsulation of PE.25 cells. TGFβ presentation is shown to be tunable by varying the concentration of thiolated-TGFβ present in monomer solutions. Finally, hMSCs are encapsulated in tethered TGFβ hydrogels, and we study how such tethered growth factor biomaterials promote the chondrogenic differentiation of hMSCs, as indicated by glycosaminoglycan (GAG) production and collagen type II secretion.

**Specific Aim 4:** Use PEG hydrogel platforms to study biomechanical and biochemical factors contributing to myofibroblast activation of valvular interstitial cells.

Based upon the methods used in specific aim 3, aim 4 focuses on the covalent tethering of bioactive TGFβ into step-growth PEG hydrogel materials. PEG norbornene-co-PEG thiol hydrogels are formed using a photoinitiated step-growth reaction. Gels are synthesized through the solution polymerizations of thiol:ene mixtures; elasticity of the resulting PEG hydrogels is tuned by varying the ratio of ene:thiol in the monomer solutions. After formation, gels are patterned with covalently tethered thiol-TGFβ using a sequential thiol-ene reaction. Bioactive TGFβ surface density is confirmed via a modified surface ELISA and reporter cell assays. Gels are seeded with valvular interstitial cells (VICs); VICs are seeded on low- (< 15 kPa substrate stiffness activation threshold), intermediate, and high (> 15 kPa) elasticity gels, and activation levels are characterized for control gels and those with surfaces patterned with TGFβ. VIC activation is quantified by monitoring alpha smooth muscle actin, a hallmark of the activated myofibroblast phenotype. Finally, TGFβ is photolithographically patterned onto PEG hydrogel
surfaces, which are subsequently used as a culture platform for VICs, demonstrating the utility of this platform for spatial control of cell activation.

Collectively, the objectives of this thesis are constructed to demonstrate the development of synthetic hydrogel platforms as tools for tailoring the cellular presentation of bioactive proteins. This goal is realized through characterization of bioactive protein recovery from polymerization reactions and by covalently functionalizing a hydrogel scaffold with tethered proteins. The resulting material platforms are capable of providing persistent, tailorable protein-mitigated cell signaling in two- and three-dimensional culture.

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Chapter 3

Affinity Peptides Protect Transforming Growth Factor Beta During Encapsulation in Poly(ethylene glycol) Hydrogels

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3.1 Abstract

Transforming growth factor beta (TGFβ1) influences a host of cellular fates, including proliferation, migration, and differentiation. Due to its short half-life and cross reactivity with a variety of cells, clinical application of TGFβ1 may benefit from a localized delivery strategy. Photoencapsulation of proteins in polymeric matrices offers such an opportunity; however, the reactions forming polymer networks often result in lowered protein bioactivity. Here, PEG-based gels formed from the chain polymerization of acrylated monomers were studied as a model system for TGFβ1 delivery. Concentrations of acrylate group ranging from 0 to 50 mM and photopolymerization conditions were systematically altered to study their effects on TGFβ1 bioactivity. In addition, two peptide sequences, WSHW (K_D = 8.20 nM) and KRIWFIPRSSWY (K_D = 10.41 nM), that exhibit binding affinity for TGFβ1 were introduced into the monomer solution prior to encapsulation, to determine if affinity binders would increase the activity and release of the encapsulated growth factor. The addition of affinity peptides enhanced the bioactivity of TGFβ1 *in vitro* from 1.3- to 2.9-fold, compared to hydrogels with no peptide.
Further, increasing the concentration of affinity peptides by a factor of 100 to 10,000 relative to the TGFβ₁ concentration increased fractional recovery of the protein from PEG hydrogels.

3.2 Introduction

Strategies to direct cellular functions in biomaterials via spatial and temporal delivery of proteins, such as growth factors, chemokines, and cytokines, are of growing interest in tissue engineering applications. These biomacromolecules can control cell differentiation, proliferation, migration, and even apoptosis. However, dosing and targeting of proteins to specific cell populations can pose significant challenges. For example, growth factors are eminently potent and can elicit a variety of cellular responses at picomolar concentrations. Further, many factors are cross-reactive across a multitude of cells and tissue types and are known to have short half-lives in vivo. To overcome some of these limitations, a biomaterial delivery platform was explored to facilitate greater control over the bioactivity and availability of growth factors, particularly transforming growth factor beta (TGFβ₁), delivered locally to targeted cell populations or tissues.

TGFβ₁, a member of the TGF superfamily, regulates many cellular process including proliferation, differentiation, chemotaxis, and tumorigenesis. TGFβ₁ is known to play a crucial role in promoting chondrogenic differentiation of human mesenchymal stem cells, guiding the organization of endothelial cells in angiogenesis, and regulating the extracellular matrix production of valvular smooth muscle cells. Since many cells express TGFβ₁ receptors, a local delivery platform is often required for spatial and temporal control over its dosage. One method for controlling the delivery of growth factors is through encapsulation in polymeric matrices, such as poly(ethylene glycol) (PEG). PEG hydrogels have been used to deliver a variety of
growth factors; however, a great challenge facing PEG hydrogels crosslinked by chain growth polymerizations is the potential for irreversible protein damage.\textsuperscript{13}

For many protein delivery applications, direct encapsulation of growth factors (i.e., through the inclusion of the target protein in the monomer precursor solution) is desirable due to its simplicity in preparation and a facile control of the total growth factor payload. Photoinitiated reactions are commonly used in cell encapsulation schemes, due to their mild reaction conditions – specifically physiological pH, temperatures, and osmolarity. While these characteristics render a photoinitiated polymerization system desirable for the formation of cell-laden hydrogels, they are known to create adverse reactions to protein therapeutics, which are usually unstable and can be easily denatured. For example, growth factors present in the formation of hydrogels are susceptible to damage during the reaction, primarily due to the presence of highly reactive radical species\textsuperscript{14} generated by cleavage of photoinitiator species. In addition to initiating polymerization reactions, these free radicals may undergo a number of non-specific side reactions with functional groups associated with amino acids, including phenols, thiols, and disulphides,\textsuperscript{15} leading to either direct conjugation of the growth factor to the polymer backbone or loss of protein conformation, and therefore, bioactivity.\textsuperscript{13} The development of an encapsulation scheme to ameliorate potential radical damage would, therefore, be desirable to enhance the efficacy of polymeric growth factor delivery platforms.

A number of polymeric materials have been utilized as protein delivery vehicles, including alginate\textsuperscript{16}, collagen\textsuperscript{17}, PLGA\textsuperscript{18}, and PEG\textsuperscript{18}. Recent work demonstrates the use of novel polymeric materials incorporating affinity ligands for sustained protein release through mixed-mode, thiol-acrylate polymerizations.\textsuperscript{19-21} These systems utilize ligands that non-covalently and reversibly interact with the target protein, with release being tuned by both diffusion and the
binding kinetics unique to the ligand-protein pair. Specific peptide-ligand systems, where the peptide ligand has affinity for a unique protein, have been shown effective for controlled release of bFGF\textsuperscript{19} and sequestration of MCP-1\textsuperscript{20} and TNFα.\textsuperscript{21} Non-specific ligands such as heparin and alginate sulfate employ electrostatic affinity interactions present on numerous proteins;\textsuperscript{22} such ligands, when added to hydrogel systems have been utilized to govern release of many growth factors, including bFGF,\textsuperscript{23} NGF,\textsuperscript{24} VEGF,\textsuperscript{25,26} PDGF-BB,\textsuperscript{26,27} and TGFβ\textsubscript{1}.\textsuperscript{26-28}

We hypothesized that the presence of free radicals generated during photoinitiated polymerizations would induce TGFβ\textsubscript{1} structural and functional damage, and that the inclusion of affinity binding peptides during photopolymerization could prevent some of this damage. Using photocrosslinked PEG hydrogels as a platform, we systematically studied the influence of photopolymerization conditions on TGFβ\textsubscript{1} bioactivity and availability. Quantification of released TGFβ\textsubscript{1} was determined by ELISA, while confirmation of TGFβ\textsubscript{1} bioactivity was achieved via a TGF-receptor reporter cell line. Further, we analyzed the binding affinity of the TGFβ\textsubscript{1} peptide ligands, Trp-Ser-His-Trp\textsuperscript{29} and Lys-Arg-Ile-Trp-Phe-Ile-Pro-Arg-Ser-Ser-Trp-Tyr,\textsuperscript{30} using surface plasmon resonance studies. These affinity peptides were included in monomer solutions during photoencapsulation of TGFβ\textsubscript{1}, and the enhancement of TGFβ\textsubscript{1} recovery from photopolymerized PEG hydrogels was examined. The dose dependence of the peptide: TGFβ\textsubscript{1} ratio on protein recovery and bioavailability from PEG hydrogels was also studied and quantified for both affinity sequences.
3.3 Materials and Methods

Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise noted.

PEGDA synthesis

Poly(ethylene glycol) diacrylate (PEGDA) monomers were prepared as previously described. Briefly, hydroxyl-terminated poly(ethylene glycol) (M<sub>n</sub> = 4600, 6000, or 10,000 Da) was reacted with acryloyl chloride in the presence of triethylamine under argon for overnight. The product solution was filtered through neutral alumina oxide and stirred for 2 hours in sodium carbonate. After an additional filtering step, excess toluene was removed under reduced pressure and subsequently precipitated into cold ethyl ether. ¹H NMR revealed a degree of acrylation of at least 95% for all material used in this study.

Solid phase peptide synthesis

All peptides were synthesized using a solid phase peptide synthesizer (Applied Biosystems 433A) and standard Fmoc chemistry. Peptide cleavage solution was formed by dissolving 250 mg dithiothreitol and 250 mg phenol in a solution of 95% trifluoroacetic acid (TFA), 2.5% triisopropysilane (TIS), and 2.5% deionized water. Synthesized peptides were cleaved in the solution for 2 hours. Cleaved peptides were precipitated in cold ethyl ether and desiccated overnight, followed by reverse-phase HPLC (Waters Delta Prep 4000) purification. The collected fractions of purified peptides were identified by matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) mass spectrometry.
**TGFβ1 photodestruction studies**

Recombinant human TGFβ1 solutions (Peprotech) (final concentration: 2 nM) were prepared in PBS, in the presence of 1 mM lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) initiator and varying concentrations of poly(ethylene glycol) monoacrylate (Mn = 2000 Da). Solutions were exposed to UV light (Omnicure 365 nm) at an intensity of 10 mW/cm² for 3 minutes. Following UV exposure, TGFβ1 solutions were analyzed with a Human/Mouse TGFβ1 ELISA kit (eBioscience) to determine the recovery of intact TGFβ1.

**TGFβ1 release from PEG hydrogels**

Human TGFβ1 at a final concentration of 25 nM was photo-encapsulated in 10 wt% PEG (Mn = 10,000 Da) hydrogels. For affinity peptide formulations, TGFβ1 binding peptides WSHW or KRIWFIPRSSYW were also incorporated. Peptides were included at a molar ratio, R, relative to TGFβ, equal to 100, 1,000, or 10,000. Growth factor release studies were conducted in release buffer (1 mM EDTA and 0.05% BSA in PBS) in scintillation vials pre-treated with SigmaCote to reduce non-specific protein absorption on the wall of the vials. Supernatants were collected at predetermined time points and replaced with fresh release buffer. Concentrations of released TGFβ1 were determined by ELISA.

**TGFβ1 bioactivity assay**

TGFβ1 bioactivity was confirmed with PE.25 cells stably transfected with a luciferase reporter gene. The assay was performed as reported previously. Briefly, PE.25 cells were plated in 12 well plates (200,000 cells/well) and incubated in serum-free DMEM media.
PEGDA hydrogels (Mₙ = 10,000 Da) encapsulated with 25 nM TGFβ₁ were placed in co-culture with cells for 24 hours at 37° C, and 5% CO₂. Cells were lysed in lysis buffer (Promega) and frozen at -80°C for greater than 2 hours. Lysate was centrifuged at 15,000 RPM at 4°C for 10 minutes and supernatant collected and added to luciferase substrate (Promega). Luminescence was measured using Perkins-Elmer 1420 spectrophotometer.

**Surface plasmon resonance binding studies**

A Biacore 3000 instrument (GE Healthcare) and research grade carboxymethyl-dextran functionalized (CM5) biosensor chips (GE Healthcare) were used for all studies. The flowcell surfaces were equilibrated in HBS-EP running buffer and preconditioned with NaOH, HCl, SDS and H₃PO₄. Flowcell surfaces were activated with a solution of 0.25 M n-hydroxysuccinimide and 0.5 M N-ethyl-N’-(dimethylaminopropyl) carbodiimide hydrochloride, followed by injection of 6.2 mM N-Phenyldiethanolamine in 0.1 M borate buffer. Flowcells were then injected with ligand-functionalized affinity peptides in acetate buffer, and all surfaces were then deactivated with a solution of l-cysteine/NaCl in 100 mM sodium formate buffer.

After allowing the flowcell surfaces to equilibrate with HBS-EP running buffer, solutions of varying concentrations of TGFβ₁, diluted in HBS-EP buffer, were injected using kinetic analysis injection protocols through Biacontrol software. All data was analyzed using Scrubber2 software (BioLogic Software).

**Statistics**

All data are reported as a mean ± s.e.m., based on three repeats per experimental condition, unless otherwise noted.
3.4 Results and Discussion

Effect of network crosslinking density on TGF\(\beta_1\) release

Varying crosslinking density (or mesh size) in hydrogel matrices provides a facile method to control solute diffusivity. As an example, the mesh size of PEG hydrogels can be tailored by varying the molecular weight of PEGDA at a defined weight content. Figure 1 shows fractional release of TGF\(\beta_1\) encapsulated in 10-wt% PEG hydrogels of varying molecular weights. Hydrogels of PEGDA 4,600 Da and 6,000 Da released less than 5% of the encapsulated protein over a two-day period, while 10 wt% PEGDA 10,000 Da gels released approximately 25% of the TGF\(\beta_1\) payload over the same time frame. To determine if the lower TGF\(\beta_1\) release from PEGDA 4,600 Da and 6,000 Da gels was due to lower hydrogel mesh sizes that reduce apparent protein diffusivity, the mesh size of these gels was estimated from equilibrium swelling ratios using a modified Flory-Rehner method\(^3\) (Table 3.1):

Figure 3.1. Fractional release of TGF\(\beta_1\) as a function of time when entrapped in PEG gels formed from the solution polymerization of PEGDA monomers of varying molecular weight. All gels were formed from 10 wt% monomer systems. The final network mesh size affects TGF\(\beta_1\) release. While PEG 4,600 and PEG 6,000 gels had no appreciable TGF\(\beta_1\) release, PEG 10,000 gels had 25% fractional release over 2 days.
Table 3.1. Equilibrium swelling and calculated average mesh size of hydrogels formed from the solution polymerization of poly(ethylene glycol) diacrylate monomers of varying molecular weight.

<table>
<thead>
<tr>
<th>PEGDA Molecular Weight (Da)</th>
<th>Mass Swelling Ratio, q</th>
<th>Volumetric Swelling Ratio, Q</th>
<th>Mesh Size, ( \xi ), (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4,600</td>
<td>17 ± 2</td>
<td>18 ± 2</td>
<td>200 ± 20</td>
</tr>
<tr>
<td>6,000</td>
<td>24 ± 1</td>
<td>25 ± 1</td>
<td>300 ± 20</td>
</tr>
<tr>
<td>10,000</td>
<td>29 ± 2</td>
<td>31 ± 2</td>
<td>380 ± 40</td>
</tr>
</tbody>
</table>

While the hydrodynamic radius of TGF\( \beta_1 \) has not been reported in the literature, proteins of similar molecular weight, including chymotrypsinogen\(^{34}\) and enhanced green fluorescent protein\(^{35}\), have reported radii on the order of 28 – 35 Å. However, since no appreciable amount of TGF\( \beta_1 \) encapsulated in PEGDA 4,600 and 6,000 was released over a two day period, mesh size was not likely the principal determinant of TGF\( \beta_1 \) release in this system. In comparing the three hydrogel formulations used, the photoinitiator concentration and UV exposure conditions were identical, as were the monomer concentrations relative to the TGF\( \beta_1 \) concentration. Due to the use of a constant weight/volume formulation, acrylate concentrations were not held constant (Table 2), and the effect of this factor on TGF\( \beta_1 \) release from the gels warranted further investigation, particularly since the rate of polymerization scales directly with the acrylate concentration to a first approximation.
Table 3.2. Calculated acrylate concentrations in formulations of various molecular weight PEGDAs at 10 wt%, the concentrations used to synthesize the hydrogel formulations.

<table>
<thead>
<tr>
<th>PEGDA Molecular Weight (Da)</th>
<th>Acrylate Concentration at 10 wt% monomer (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4,600</td>
<td>43.5</td>
</tr>
<tr>
<td>6,000</td>
<td>33.3</td>
</tr>
<tr>
<td>10,000</td>
<td>20</td>
</tr>
</tbody>
</table>

*Effect of acrylate concentration on TGFβ1 recovery in solution*

To characterize the effect of acrylate concentration on TGFβ1 recovery during photoinitiated reactions, a PEG monoacrylate (PEGMA) monomer was selected. At low concentrations, similar to those used for hydrogel formation with the diacrylated PEG, monoacrylates do not form a crosslinked polymer when exposed to UV radiation in the presence of photoinitiators. Photoinitiated radicals can propagate through the acrylate group, and polyacrylate kinetic chains are formed via a chain polymerization, but the polymer remains soluble. This aspect makes PEGMA ideal for use in modeling the PEGDA – protein encapsulation reaction scheme\textsuperscript{13,36}. Proteins photoencapsulated in PEGDA may be covalently conjugated to the polymer, resulting in a loss of soluble protein in solution. Alternatively, irradiated solutions of PEGMA and TGFβ1 may lead to radical mediated damage through chain transfer, and the solutions can be subsequently assayed for protein concentration or bioactivity. Reductions in either factor are attributed to the effects of reaction conditions.

Solutions of TGFβ1 and photoinitiator, with varying concentrations of PEGMA, were exposed to UV dosages identical to that used for photoencapsulation with diacrylate PEGDA. The influence of the polymerization conditions on TGFβ1 recovery after UV exposure was tested via ELISA on diluted solution samples, which showed increased growth factor recovery with
increasing acrylate concentration. Maximum post-irradiation recovery of TGFβ1 greater than 90% was found for acrylate concentrations above 40 mM (Figure 3.2A). TGFβ1 recovery data via ELISA (Figure 3.2a) supports results previously published on the so called “protective effect” that increasing monomer concentrations afford proteins\textsuperscript{13}.

Figure 3.2. TGFβ\textsubscript{1} recovery from photopolymerized acrylate solutions. (a) In non-gelling monoacrylate solutions, TGFβ\textsubscript{1} recovery is highest at high acrylate concentrations, as measured by ELISA. (b) Bioactivity assays across the same acrylate concentration range show a maximum at intermediate concentrations, as determined by reporter cell assay. Error bars represent standard error (n=4).

Interestingly, bioactivity after UV exposure was maximized at 20 mM acrylate concentration, with lower activity at both higher and lower acrylate concentrations (Figure 3.2B). While loss of bioactivity of TGFβ\textsubscript{1} at low acrylate concentration is consistent with data from the ELISA assays, reduced bioactivity for samples with high acrylate concentrations is not. One explanation for this observation is potential PEGylation of the growth factor, resulting in a loss of bioactivity and increased hydrophilicity. Direct detection of PEGylated TGFβ\textsubscript{1} presents a challenge for traditional mass spectroscopic techniques, due to the biologically relevant nanomolar concentration range used. This concentration is several orders of magnitude below the limit of detection for HPLC, NMR, GC, and MALDI methods. PEGylated BMP-2 has been
characterized qualitatively using SDS-PAGE\textsuperscript{37}, but this method is limited by low solubility of hydrophobic proteins, such as TGFβ\textsubscript{1}, in SDS solutions. In the referenced work, PEGylation of BMP-2 was confirmed by SDS-PAGE at a protein concentration of 1 mg/mL, several orders of magnitude higher than that utilized for the present work. For solution studies using PEG monoacrylate, any PEGylated growth factor remains in the reaction solution and is potentially detectable by ELISA techniques. However, in diacrylate systems, PEGylated growth factors could be covalently conjugated to the hydrogel polymer. Any conjugation would lead to significant reduction in the total fractional release of soluble, bioactive TGFβ\textsubscript{1} from the polymer. This mechanism may explain, in part, the lower fractional release of TGFβ\textsubscript{1} from PEGDA 4,600 and PEGDA 6,000 hydrogels.

*Surface plasmon resonance studies confirm TGFβ\textsubscript{1}:peptide affinity*

Affinity peptides have previously been used to successfully control the release of encapsulated proteins.\textsuperscript{18} Further, a small soluble affinity ligand has been previously used to protect photoencapsulated bovine serum albumin in PEG hydrogels.\textsuperscript{36} Here, we aimed to test whether inclusion of affinity peptides in monomer solutions could help protect proteins from radical mediated damage and/or conjugation during photoencapsulation reactions. First, surface plasmon resonance (SPR) was used to characterize the binding affinity between each peptide sequence and TGFβ\textsubscript{1}. SPR technology allows precise, label-free measurement of the formation of affinity-binding complexes between two interacting macromolecules\textsuperscript{38} and provides a useful way to analyze the affinity interactions between peptides and TGFβ\textsubscript{1}. Two reported TGFβ\textsubscript{1} binding peptides were synthesized with a terminal cysteine separated from the binding sequence by two glycine spacers (CGGWSHW\textsuperscript{29} and CGGKRIWFIRPSSWY\textsuperscript{30}) then covalently linked to a dextran-functionalized SPR flowcell surface using standard ligand-thiol coupling chemistry.
After equilibrating the chip in HBS-EP running buffer, TGFβ₁ solutions of varying concentration, from 5 nM to 100 nM, were injected across flow cells, and the normalized response, proportional to the amount of peptide: TGFβ₁ complex formed on the chip surface, is reported in Figure 3.3.

Figure 3.3. SPR sensorgrams for TGFβ₁ injection over surfaces functionalized with immobilized peptides, CGGWSHW (a) and (b) CGGKRIWFIPRSSWY. TGFβ₁ was injected at 100, 50, 20, 10, 5, and 0 nM in HBS-EP buffer at a flowrate of 50 µL/min. Sensorgrams represent average signal for three injections.

Both the WSHW (Figure 3.3a) and KRIWFIPRSSWY (Figure 3.3b) functionalized flowcells exhibit the formation of affinity complexes with TGFβ₁, and show binding in a dose-dependent manner, confirming peptide: TGFβ₁ affinity interaction. Analysis of the association and dissociation regimes of the sensogram yielded $k_d$, the peptide:TGFβ₁ dissociation rate constants, and $K_D$, equilibrium dissociation constants as shown in Table 3.3. Each peptide sequence was found to have affinity binding capacity for TGFβ₁ in the nanomolar range, qualifying each as a strong binder; however, the $K_D$ values for the two peptides do not differ significantly.
Table 3.3. Affinity peptide:TGFβ₁ kinetic parameters calculated from SPR analysis.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>$k_d \times 10^4$ (s⁻¹)</th>
<th>$K_D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGGWSHW</td>
<td>9.85</td>
<td>8.20</td>
</tr>
<tr>
<td>CGGKRIWFIPRSSWY</td>
<td>8.22</td>
<td>10.41</td>
</tr>
</tbody>
</table>

Affinity peptides do not inhibit bioavailability of TGFβ₁

After SPR confirmation of peptide TGFβ₁ affinity, an inhibition study was conducted to determine if peptides incubated with TGFβ₁ would interfere with extracellular TGF receptors, preventing growth factor signaling. The WSHW and KRIWFIPRSSWY sequences were originally reported as TGFβ₁ inhibitors, but inhibition was demonstrated through growth factor pull-down studies, where solutions of growth factor are incubated with peptide tethered to a solid phase resin. A bioactivity assay was required to investigate whether soluble peptides, complexed to TGFβ₁ in solution, would interfere with TGF receptor signaling. PE.25 cells, transfected with a luciferase reporter gene for SMAD signaling, were incubated with solutions of WSHW, KRIWFIPRSSWY, and TGFβ₁ (Figure 3.4). At a 10,000 molar excess and a peptide with a nanomolar dissociation constant, over 99% of the growth factor in solution will exist in the peptide:TGFβ₁ affinity complex. Luciferase activity of the cell lysate was insignificant in TGFβ₁- conditions for both peptide solutions and a control, indicating affinity peptides are incapable of binding TGFβ₁ receptors. Luciferase activity of TGFβ₁+ solutions was three orders of magnitude greater than that of respective TGFβ₁- solutions, with no statistical difference between the activity of the peptide solutions and that of the control media. Thus, the peptide:TGFβ₁ affinity complex does not inhibit the growth factor receptor signaling, and TGFβ₁
incubated with peptides in monomer solutions retains its bioactivity in the absence of photoencapsulation.

![Figure 3.4](image)

Figure 3.4. Affinity binding peptides WSHW or KRIWFIPRSSWY do not inhibit TGFβ₁ receptor signaling in media supplemented with TGFβ₁, and peptides do not elicit a response from PE.25 cells in TGFβ₁ deficient media. Error bars represent standard error (n=4).

**Soluble affinity peptides protect TGFβ₁ during UV exposure**

To further explore the effect of soluble peptides on protecting TGFβ₁ during photopolymerization reactions, a monoacrylate solution study was employed, similar to that previously described. Solutions of PEGMA (Mₙ = 2,000), photoinitiator, and TGFβ₁ were exposed to UV radiation, and subsequently assayed via ELISA for TGFβ₁ concentration, as were solutions containing WSHW or KRIWFIPRSSWY (R=1,000) (Figure 3.5). For PEGMA solution exposed to UV radiation in the absence of affinity peptides, TGFβ₁ recovery was 75% of the pre-exposure concentration, while solutions including WSHW or KRIWFIPRSSWY peptides had recovery of approximately 100%, and not significantly different from non-UV exposure condition (t-test, p < 0.05.) These results confirm affinity peptides offer a protective effect for the encapsulated proteins during photopolymerization reactions. Further, the inclusion
of these affinity peptides in monomer solutions should presumably increase the total fractional release of encapsulated growth factor from PEGDA hydrogels, as their presence in acrylate solutions provides an increase in recoverable TGFβ₁.

Figure 3.5. Affinity Peptides Protect TGFβ₁ During Photoencapsulation. Soluble affinity peptides, added to monomer solution of 20 mM PEG (Mₙ = 2,000 Da) monoacrylate and photoinitiator, increase the amount of recovered TGFβ₁ after UV exposure. * indicates p<0.05 (n=4) for each peptide concentration, relative to solution containing no peptide. Error bars represent standard error (n=4).

Affinity peptides increase fractional release of encapsulated TGFβ₁

To characterize the effect of affinity peptides on TGFβ₁ release from PEG hydrogels, the growth factor was encapsulated in monomer solutions of PEGDA Mₙ = 10,000 with or without affinity peptides. Control gels (no peptide) and affinity gels (WSHW or KRIWFIPRSSWY at R=1000), were monitored over a four-day time frame (Figure 3.6a).
Figure 3.6. Affinity peptides increase release of encapsulated TGFβ₁ from PEG (Mₙ = 10,000 Da) hydrogels. (a) Hydrogels encapsulated with TGFβ and affinity peptides (R=1000) show greater release than gels encapsulated with growth factor alone. Error bars represent standard error (n=4). (b) When peptides are encapsulated with TGFβ₁ in hydrogels, a higher cellular response is seen at 24 hours, relative to hydrogels without TGFβ₁ or those with TGFβ₁ and no peptide. * represents p<0.05 (n=4) relative to TGFβ₁ positive gels with no affinity peptides.

Of the three conditions, control gels exhibited the lowest fractional release of growth factor, with only 12.7 ± 1.2% of the TGFβ₁ payload released by day four. Affinity peptide gels released a larger fraction of the encapsulated growth factor; WSHW gels released 30.4 ± 5.3% and KRIWFIPRSSWY gels with 60.3 ± 5.8% of encapsulated TGFβ₁ over the same time interval. Interestingly, the release from KRIWFIPRSSWY peptide gels is much greater than that of gels with WSHW, although the two peptides exhibit similar dissociation constants (Table 3.3). Since the two affinity peptides differ in size, the difference in fractional release between the two affinity systems may be partially attributable to changes in the solubility of the TGFβ₁-peptide complex, relative to unbound growth factor. However, since TGFβ₁ consists of a 112 amino acid chain, and the affinity peptides are four or twelve amino acids, their impact on solubility is expected to be minor. While SPR techniques quantify binding strength between TGFβ₁ and a given peptide, the photoencapsulation reaction is complicated by the inclusion of PEGDA.
monomer and initiator species. To confirm that differences in release between KRIWFIPRSSWY and WSHW gels were not due to bulk material differences, the shear modulus was measured for equilibrium swollen PEGDA gels to elucidate any potential differences in the crosslinking density. Rheometric data showed inclusion of either affinity peptide in monomer solution did not significantly affect the swollen shear modulus, $G'$, of the resulting polymer. Thus, increased TGFβ1 release from peptide gels was not attributed to bulk differences in hydrogel crosslinking density. These results provide confirmation that affinity peptide sequences WSHW and KRIWFIPRSSWY increase the amount TGFβ1 recovered from PEGDA hydrogels.

The presence of soluble peptides (R=1000) in monomer solutions of TGFβ1 and PEGDA $M_n = 10,000$ also increased bioactive growth factor release over a 24 hour period, as seen in Figure 3.6b. PE.25 cells incubated with control gels with no affinity peptide had luciferase activity twice that of negative control conditions (TGFβ1-), while cells co-cultured with gels encapsulated with WSHW (R=1000) had three times higher activity, and KRIWFIPRSSWY gels produced 5 times greater luciferase activity in the reporter cells. This result correlates to the trend in amount of TGFβ1 released (as measured with ELISA) over the same 24-hour time frame in Figure 3.6a. Soluble affinity peptides, when included in monomer solutions prior to polymerization, then are shown to increase the amount of bioactive TGFβ1 released from PEG hydrogels.

Further, fractional release of TGFβ1 was increased when the peptide concentration in the monomer formulation was increased (Figure 3.7). The growth factor was encapsulated at 25 nM in PEDGA $M_n = 10,000$ for all studies, and the relative molar ratio of soluble peptide:TGFβ1 was varied from a low concentration of 2.5 μM (R = 100) to a maximum peptide concentration of 250 μM (R = 10,000). Gels encapsulated with affinity peptides WSHW or KRIWFIPRSSWY
had the highest fractional release over a two-day timespan with a ratio of \( R = 10,000 \), and lower fractional release was observed at lower ratios. At \( R = 10,000 \), the inclusion of the WSHW peptide resulted in 58.8 ± 4.8% recovery, while KRIWFIPRSSWY (\( R=10,000 \)) gel exhibited complete release (115 ± 15.5%) of encapsulated TGF\( \beta_1 \) over a two day timespan. In conjunction with the monoacrylate studies on TGF\( \beta_1 \) recovery, these findings indicate that the presence of affinity peptides can be used to increase the amount of soluble and bioactive TGF\( \beta_1 \) in encapsulated hydrogels, resulting in more predictable delivery and higher total fractional release.

Figure 3.7. Increased concentration of affinity peptides increases release of encapsulated TGF\( \beta_1 \) from PEG (\( M_n = 10,000 \) Da) hydrogels. For both a) WSHW and b) KRIWFIPRSSWY peptides, high concentrations of peptide (\( R=10,000 \)) results in maximum release of encapsulated TGF\( \beta_1 \). For KRIWFIPRSSWY, 100% fractional release is achieved in 1 day at \( R=10,000 \), while \( R=100 \) gels only released 6.4 ± 1% over a 2 day time span. For WSHW gels, \( R=10,000 \) gels achieved 58.8 ± 5% release in 2 days, while \( R=100 \) gels only released 10.6 ± 1% of their payload in the same time. Error bars represent standard error (\( n=4 \)).

3.5 Conclusion

Conditions to maximize release of the human cytokine TGF\( \beta_1 \) from photopolymerized PEG diacrylate hydrogel encapsulation were studied systematically. In solution studies, high acrylate concentration, greater than 20 mM, showed an increase in recoverable TGF\( \beta_1 \), but a lowered bioactivity via cell activity assays. Inclusion of affinity binding peptide sequences in
monomer solutions, prior to photoencapsulation, allowed higher total release of TGFβ₁ from
PEG hydrogels, as well as increased bioactivity of released protein. The inclusion of soluble
peptides provides a facile method for increasing the net recovery of encapsulated TGFβ₁ in
applications demanding localized and sustained delivery, such as tissue regeneration and wound
healing.

3.6 Acknowledgements

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Chapter 4

Thiol-ene photopolymerizations provide a facile method to encapsulate proteins and maintain their bioactivity

published in *Biomacromolecules*

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4.1 Abstract

Photoinitiated polymerization remains a robust method for fabrication of hydrogels, as these reactions allow facile spatial and temporal control of gelation and high compatibility for encapsulation of cells and biologics. The chain-growth reaction of macromolecular monomers, such as acrylated PEG and hyaluronan, is commonly used to form hydrogels, but there is growing interest in step-growth photopolymerizations, such as the thiol-ene “click” reaction, as an alternative. Thiol-ene reactions are not susceptible to oxygen inhibition and rapidly form hydrogels using low initiator concentrations. In this work, we characterize the differences in recovery of bioactive proteins when exposed to similar photoinitiation conditions during thiol-ene versus acrylate polymerizations. Following exposure to chain polymerization of acrylates, lysozyme bioactivity was approximately 50%; after step-growth thiol-ene reaction, lysozyme retained nearly 100% of its pre-reaction activity. Bioactive protein recovery was enhanced 1000 fold in the presence of a thiol-ene reaction, relative to recovery from solutions containing identical primary radical concentrations, but without the thiol-ene components. When the cytokine TGFβ was encapsulated in PEG hydrogels formed via the thiol-ene reaction, full protein bioactivity was preserved.
4.2 Introduction

Poly(ethylene glycol) (PEG) is utilized for a number of biomaterial applications, including anti-thrombotic and anti-fouling surfaces\(^1,2\), implantable medical devices\(^3,4\), drug delivery\(^3,5-7\), and three-dimensional cell scaffolds\(^8-10\). The hydrophilic nature of PEG minimizes non-specific interactions with many biomacromolecules, providing a material platform that is highly resistant to protein adsorption\(^11,12\). PEG is easily modified with functional end groups that can be subsequently crosslinked to form covalently linked networks. There is growing interest in the use of PEG hydrogels formed from such reactions, especially photoinitiated crosslinking reactions, that can be performed in the presence of cells or biomolecules in-situ. In the case of cell encapsulation, a variety of cytocompatible photopolymerization conditions have been identified that proceed at physiological temperature and osmolarity\(^13-16\), but conditions for encapsulation of proteins while maintaining activity are more stringent\(^17,18\).

A common approach to forming PEG hydrogels is the chain polymerization of multi(meth)acrylated PEG monomers. This acryl homo-polymerization proceeds to high conversion in aqueous environments, with rapid gel formation and development of a network structure characteristic of radically mediated chain growth polymerizations\(^19,20\). Photoinitiation is often used to form PEG gels, which allows spatial and temporal control of the polymerization process. Hydrogel formation using photoinitiated polymerization of (meth)acrylated PEG monomers is particularly favorable for the encapsulation of cells, proteins and other biologically relevant molecules, as this approach allows for cytocompatible reaction temperature and facile maintenance of sterile conditions\(^14\). Furthermore, a number of water-soluble photoinitiating species are commercially available, and the reaction exhibits low cytotoxicity at the wavelengths
and light dosages typically required for hydrogel formation\textsuperscript{13, 14}. However, the photoencapsulation of proteins and biologics can be more challenging and appropriate reaction conditions more difficult to identify\textsuperscript{17, 18, 21, 22}.

While robust, the use of a radically-mediated polymerizations poses additional challenges when forming hydrogels via solution polymerization of (meth)acrylated monomers. For instance, radical mediated chain-growth polymerizations are susceptible to oxygen inhibition\textsuperscript{23-25}, which results in longer polymerization times and requires increased irradiation dosing. Further, when used for encapsulation of biomacromolecules, the increased radical generation, lifetime, and exposure time can lead to undesired side effects, namely damage of the encapsulant\textsuperscript{17, 18}. A number of amino acids have reported antioxidant potential, including tyrosine, tryptophan, and cysteine among others\textsuperscript{26, 27}, although cysteine is typically present in an oxidized state in the form of disulphide bridges, which has a lowered antioxidant potential\textsuperscript{28}. Radical transfer from propagating polymeric chains to biomacromolecules can result in changes to protein secondary and tertiary structure\textsuperscript{17}, chain scission\textsuperscript{27, 29}, or protein-polymer conjugation. Several approaches have been shown to ameliorate this protein damage in (meth)acrylate chain-growth reactions. For instance, higher concentrations of acrylate monomer are effective in protecting lysozyme during photoinitiated polymerization\textsuperscript{17}, and peptide affinity ligands included in pre-polymer solutions protect the cytokine TGFβ during encapsulation in PEG diacrylate hydrogels\textsuperscript{18}. While much effort has focused on strategies to minimize damage to encapsulated biologics during photoinitiated radical polymerization of PEGs, we sought to investigate the potential benefits of using different PEG precursors that undergo a radical mediated photopolymerization.
In particular, there is a growing interest in “click” based thiol-ene photopolymerization\textsuperscript{30-33}. The thiol-ene reaction proceeds via a radical mediated mechanism, but by proper choice of the ene functionality, gel formation occurs via a step-growth mechanism. As a result, even with similar photoinitiation conditions, the radical concentrations and lifetimes can be substantially different during the evolution of PEG gels formed via acrylate chain polymerization versus thiol-ene step polymerizations. For example, PEG functionalized with terminal norbornene groups and reacted with bis(thiol) crosslinkers has been successfully copolymerized through photoinitiation to create hydrogel platforms for a number of biomaterials applications, including encapsulation of fibroblasts,\textsuperscript{10} pancreatic beta cells\textsuperscript{34}, human mesenchymal stem cells\textsuperscript{35}, primary valvular interstitial cells\textsuperscript{36} and therapeutic proteins\textsuperscript{37}. The thiol-ene reaction involves two steps: first, an initiator radical is transferred to a thiol, creating a thiyl radical that propagates across a carbon-carbon double bond. Second, the carbon-radical rapidly undergoes chain-transfer to a new thiol, regenerating the thiyl species and allowing for a cycle of coupling reactions that form the macroscopic network (Scheme 1B). Relative to (meth)acrylate chain growth, the thiol-ene reaction is less susceptible to oxygen inhibition\textsuperscript{30}, and differs in both the reactivity of the propagating radical species and radical lifetime. While many measurement of acryl radical concentrations during photopolymerization have been reported\textsuperscript{38-40}, no such measurement has yet been published for thiol-ene polymerizations and it is often implied that part of the reason for this lack of quantification is the very low radical concentrations\textsuperscript{30}. Further, the rapid polymerization of thiol-norbornene crosslinked polymers at physiological conditions makes these monomer systems an excellent choice for many in-situ forming hydrogel applications.

We speculated that the lower radical concentration and rapid polymerization of the thiol-ene step-growth reaction might improve protein bioactivity during encapsulation. In this work,
we systematically compare protein activity during photoinitiated polymerization of PEG precursors utilizing two polymerization schemes: (i) acrylate chain-growth and (ii) thiol-ene step-growth reactions. In both the cases, polymerizations are photoinitiated using a water-soluble initiator, lithium acylphosphinate (LAP), and conducted in the presence of two proteins, lysozyme and TGFβ, to study the protein bioactivity during these radically-mediated photopolymerizations. We investigate loss in protein bioactivity as a result of exposure to photoinitiated radicals, and characterize the differences in bioactivity when acrylates versus thiol-ene functional groups are polymerized, using the same initial functional group concentrations. We show that at high extents of reaction, the thiol-ene step-growth reaction affords significantly higher levels of recovery of bioactive protein relative to that observed following chain-growth acrylate homopolymerization. We correlate loss of protein activity to the concentration of radicals generated, and show that, during a thiol-ene polymerization, protein activity is preserved over a much broader range of photopolymerization conditions.

4.3 Experimental Section

Materials

All chemicals were purchased from Sigma-Aldrich unless noted otherwise.

Synthesis of 4-arm PEG norbornene

4-arm PEG norbornene (PEG-4-NB) was synthesized as detailed elsewhere. Briefly, 5-norbornene 2-carboxylic acid was added at 10x excess (basis: PEG hydroxyl groups) with 5x excess dicyclohexylcarbodiimide in dichloromethane, and the solution stirred for 30 minutes at
room temp. Separately, 4-arm PEG (M<sub>n</sub> 10,000) (JenKem USA) was dissolved in DCM, with 5x pyridine and 0.5x 4-(dimethylamino) pyridine (DMAP), and then added to the DCC/norbornene solution. The reaction mixture was stirred overnight at room temperature under argon. The product was precipitated into ice-cold ethyl ether, and subsequently washed using soxhlet extraction into ethyl ether.

**Synthesis of PEG diacrylate**

Linear PEG diacrylate was synthesized as detailed previously\textsuperscript{41}. Briefly, PEG (M<sub>n</sub> 4,600) was dissolved in toluene and reacted with 4x acryoyl chloride (basis: PEG hydroxyls) in the presence of 4x triethylamine. The mixture was stirred at room temperature overnight under argon. Product was washed in DCM and precipitated in cold ethyl ether.

**In-Situ Dynamic Rheology during Photopolymerization**

Rheometrical measurements were carried out on an Ares TA rheometer using a parallel plate geometry. Hydrogels were formed using 10 wt% solutions of PEG-4-NB (M<sub>n</sub> 10,000) reacted with linear PEG dithiol (Sigma), or PEGDA (M<sub>n</sub> 4,600) at atmospheric (non-purged) conditions. Approximately 30 seconds after beginning measurement, UV light (\(\lambda = 365\text{nm}, I_0 = 10\ \text{mW/cm}^2\)) was introduced to the monomer solutions through a quartz plate, and modulus measurements were recorded in situ at 10% strain, 100 rad/s. These settings were used after confirming that they were within the linear range, using strain sweeps on monomer solutions and the final crosslinked polymer.
Lysozyme-monomer photopolymerization studies

All monomer solutions were prepared with lysozyme (Worthington Biochemical) at a concentration of 1 µM, and photopolymerization was initiated using an Omnicure lamp (λ = 365 nm) under optically thin conditions (100 µL monomer/sample). Non-gelling acrylate polymerizations were conducted using PEG monoacrylate (Mₙ=2,000) (Monomer-Polymer and Dajac Labs) at a concentration of 40 mM in PBS, with 1 mM LAP initiator. Four-arm PEG norbornene (Mₙ=10,000) was reacted at 10 mM (40 mM norbornene) with a stoichiometric cysteine concentration to create a non-gelling thiol-ene monomer system. Thiol-ene polymerization reactions were initiated with 0.1, 1, or 10 mM LAP. Following photopolymerization, protein/polymer solutions were assayed for enzymatic activity as described below.

Lysozyme bioactivity assay

Lysozyme from chicken embryo (Worthington Biochemical) was reconstituted at 50 mg/mL in deionized water, and further diluted to an appropriate working range (150-450 U/mL) in deionized water. The substrate micrococcus lysodeiktus (Worthington Biochemical) was reconstituted in deionized water at 0.6 – 1.0 mg/mL. For measurements of native bioactivity, solutions of lysozyme and substrate were mixed at a 1:1 ratio and changes in absorbance at 450 nm were measured on a Biotek Hybrid H1 spectrophotometer. Changes in absorbance were plotted versus time and correlated to changes in relative bioactivity.

TGFβ bioactivity assay
TGFβ bioactivity was quantified as described elsewhere\textsuperscript{42}, using a mink lung epithelial cell line (PE.25) permanently transfected with a luciferase reporter for SMAD2 gene activity such that the cells produce luciferase upon culture with bioactive TGFβ. Briefly, PE.25 cells were plated in 24-well TCPS plates (100,000 cells/well) in serum-free DMEM and incubated overnight at 37°C, 5% CO\textsubscript{2} prior to culture with monomer solutions.

Non-gelling monomer solutions were formulated using either PEG monoacrylate or PEG 4-norbornene/cysteine (500 µL/sample). Each monomer solution was prepared to yield 40 mM reactive group concentration and TGFβ (Peprotech) at 20 nM. Photopolymerization was initiated using 1 mM LAP at $I_o = 10$ mW/cm\textsuperscript{2} ($\lambda = 365$ nm) in a sterile hood. Prior to and following photopolymerization, 100 µL of the protein/polymer solution was diluted 1:1000 in serum-free DMEM media, and PE.25 cells were cultured in such for 18 hours. Cells were lysed and analyzed using Glo-Lysis reagents (Promega), and luciferase production was quantified using a Biotek Hybrid H1 spectrophotometer.

Encapsulation and recovery of model proteins from crosslinked thiol-ene hydrogels

Monomer solutions were formulated with 1 mM LAP, 4-arm PEG norbornene and linear PEG dithiol ($M_n=1,500$). Lysozyme, chymotrypsinogen (Worthington Biochemical), collagenase 3 (Worthington Biochemical), and bovine serum albumin were encapsulated at 100 µg/gel (gel volume = 50 µL), and human serum was encapsulated at 4% v/v (gel volume of 50 µL). Gels were formed by exposing the solutions to $I_o = 10$ mW/cm\textsuperscript{2} ($\lambda = 365$ nm) for 5 seconds, then immediately placed into 2 mL PBS. After 24 hours incubation at 4°C, the supernatant was assayed for protein concentration using MicroBCA (Pierce) as per the manufacturer’s instructions.
Encapsulation and recovery of bioactive TGFβ from crosslinked thiol-ene hydrogels

A monomer solution of 4-arm PEG norbornene (Mₙ=10,000), linear PEG dithiol (Mₙ=2000), 1 mM LAP, and 20 nM TGFβ was used to form crosslinked PEG hydrogels. 100 µL of this monomer solution was crosslinked by exposure to light (I₀ = 10 mW/cm², λ = 365 nm) for 10 seconds and immediately placed in 10 mL serum-free medium. Alternatively, 100 µL of a monomer solution with 1 mM LAP and 20 nM TGFβ was placed directly into 10 mL serum-free medium (in the absence of polymerization). Both media were incubated overnight at 37°C, 5% CO₂, and then incubated with PE.25 cells for 18 hours under sterile conditions. The cells were lysed and analyzed for luciferase activity as described above.

Statistical analysis

All data were plotted and analyzed using Graphpad Prism 5.0 software. Error bars are plotted as standard error measurement for three replicate conditions, unless otherwise noted.

4.4 Results and Discussion.

Network formation of thiol-ene and acrylate hydrogels

To compare the formation of hydrogel networks prepared from acrylate and thiol-ene reactions on protein activity, some measure of the light dosage needed to completely react the monomer functional groups via the respective mechanisms was required. While direct monitoring of functional group conversion with spectroscopic methods was difficult because of their dilute concentration, we found in-situ rheology under UV exposure to be a highly sensitive method to monitor shear modulus development during photopolymerization. Others⁴³-⁴⁵ have
shown that the plateau in the modulus correlates well with approximate reaction times for complete photopolymerization of hydrogels. Figure 1A shows a plot of shear modulus ($G'$) vs. reaction time for monomer solutions irradiated at $I_o = 10$ mW/cm$^2$ ($\lambda = 365$ nm). Initial functional group concentrations for both acrylate and thiol-ene systems were 40 mM, corresponding to an approximate 10-wt% monomer solution. The initial concentrations were set equal to make comparisons between the two systems, as both the reaction time and protein stability depend on the functional group concentration. Both the polymerizations were photoinitiated with LAP at an initial concentration of 1 mM. As observed in Figure 1a, the step-growth thiol-ene reaction proceeds rapidly, achieving a shear modulus on the order of 10 kPa after less than 10 seconds of light exposure.

Scheme 4.1. Monomer and polymer structure. A) PEG diacrylate, B) PEG 4-arm norbornene, C) PEG dithiol. Upon polymerization, PEG diacrylate forms a chain-growth network as depicted in (D), while the thiol-ene reaction forms a step growth network (E).
Figure 4.1. In-situ rheology during photopolymerization shows evolution of mechanical properties for hydrogels formed via chain-growth acrylate and step-growth thiol-ene polymerizations. A) Hydrogel formation for polymerization initiated at 10 mW/cm² (λ = 365 nm), in the presence of 1 mM LAP. For equal initial functional group concentrations (40 mM), the thiol-ene reaction reached a maximum shear modulus of 10 kPa in less than ten seconds. B) For a constant thiol-ene initial functional group concentration of 40 mM, polymerization was initiated using an intensity of 10 mW/cm² (λ = 365 nm) while the LAP initiator concentration was varied from 0.1 mM to 10 mM. 10 and 1 mM LAP concentrations promoted complete crosslinking in less than ten seconds, but the polymerization was much slower with only 0.1 mM initiator and required ~60 s of light exposure for complete gel formation.

In contrast, the diacrylate chain-growth reaction requires over 300 seconds of light exposure to asymptotically approach a maximum modulus value, although after 180 seconds the shear modulus was within ~95% of the polymer’s final $G'$ of approximately 10 kPa. Further, a significant lag time in elastic modulus evolution was observed (i.e., ~30 seconds), and is likely attributable to oxygen inhibition of the acrylate reaction, which is negligible in thiol-ene reactions\textsuperscript{30, 46}. The need to generate more radicals to overcome inhibition can become problematic for radically sensitive applications like cell or protein encapsulation. This is
noteworthy, as a hydrogel formed via the thiol-ene necessitates shorter polymerization times and therefore, fewer photoinitiated radical species are generated (Table 4.1).

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>[LAP], mM</th>
<th>Time to reach 95% of $G'_{\text{max}}$, (s)†</th>
<th>Total initiator radicals generated, mM‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylate</td>
<td>1</td>
<td>180</td>
<td>1.82</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>60</td>
<td>0.11</td>
</tr>
<tr>
<td>Thiol-ene</td>
<td>1</td>
<td>5</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1</td>
<td>0.27</td>
</tr>
</tbody>
</table>

† as measured using rheology during photopolymerization
‡ as calculated using $R_i = \frac{2f\Phi e_{\lambda} I_o [LAP]}{N_A h \nu I_o [LAP]}$, where $f$ is initiator efficiency, $\Phi$ is the number of radicals formed per photon absorbed, $e_{\lambda}$ is the initiator molar absorbptivity at a given wavelength, $I_o$ is incident light intensity, $N_A$ is Avogadro’s number, $h$ is Planck’s constant, and $\nu$ represents the frequency of initiating light. The photoinitiator concentration [LAP] is represented as a function of exposure time.

Since the thiol-ene reaction is very rapid at typical photoinitiator concentrations used to make PEG-acrylate gels, we next investigated the effect of LAP initiator concentration on the polymerization time required to form PEG hydrogels using the thiol-ene reaction. By varying the LAP concentration used to photoinitiate the reaction at a constant light intensity ($\lambda = 365$ nm, $I_o = 10$ mW/cm$^2$), the total time required for reaching a maximum shear modulus can be tuned (Figure 1B). At both 1 and 10 mM LAP concentration, the thiol-ene hydrogel forms rapidly, and in less than 10 seconds of UV exposure, $G'$ has reached a maximum of approximately 10 kPa. Only at the lowest initiator concentration tested, 0.1 mM LAP, does the thiol-ene polymerization
require significantly longer exposure times of 60 s. Despite this longer exposure time, the 0.1 mM LAP condition still generates a lower total number of radicals than the 1 and 10 mM LAP conditions (Table 4.1). Interestingly, over three orders of magnitude in LAP concentration range, the thiol-ene reaction can be utilized to form hydrogels with lower irradiation doses than that required to form similar PEG diacrylate networks, suggesting the thiol-ene polymerization may be advantageous for encapsulation of proteins or cells with known radical susceptibility.

*Loss of protein bioactivity from initiator radical species*

After determination of the timescale for development of hydrogel networks using acrylate and thiol-ene reactions, it was necessary to determine similar ranges for the timescale and light doses to observe radically-mediated protein damage. Lysozyme, an enzyme that lyses the bacterial cell wall as part of the innate immune system, was used as a model for screening protein bioactivity under various reaction conditions. Native lysozyme bioactivity was measured and subsequently used as a benchmark for relative comparison. Solutions of lysozyme were then prepared including LAP at a concentrations of 0.1 and 1 mM and irradiated with UV light (λ = 365 nm) at two intensities, 1 and 10 mW/cm² respectively, for a total of 60 seconds. Results are shown in Figure 4.2. At the highest light intensity of 10 mW/cm² (Figure 4.2A), protein inactivation was rapid.
Figure 4.2. Protein destruction via photo generated initiator radicals. Solutions of protein and LAP were exposed to light for various times and subsequently assayed for bioactivity relative to native protein.  A) Loss of bioactivity in the presence of 1 or 0.1 mM LAP, exposed to $I_o = 10$ mW/cm$^2$ of 365 nm light for various times; B) Loss of bioactivity for identical exposure times, but at a lower light intensity of 1 mW/cm$^2$.  C) Loss of protein activity data plotted versus total concentration of radicals generated, with a trendline added for visualization.  Loss of protein bioactivity was rapid above a critical radical concentration of ~0.002 mM.

In particular, for the 1 mM LAP condition, 15 seconds of light exposure resulted in complete loss of protein function.  Lowering the LAP concentration to 0.1 mM slowed this protein destruction; after 60 seconds of exposure, approximately 75% of activity was lost.  The total number of radicals generated can be further lowered by reducing the light intensity, as shown in Figure 4.2B.  As expected, when the incident light intensity is reduced to 1 mW/cm$^2$, a LAP concentration of 1 mM results in 75% protein inactivation after 60 seconds of light exposure, since the radicals generated for this condition should be identical to that of $I_o = 10$ mW/cm$^2$ at a LAP concentration of 0.1 mM.  For the mildest condition tested, $I_o = 1$ mW/cm$^2$, with LAP at 0.1 mM, 60 seconds of light dosage resulted in ~ 25% loss of protein function, signifying that at lower radical concentrations, lysozyme exhibits some functional stability.

In order to characterize this protein damage in terms of radicals generated, the four protein activity data sets were plotted as a function of total photoinitiated radicals generated in Figure 4.2C.  The loss of protein activity collapses along a characteristic sigmoidal curve, with a critical threshold of ~0.002 mM radicals.  Below this concentration, there is little to no loss of
lysozyme function. Above this plateau concentration, relative protein bioactivity rapidly declines, and total loss of bioactivity is achieved above a concentration of ~ 0.5 mM radicals generated. This is quite interesting to note, as the concentration of dissolved oxygen in acrylic monomer solutions has reported on the order of 0.5 – 2 mM\textsuperscript{48,49}. One potential cause for this 0.5 mM radical threshold is the formation of reactive oxygen species, effectively consuming primary photogenerated radical species to protein in-situ lysozyme.

*Protein damage in the presence of photoinitiated acrylate and thiol-ene polymerizations*

Next, solution polymerizations were used to study the loss of lysozyme bioactivity when the protein was present in-situ during radically-mediated acrylate and thiol-ene reactions. Model formulations were selected to avoid gel formation and allow for ease of protein recovery. Non-gelling monomer systems were formulated at 40 mM functional group concentration, approximately equal to those used for hydrogel formation (Figure 4.1). The acrylate chain-growth reaction was modeled using PEG-monoacrylate, while the thiol-ene reaction was characterized using 4-arm PEG norbornene in conjunction with cysteine, a mono-functional thiol. Relative protein bioactivity was measured for monomer/protein systems with no UV exposure, and compared to that of a native protein solution (Figure 4.3A.) Both acrylate and thiol-ene monomer solutions, each with a LAP concentration of 1 mM, were then exposed to light ($I_o = 10 \text{ mW/cm}^2, \lambda = 365 \text{ nm,}$.) Exposure times from Table 1 were used, in order to mimic the total number of radical generated during photoinitiation that are required for full network development of the target hydrogel formulations. It should be noted that these solutions were exposed to light in optically thin conditions, and that for 365 nm light at $I_o = 10 \text{ mWcm}^2$, more than 180 seconds are required to completely consume the initial LAP (supplemental
information). Lysozyme exposed to acrylate chain-growth (180 seconds) exhibits a 50% reduction in bioactivity, relative to a non-irradiated monomer solution. This result agrees well with previously published work\textsuperscript{17,18} showing a “functional group protective effect.” Namely, the higher concentration of reactive groups relative to protein concentration, typically a difference of several orders of magnitude, provides limited protection to proteins present \textit{in-situ} during polymerization.

Figure 4.3. Loss of protein bioactivity upon exposure to photoinitiated radical species. Solutions of monomer, protein and LAP (1 mM) were assayed for bioactivity before and after exposure to light at $I_o = 10 \text{ mW/cm}^2$ ($\lambda = 365 \text{ nm}$). Acrylate and thiol-ene monomers, both at 40 mM functional group concentrations, were irradiated based on exposure times required for full shear modulus development. A) For the acrylate reaction (180 s), lysozyme destruction is approximately 50%, while the thiol-ene step-growth reaction (10 s) preserves 100% of protein activity. Results are presented as average activity ± s.e.m. (n=5). B) After exposure to the acrylate chain-growth reaction, TGFβ loses all bioactivity, as measured by a reporter cell assay, while the thiol-ene step-growth reaction preserves 100% of protein activity. Results are presented as average activity ± s.e.m. (n=4).

Interestingly, the thiol-ene reaction significantly increased the recovery of bioactive protein; after 10 seconds of light dosage, the relative lysozyme bioactivity was identical to that of
a solution receiving no light dose. We postulate that this protein protection may be due to two factors. First, the rapid conversion of the thiol-ene reaction allows for shorter light exposure times and a lower total number of radicals generated, as discussed previously. Secondly, protein protection may be afforded due to the reactivity of the propagating radical species itself. In a (meth)acrylate chain-growth reaction, a vinyl carbon radical is propagated, while in the thiol-ene step-growth mechanism, each propagation step results in both consumption and regeneration of a thiyl radical. Our findings suggest that these thiyl radical species may be less destructive to proteins in-situ, or that the thiol-ene reaction is less promiscuous than the (meth)acryl chain-growth mode of polymerization.

To confirm protein protection results with the model protein lysozyme, we devised a study to measure the relative protection afforded by the thiol-ene and acrylate reactions using a more biologically significant protein. The cytokine TGFβ is implicated in a number of cellular processes, and like many signaling proteins, exhibits bioactivity at very low concentrations, on the order of pico- to nanomolar\(^50\). TGFβ was included in acrylate and thiol-ene monomer solutions, at a concentration of 20 nM. As a control, TGFβ/monomers were diluted in culture medium and incubated with a reporter cell line (PE.25) for 18 hours. Monomer/protein solutions were also exposed to light (\(I_0=10\) mW/cm\(^2\), \(\lambda=365\)nm) for times appropriate for gel crosslinking (Table 4.1), and subsequently diluted in culture medium. Following incubation, cells were lysed and the lysate assayed for luciferase activity, a measure of bioactive TGFβ concentration in the medium (Figure 4.3B). Non-irradiated solutions of acrylate and thiol-ene monomers had a similar luciferase activity, indicating that the monomers had no innate effect on the cell reporter assay. Following polymerization, however, relative TGFβ bioactivity was distinctly higher for proteins in the thiol-ene monomer formulations, while TGFβ exposed to the acrylate chain-
growth reaction retained no detectable bioactivity. This finding is in contrast to the results reported in Figure 3, where the acrylate polymerization resulted in only 50% loss of lysozyme activity. The higher damage could be due to differences in protein molecular weight (TGFβ is 25 kDa, lysozyme is 15 kDa), susceptibility of the protein active site to radical damage, or concentration of protein in the photopolymerization. Biologically relevant protein concentrations were chosen for this study, and for both lysozyme and TGFβ. In either case, protein bioactivity was maintained at higher levels following exposure to thiol-ene reaction conditions.

Characterizing protein protection afforded by the thiol-ene system

To further characterize the ability to encapsulate proteins and maintain their activity using radically-mediated thiol-ene polymerizations, we next conducted in-situ protein/polymerization studies with varying concentration of a photoinitiator species, as this approach provides a facile method to study the effect of radical concentration on protein protection during a thiol-ene polymerization. Solutions of protein and monomer were prepared, and the initiator LAP was included in the solutions at three different concentrations: 0.1, 1, and 10 mM. Protein solutions with no photoinitiator, both with and without thiol-ene monomer were also prepared, in order to determine loss of protein bioactivity, if any, due to irradiation alone. All protein solutions were exposed to light (λ = 365 nm, I_o = 10 mW/cm²) for a total of 60 seconds, and subsequently assayed for protein bioactivity. Bioactivity results were normalized to a native protein sample and are presented in Figure 4. Native protein, in the absence of thiol-ene monomer and LAP, maintained ~95% of pre-irradiation activity, a result that indicates light exposure alone has minimal negative effect on the function of lysozyme. When thiol-ene
monomer is added to a protein solution, but no photoinitiator is present, bioactivity is ~100% following light exposure. Radical damage, however, was determined to be the primary mode of protein inactivation, as seen in data for solutions containing LAP. At the lowest initiator concentration tested, 0.1 mM, protein activity was maintained at approximately 100%; there was no significant difference in relative bioactivity between monomer solutions with 0 or 0.1 mM LAP concentration (p < 0.005). At higher concentrations of LAP, however, protein protection provided by the thiol-ene polymerization became limited.

![Figure 4.4](image.png)

Figure 4.4. Protection of *in-situ* protein bioactivity by thiol-ene monomer system. Thiol-ene photopolymerizations were initiated with varying concentrations of LAP, while reactive functional group and protein concentrations were held constant. Solutions were exposed to an identical light dosage ($I_o = 10$ mW/cm$^2$, $\lambda = 365$ nm) for 60 seconds, and subsequently assayed for protein bioactivity, relative to a native protein solution. Results are presented as an average activity ± s.e.m. (n=5).

For protein-monomer solutions formulated with 1 mM photoinitiator, ~75% of pre-irradiation protein activity was maintained after polymerization; when the thiol-ene reaction was
initiated using 10 mM LAP, only 10% of protein activity remained following light exposure. This loss of protein protection by the thiol-ene system was somewhat expected, when considering the 60- second light dosage. For polymerization at \( I_o = 10 \text{ mWcm}^2 \) (\( \lambda = 365 \text{ nm} \)), 60 seconds far exceeds the time required to fully form a crosslinked hydrogel material, as reported in Table 4.1. Based on this data, we hypothesized that the protection of proteins \textit{in-situ} during a thiol-ene polymerization was due, in part, to the presence of unreacted monomer functional groups. Thus, for the lower 0.1 mM LAP concentration, no loss of protein activity was observed over a 60 second exposure time, since this is the timescale over which polymerization occurs (i.e., the shear modulus is fully developed). For the same functional group concentration and light dosage, both 1 and 10 mM LAP concentrations fully form a hydrogel in less than 10 seconds. Irradiation times beyond that necessary to reach complete polymerization would then result in radical generation in the absence of reactive groups, allowing proteins to be the primary target for radicals. In practical terms, this reinforces the importance of limiting overexposure in photocuring applications. Our hypothesis is supported by the data presented in Figure 4.4; however, to more fully characterize the timescale for protein destruction in the presence of a thiol-ene reaction, we designed a study to evaluate the light dosage conditions for \textit{in-situ} protein-polymer reactions and monitor resulting changes in bioactivity.

\textit{Effects of varying light dosage on protein destruction during thiol-ene polymerization}

Solutions were prepared with a constant concentration of thiol-ene functional groups (40 mM) and lysozyme (1 \( \mu \text{M} \)), and these solutions were exposed to light (\( I_o = 10 \text{ mWcm}^2 \), \( \lambda = 365 \text{ nm} \)) for a range of times from 0 to 180 seconds. Following photopolymerization, relative bioactivity of the protein in the reaction mixture was assayed, and reported relative to a native
protein solution. Results are plotted in Figure 4.5A. While the lower LAP concentration of 0.1 mM should exhibit the lowest protein destruction, results were somewhat unexpected. Over a three-minute exposure time, there was no effective change in lysozyme bioactivity, although this time exceeds what is required for complete polymerization and network formation. Likewise, when the thiol-ene polymerization was initiated with 10 mM LAP, solutions maintained high protein bioactivity. After 180 seconds of exposure, protein in the thiol-ene monomer system retains only 30% of pre-irradiation activity. These exposure times are much longer than that required to fully form a crosslinked hydrogel (Table 4.1), and this finding suggests that radical protection is afforded through a mechanism more complicated than that of simple functional group conversion.

Figure 5. Loss in protein activity during photoinitiated radical generation with 0.1 and 10 mM LAP, following exposure to various light doses. Non-gelling thiol-ene photopolymerizations were initiated with either 0.1 or 10 mM LAP, while functional group and protein concentrations were held constant. Solutions were exposed to light ($I_0 = 10$ mW/cm$^2$, $\lambda = 365$ nm) for 0, 2.5, 5, 10, 20, 30, 60, 120, and 180 seconds, and subsequently assayed for protein bioactivity. A) Protein bioactivity after light exposure is plotted for 0.1 and 10 mM initiator as a function of light exposure time. B) Protein bioactivity data is plotted as a function of total radical concentration. The line is included in order to guide the eye. Plateau extends to a radical
concentration of 2.5 mM. Results are plotted as average activity ± s.e.m. (n=4); error bars are smaller than the plotted symbols.

Figure 4.5B shows relative protein activity when exposed to both 0.1 mM and 10 mM LAP photoinitiation conditions, plotted as a function of total radical generation. Results are plotted and fitted with a trendline, similar to the approach in Figure 4.2C with primary radicals. Interestingly, we observe that in the presence of thiol-ene polymerization, protein protection is much higher, as observe by modest losses in protein activity occurring below a critical total generated radical concentration of 2.5 mM. This represents an increase of three orders of magnitude in activity relative to native protein solutions exposed to photoinitiator radicals in the absence of monomers (Figure 4.2C). It is also noteworthy that this critical radical concentration for the onset in loss of protein activity is significantly higher than the radical number required to form hydrogels with 0.1, 1, or 10 mM LAP initiator.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight (kDa)</th>
<th>% Recovery ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>15</td>
<td>80.5 ± 8.1</td>
</tr>
<tr>
<td>Chymotrypsinogen</td>
<td>25</td>
<td>77.9 ± 2.6</td>
</tr>
<tr>
<td>Collagenase 3</td>
<td>60</td>
<td>91.7 ± 14.7</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>66</td>
<td>47.3 ± 4.2</td>
</tr>
<tr>
<td>Human serum</td>
<td>--</td>
<td>79.7 ± 3.9</td>
</tr>
</tbody>
</table>

Table 4.2. Protein recovery from crosslinked thiol-ene hydrogels. Various proteins were encapsulated in thiol-ene hydrogels and release was monitored over a 24-hour period. Results are presented as average ± standard deviation (n=3)
Encapsulation and recovery of bioactive TGFβ from thiol-ene hydrogels. Solutions of monomer, TGFβ and LAP (1 mM) were assayed for bioactivity before and after light dosage ($I_0 = 10$ mW/cm$^2$, $\lambda = 365$ nm, 10 s). Solutions of 4-arm PEG norbornene/PEG dithiol, both at 40 mM functional group concentration, were added directly to culture medium (-UV) or were irradiated for times appropriate to fully form hydrogels (+UV). For crosslinked samples, the resulting polymer was swollen overnight in culture medium and incubated with PE.25 reporter cells. Cell lysate was assayed for luciferase activity to quantify bioactive TGFβ concentration.

Encapsulation and recovery of proteins from crosslinked thiol-ene hydrogels

To demonstrate the utility of the thiol-ene reaction to recover proteins from PEG hydrogels, a number of proteins of various molecular weights were encapsulated in gels formed from 4-arm PEG norbornene and linear PEG dithiol. LAP (1 mM) was used to initiate the photopolymerization ($I_0=10$ mW/cm$^2$, $\lambda=365$ nm) for 5 seconds (i.e., the time required to fully form the gel). Protein-loaded gels were placed in PBS for 24 hours, at which time the protein concentration that diffused into the supernatant was quantified. Recoveries of greater than 80% were measured for all encapsulated proteins, with the exception of bovine serum albumin (BSA). Interestingly, serum albumin has one non-oxidized cysteine residue that results in a free thiol$^51$, which may explain its low recovery. Finally, to access the bioactivity of proteins encapsulated using thiol-ene gel systems, TGFβ was studied. Specifically, TGFβ was included at 20 nM in a
monomer solution of 4-arm PEG norbornene and linear PEG dithiol using photopolymerization conditions that lead to high protein stability (Figure 4.3). Non-photopolymerized monomer was added directly to culture medium. For comparison, the monomer/protein formulation was also photopolymerized \((I_o = 10 \text{ mW/cm}^2, \lambda = 365 \text{ nm})\) for 10 seconds (i.e., the time required to fully form the gel (Table 4.1)), and the resulting hydrogel was added to the culture medium. Both media samples were then incubated with the PE.25 reporter cell line overnight, and cell lysate was assayed for luciferase activity. Results are plotted in Figure 4.6, showing that TGFβ encapsulated via a thiol-ene reaction had nearly identical bioactivity to that of growth factor that was simply in solution, but never exposed to the radical-mediated thiol-ene polymerization.

### 4.5 Conclusions

Hydrogels were formed via photopolymerization using acrylate chain-growth and thiol-ene step growth mechanisms, and the appropriate light doses were confirmed using in-situ rheology under UV exposure. Loss of protein bioactivity following exposure to photogenerated primary radicals was characterized using the enzyme lysozyme. Non-gelling solution polymerizations were then used to study loss of protein function during exposure to acrylate and thiol-ene photopolymerization reactions, using lysozyme and the cytokine TGFβ. While the acrylate reaction provided some marginal protection to \textit{in-situ} protein, there was no loss of protein bioactivity following exposure to the thiol-ene reaction. This may be due to the more rapid kinetics of the thiol-norbornene reaction or oxygen inhibition in the acrylate reaction, which required higher radical concentrations to proceed to completion. When lysozyme, chymotrypsinogen, collagenase, bovine serum albumin, human serum, and TGFβ were encapsulated in crosslinked thiol-ene gels and subsequently released into PBS buffer, greater
than 80% recovery was observed. Finally, TGFβ was encapsulated in PEG hydrogels formed via a thiol-ene reaction, and no statistically significant loss of bioactivity was detected relative to the non-encapsulated growth factor. Photopolymerization reactions that provide rapid gelation at low radical concentrations are highly desirable for applications that seek to encapsulate sensitive payloads, such as proteins or cells. Results of this study indicate that thiol-ene click reactions are capable of proceeding rapidly at low initiator concentrations with little to no impact on \textit{in-situ} protein bioactivity.

\textbf{4.6 Acknowledgements.}

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4.7 References:


Chapter 5

Covalently tethered transforming growth factor beta in PEG hydrogels promotes chondrogenic differentiation of encapsulated human mesenchymal stem cells

published in Drug Delivery and Translational Research

5.1 Abstract

Methods to precisely control growth factor presentation in a local and sustained fashion are of increasing interest for a number of complex tissue engineering applications. The cytokine transforming growth factor beta (TGFβ) plays a key role in promoting the chondrogenic differentiation of human mesenchymal stem cells (hMSCs). Traditional chondrogenic approaches utilize soluble delivery, an approach with limited application for clinical translation. In this work, we introduce a reactive thiol onto TGFβ and covalently tether the growth factor into PEG hydrogels using a photoinitiated thiol-acrylate polymerization mechanism. We demonstrate the bioactivity of thiolated TGFβ, before and after polymerization, using a SMAD2 reporter cell line. hMSCs were encapsulated in PEG hydrogels with and without tethered TGFβ, and subsequently assayed for glycosaminoglycan (GAG) and collagen-II production as indicators of chondrogenesis. Over a 21-day time course, tethered TGFβ promoted chondrogenesis at levels similar to a positive control using solubly dosed growth factor. These results provide evidence that tethered TGFβ materials can be successfully used to promote chondrogenic differentiation of MSCs.
5.2 Introduction

There is growing interest in methods to sequester and present bioactive therapeutic proteins to cells immobilized in three-dimensional matrices, specifically for application in the areas of stem cell culture and regenerative medicine\(^1\). Numerous biological processes are regulated through protein signaling. For example, cytokines and chemokines are a particularly attractive target for tissue engineering applications, since these biomolecules are directly involved in controlling critical cell functions like proliferation, differentiation, and chemotaxis. Typically, these proteins are potent at concentrations as low as nano- to picomolar\(^2-4\), and many of them function via interaction with extracellular surface receptors\(^5\), which then regulate changes in gene expression. Growth factors are commonly dosed solubly via culture media \textit{in vitro}; however, \textit{in vivo}, these proteins are sequestered in the extracellular matrix, and these sequestered factors are then available to nearby cells.

Hydrogel scaffolds are increasingly utilized to reconstruct and study such complex three-dimensional interactions. The cellular microenvironment plays a key role in providing diverse cues that direct cell function \textit{in-vivo}\(^6,7\). The extracellular matrix itself provides not only a niche for cell attachment, but also acts as a storage depot for signaling proteins\(^2,6,7\), and there is growing interest in strategies that recapitulate aspects of these functionalities using synthetic hydrogel scaffolds. Sequestered protein approaches also address practical implications for growth factor delivery. Growth factors are typically cross-reactive with multiple cell types and are reported to have short serum half-lives\(^3\), limitations that often necessitate localized presentation, rather than systemic dosing. Additionally, immobilizing such proteins allows control of total dose delivered, and can increase the safety and persistence of signaling. Indeed,
immobilizing growth factors in a bioactive, physiologically relevant context is an important step towards clinical implementation for regenerative medicine as a whole. Recently, a number of growth factors have been immobilized into synthetic substrates, including VEGF, PDGF, and TGFβ and shown to maintain bioactivity while tethered. Covalent incorporation of such potent cell-directing proteins into hydrogels suggests an attractive approach to design cell delivery scaffolds for locally directing and guiding cell properties important for tissue regeneration.

In this regard, we were particularly interested in modifying PEG hydrogels with proteins, as these systems are broadly explored for cell delivery applications and the low fouling properties of PEG render it useful for examining the effects of particular protein signals on encapsulated cell function. One widely used approach for forming PEG hydrogels is through the photoinitiated chain polymerization of di(meth)acrylated PEG monomers. Photopolymerization allows for precise spatial and temporal control of polymerization and can be carried out at physiological temperature in aqueous conditions. The resulting crosslinked PEG hydrogels have enjoyed wide utilization for encapsulation of numerous cells types with high cell survival reported following photoencapsulation. However, less explored is the mixed-mode thiol-acrylate photopolymerization that provides a facile method for incorporating thiol-functionalized molecules during polymerization of acrylate functional groups. Our group has successfully used this approach to incorporate a number of peptide functionalities into PEG hydrogels, including adhesive motifs, affinity binding ligands, and enzyme-cleavable peptides; however, proteins have been less explored. We hypothesized that a thiolated growth factor, such as TGFβ, could be incorporated into PEG hydrogels via a thiol-acrylate photopolymerization while maintaining its activity and accessibility for cell-binding.
Specifically, we were interested in exploring how this method might be useful for locally promoting the chondrogenesis of human mesenchymal stem cells (hMSCs) in the absence of exogenously delivered TGFβ.

In this work, TGFβ was thiolated and covalently incorporated into PEG diacrylate hydrogels using a photoinitiated thiol-acrylate polymerization scheme. We confirmed that thiolated TGFβ had bioactivity similar to that of the native, unmodified growth factor, using a cell reporter assay for SMAD2 signaling. By varying the concentration of thiolated TGFβ in the monomer solution, the total growth factor concentration in the final gel was readily controlled, as determined by a modified surface ELISA. Bioactivity of the tethered growth factor and the ability for it to signal encapsulated cells was further confirmed using the SMAD2 reporter cell line. Finally, we demonstrate the potential of this material platform by encapsulating hMSCs in order to promote chondrogenic differentiation. MSCs in tethered TGFβ hydrogels produced ECM proteins indicative of chondrogenic differentiation. Namely, MSC cultured in gels with tethered TGFβ produced GAG and collagen-II at levels similar to or exceeding that of positive controls, where TGFβ was dosed solubly in the culture media. We believe these results indicate the clinical potential of tethered growth factor biomaterial platforms as a cell delivery system for tissue engineering applications demanding tunable control of bioactive protein signals in a local and sustained manner.
5.3 Materials and Methods

Cell culture and expansion

Human mesenchymal stem cells (hMSCs) were isolated from bone marrow aspirate (Lonza Biosciences) as detailed elsewhere\(^2^6\). Cells were cultured in growth medium consisting of low glucose DMEM supplemented with 10% fetal bovine serum, 1 µg/mL amphotericin B, 50 U/mL penicillin, 50 µg/mL streptomycin and 20 µg/mL gentamicin. Cultures were maintained at 5% CO\(_2\) and 37°C, and hMSCs were passaged up to three times prior to encapsulation studies.

PE.25 cells were cultured in growth medium identical to that used for hMSCs. For analysis of TGF\(\beta\) bioactivity, plated cells were cultured in serum-free DMEM supplemented with 1 µg/mL amphotericin B, 50 U/mL penicillin, 50 µg/mL streptomycin and 20 µg/mL gentamicin, augmented with either native (non-thiolated) or thiolated TGF\(\beta\) at 5 ng/mL.

Monomer synthesis

Poly(ethylene glycol) (\(M_n = 4,600\)) diacrylate (PEGDA) was synthesized as previously described\(^2^4\). Briefly, poly(ethylene glycol) (PEG) was dissolved in toluene and reacted with acryoyl chloride in the presence of triethylamine overnight under argon. The product was filtered through alumina oxide and neutralized with sodium carbonate. The filtrate was then precipitated in cold ethyl ether. Proton NMR was used to verify yield; typical functionalization for the PEG material used in this study was greater than 95%.
Growth factor incorporation into PEG hydrogels

2-iminothiolane (Pierce) was purchased and used to thiolate TGFβ (isoform 1) (Peprotech) according to the manufacturer’s recommendations. Following the reaction, thiolated-TGFβ was aliquoted until used. Thiolated-TGFβ was then reacted into PEG hydrogels via thiol-acrylate polymerization with the PEGDA monomer at concentrations of 0, 1, 10, or 100 nM. The hydrogels with the tethered growth factor were incubated overnight in PBS, and the TGFβ surface density was measured using a modified ELISA as described elsewhere\textsuperscript{12}.

Briefly, gels were incubated overnight in a blocking solution of 1% BSA in PBS containing 0.05% Tween-20 (PBS-T). Gels were washed 3x in PBS-T prior to incubation with a biotinylated anti-TGFβ antibody (eBioscience) for one hour at room temperature. Gels were washed 3x in PBS-T, and incubated with avidin-HRP (eBioscience) for 30 minutes, and washed 3x in PBS-T. After washing overnight in PBS-T, gels were incubated with 3, 3’, 5, 5’ tetramethylbenzidine substrate until color developed, at which time the reaction was stopped using 2N sulfuric acid. The solutions were then measured for optical density at 450 nm using a Bioteck H1 spectrophotometer.

Bioactivity of thiolated TGFβ

PE.25 cells were plated in 12-well plates at a density of 200,000 cells/well, then incubated with serum-free DMEM media augmented with 1 ng/mL native (non-thiolated) TGFβ (Peprotech) or thiolated TGFβ. Cells were incubated overnight at 37°C, then analyzed using Glo-Lysis components from Promega.

Alternatively, PE.25 cells were encapsulated in PEG gels using a formulation of 10 wt% PEGDA, 1 mM LAP initiator, 1 mM CRGDS peptide and 0, 12.5 nM, 25 nM, or 50 nM
thiolated-TGFβ. PE.25 cells were suspended at densities of 1, 5, 10, and 25 million cells/mL of solution, and cell-laden hydrogels were formed at a volume of 40 µL (O.D. ~ 5 mm, thickness ~ 2 mm) using photoinitiation ($I_0 \sim 3.5$ mW/cm$^2$ at $\lambda = 365$ nm) for 180 seconds. Immediately following encapsulation, hydrogels were placed into growth medium in 48-well plates and incubated overnight at 37°C, 5% CO$_2$. Following incubation, hydrogels were placed in Glo-Lysis buffer (Promega) for not less than 10 minutes and frozen at -70°C for greater than 2 hours. Lysate was incubated with luciferase substrate (Promega) and luminescence was monitored on a Biotek H1 Hybrid spectrophotometer.

**hMSC encapsulation in PEGDA hydrogels**

hMSCs were encapsulated at $10 \times 10^6$ cells/mL in 10-wt% PEGDA ($M_n = 4,600$) solution, with 2 mM CRGDS adhesive peptide and 1 mM LAP photoiniator. Thiolated TGFβ concentrations of 0, 1, 10, or 100 nM were used to form tethered hydrogels. Polymerization was initiated with light ($I_0 \sim 3.5$ mW/cm$^2$, $\lambda = 365$ nm) under sterile conditions to create 40 µL gels (O.D. ~ 5 mm, thickness ~ 2 mm), and cell-laden gels were immediately placed into experimental culture medium. Growth medium (described previously) was used for a negative control, and chondrogenic medium (high glucose DMEM, 1 µg/mL amphotericin B, 50 U/mL penicillin, 50 µg/mL streptomycin, 20 µg/mL gentamicin, 100 nM dexamethasone, ITS+premix (6.25 µg/mL bovine insulin, 6.25 µg/mL transferrin, 6.25 µg/mL selenous acid, 5.33 µg/mL linoleic acid, 1.25 µg/mL bovine serum albumin), 100 µg/mL sodium pyruvate, 50 µg/mL ascorbic acid 2-phosphate) with 5 ng/mL soluble TGFβ was used as a positive control for chondrogenesis. Hydrogels with tethered TGFβ (1, 10 or 100 nM) were cultured in
chondrogenic medium, but in the absence of soluble TGF\(\beta\) added to the media. Samples were harvested at day 0, 7, 14, and 21 for analysis of chondrogenesis.

**DNA quantification of encapsulated hMSCs**

Immediately following photoencapsulation, cell-laden hydrogels (n=3) were placed into an enzymatic digest buffer (125 \(\mu\)g/mL papain (Worthington Biochemical), 10 mM cysteine) overnight at 60°C, then frozen prior to analysis. Samples were similarly harvested at days 7 and 14, and these solutions were then assayed for DNA content using a Picogreen assay (Invitrogen) to quantify changes in cell number between various culture conditions.

**Glycosaminoglycan production assay**

Dimethyl methylene blue (DMMB) assay was used to quantify glycosaminoglycan (GAG) production in hydrogel scaffolds. Cell-laden gels were harvested at pre-determined timepoints (day 0, 7, and 14) and incubated overnight at 60°C in 125 \(\mu\)g/mL papain, 10 mM cysteine. Samples were then incubated with DMMB dye and analyzed for chondroitin sulfate content by measuring absorbance at 525 nm using a Biotek H1 spectrophotometer and comparing to a standard series for quantification.

**Histological Analyses**

Samples were harvested after 20 days of culture and fixed in 4% formalin, 30% sucrose at 4°C for 48 hours. Following fixing, gels were frozen and cryosectioned at 30 \(\mu\)m using a Leica CM1850 Cryostat. Samples were stained for Safranin-O on a Leica Autostainer XL and imaged in bright field (40x) on a Nikon inverted microscope. For immunostaining, sections were
blocked in 10% goat serum with 1% BSA, then incubated with antibodies for collagen type II (abcam) and CD105 (Sigma) for 2 hours. Alexa Fluor conjugated secondary antibodies (Invitrogen) and DAPI were used to fluorescently label proteins of interest. Images were collected on a Zeiss LSM710 scanning confocal microscope. Quantification of collagen-II or CD-105 positive cells was performed on 10x images (3/sample, n=3 samples) by counting the number of cells positive for either collagen-II or CD-105, then normalizing to total cell nuclei using DAPI.

Statistical analysis

All data were plotted and analyzed using Graphpad Prism 5.0 software. Error bars are plotted as the standard deviation for three replicate conditions, unless otherwise noted. Statistical differences were calculated using a students t-test.

5.4 Results and Discussion

Activity of thiolated TGFβ in PEG hydrogels

To introduce a functional group on native TGFβ for subsequent conjugation to PEG, the protein was thiolated via the reaction of 2-iminothiolane with primary amines, such as that found on the N-terminus. During photopolymerization of PEG hydrogels, this thiol can be covalently tethered into the network via chain transfer from a propagating radical on a growing polyacrylate kinetic chain, creating a pendant protein presentation\textsuperscript{28, 29}. For confirmation of TGFβ activity following thiolation, a PE.25 cell assay was utilized. This line has been permanently transfected with a luciferase reporter for SMAD2 activation, a indicator of TGFβ bioactivity\textsuperscript{27}. PE.25 cells
were plated and incubated in medium containing either native (non-thiolated) or thiolated TGFβ at identical concentrations. Following an incubation period of 18 hours, the cells were lysed and assayed for luciferase activity, which represents relative TGFβ bioactivity (Figure 5.1A). Results indicate that thiolation with 2-iminothiolane did not significantly decrease the bioavailability of TGFβ ($p<0.005$).

Thiolated TGFβ was then added to a PEGDA ($M_0 = 4,600$) monomer (10-wt %) solution at 0, 12.5, 25, 50 or 100 nM, and the solutions were irradiated ($I_0 \sim 3.5$ mW/cm$^2$, $\lambda = 365$ nm) for 180 seconds. The resulting hydrogels were then assayed for detectable TGFβ concentration using a modified surface ELISA$^{12}$; results are presented in Figure 1B. The ELISA confirms TGFβ incorporation into the gel, and results show a linear response for TGFβ in the 0-100 nM concentration range. However, as this method only utilizes antibody recognition, further confirmation of bioactivity of the tethered TGFβ in the PEG hydrogel platform was required, using an encapsulation study with the PE.25 reporter cell line.
Figure 5.1. TGFβ can be covalently incorporated into PEG hydrogels. A) Results from a PE.25 cell assay show that thiolated TGFβ provides identical bioactivity to that of native (non-thiolated) growth factor. B) Surface ELISA results show detection of tethered TGFβ in PEG hydrogels, up to a concentration of 100 nM. Monomer solutions containing 0, 12.5, 25, 50, or 100 nM thiolated TGFβ were photopolymerized (I₀ = 3.5 mW/cm², λ = 365 nm) and allowed to equilibrate in 1% BSA. A surface ELISA showed a linear correlation between detectable TGFβ and its concentration in the initial monomer solution, providing a facile method to control the total protein payload. * denotes values statistically non-zero (p < 0.05), and ** denotes statistical differences between concentrations (p < 0.05). Both data sets are presented as mean ± s.d. (n = 5).

**Tethered TGFβ bioactivity in 3D culture**

After confirming the tunability of TGFβ concentration in PEG hydrogels, we next wanted to investigate the bioactivity of TGFβ tethered in a 3D culture system using the thiol-acrylate polymerization reaction. PEG diacrylate monomer (10-wt%) was formulated with varying concentrations of TGFβ: 0, 12.5, 25, and 50 nM. Concurrently, the seeding density of PE.25 cells was varied, with cells encapsulated at 1, 5, 10, or 25 million cells/mL. This design was chosen for a number of reasons. First, the effect of varying concentration of tethered TGFβ on cellular response was characterized, to provide insight into its bioactivity following the
photoinitiated thiol-acrylate reaction used to form these PEG hydrogels. Secondly, this approach allowed us to test the bioavailability of the scaffold-tethered protein to cell binding and ultimately response. While this PEG scaffold is non-degradable, this approach provides confirmation that tethered TGFβ is accessible to encapsulated cells. This observation is similar to the effects of adhesive peptides incorporated into non-degradable gels, where embedded cell can bind with the tethered ligand in the immediate pericellular space. Peptide signals often promote survival, but in a dose dependent manner and often with a threshold. Thus, the amount of bioactive TGFβ assayed should be a function of both TGFβ concentration and cell density. Results are presented in Figure 5.2.

For each tethered TGFβ concentration, an increased luciferase activity was observed over the lowest cell seeding densities of 1, 5, and 10 million cells/mL. For gels formed with 12.5 nM TGFβ, there was no statistically significant increase in luciferase activity when the cell seeding density was further increased from 10 to 25 million cells/mL. This result suggests that for the PEGDA hydrogels, at 10 MM cells/mL, tethered TGFβ is the limiting factor for signaling, possibly due to an excess cell-receptor concentration (i.e., a maximum cell density) or through limitations in receptor access to the tethered growth factor. Increasing the concentration of tethered TGFβ for higher cell densities would be expected to result in increased levels of luciferase activity. This hypothesis was confirmed for hydrogels containing 25 and 50 nM tethered TGFβ. Luciferase activity was 7,800 AU for 25-nM hydrogels encapsulated at 10 MM cells/mL and increased to 12,600 AU at the higher 25 MM cells/mL seeding density. Likewise, 50-nM hydrogels had luciferase activity of 12,100 AU at 10 MM cells/mL, which increased to just over 21,800 AU at the higher density of 25 MM cells/mL. Interestingly, in both the 25 nM and 50 nM TGFβ concentrations, increasing cell density by a factor of 2.5 resulted in a similar
increase (165 - 180%) in bioactive TGFβ. These results with the PE.25 cell line demonstrate the feasibility of presenting tethered TGFβ as a bioactive signal to encapsulated cells, so we next sought to devise a study to test the ability of these materials to promote a more biologically relevant and complex response – chondrogenic differentiation of mesenchymal stem cells (MSCs).

Figure 5.2. Tethered TGFβ hydrogels present a bioactive signal to encapsulated cells. PE.25 cells were encapsulated into hydrogels formed with 12.5, 25, or 50 nM tethered TGFβ. Cells were encapsulated at densities of 1, 5, 10, or 25 million cells/mL. For all TGFβ concentrations tested, increasing the cell density from 1 to 10 MM cells/mL produced increased luciferase activity, a measure of the bioactive TGFβ concentration detected by the cells. Hydrogels with 12.5 nM tethered TGFβ had no statistically significant increase in luciferase activity between 10 and 25 MM cells/mL, indicating that the amount of available tethered growth factor is limiting. At higher TGFβ concentrations (25 and 50 nM), luciferase activity increased with increasing cell number. For a given TGFβ concentration, t-test showed significant difference between all luciferase values except those indicated with *. Results are presented as mean activity ± s.d. (n = 5).
**DNA quantification of hMSCs encapsulated in tethered TGFβ hydrogels**

After confirming that tethered TGFβ maintained its bioactivity following photopolymerization, we then wanted to investigate whether such a platform could promote chondrogenic differentiation of encapsulated hMSCs. Cells were encapsulated at a density of 10 MM cells/mL in 10-wt% PEGDA hydrogels with 1 mM CRGDS peptide to promote integrin binding and survival. Immediately following photopolymerization, gels were assayed for DNA content via a Picogreen assay, and subsequently tested through culture for 14 days. Results are presented in Figure 5.3. Cells cultured in growth medium showed little increase in DNA content over 14 days, agreeing with previous work on hMSC encapsulation\(^{26}\). In contrast, samples in chondrogenic medium exhibited a two-fold increase in DNA content over the same time course. When tethered TGFβ was added to the hydrogel at the lowest concentration of 1 nM, DNA content also remained unchanged and approximated that of the profile of gels cultured in growth medium. At the higher concentrations of 10 and 100 nM TGFβ, DNA content increased and was along the same order of magnitude as chondrogenic culture samples. These finding suggested that in all cases, cell survival following photoencapsulation was robust, a finding confirmed via histological and immunostaining analyses in following sections.
Figure 5.3. Viability of hMSCs encapsulated in tethered TGFβ hydrogels. DNA content of cell-laden hydrogels was assayed over 14 days of culture, and used as a general correlative measure of cell viability. Hydrogels cultured in growth medium (used as a negative control for chondrogenesis) maintained initial cell counts, as did those incorporating 1 nM tethered TGFβ. Chondrogenic media samples (positive control) exhibited an increase in DNA of approximately 2-fold, while TGFβ tethered at 10 or 100 nM demonstrated similar increases in DNA content, suggesting high levels of viability of the encapsulated hMSCs. Results are presented as mean ± s.e.m. (n = 3).

**GAG secretion of encapsulated hMSCs**

Glycosaminoglycan (GAG) production is one key earlier indicator of a chondrocyte-like phenotype. Native cartilage is comprised primary of sulfated GAG chains and type II collagen. A DMMB assay was used to quantify secreted GAGs immobilized in the PEG hydrogel scaffold, as previously described. Results are presented in Figure 5.4A, with GAG secretion normalized to DNA (µg ChSA/ng DNA). For hydrogels cultured in growth medium, there was little to no measurable amount of GAGs. Day 0 content (for all samples) was 14.8 ± 5.6 µg/ng, and day 14 growth samples contained 13.2 ± 1.8 µg/ng. As a positive control, MSC-laden hydrogels cultured in chondrogenic medium had a marked increase in GAG production; at day 14, chondrogenic samples contained 70.2 ± 5.6 µg/ng. Interestingly, tethered TGFβ at 1 nM provided a minimal increase over growth media, as cell-laden hydrogels were
assayed to contain $22.4 \pm 0.4 \, \mu g/ng$ at day 14. However, at the same time point, both 10 nM ($54.9 \pm 1.1 \, \mu g/ng$) and 100 nM ($53.4 \pm 10.7 \, \mu g/ng$) had GAG production similar to that of the positive control, where TGFβ was delivered solubly. At day 14, each tethered TGFβ gel condition resulted in a statistically significant amount of GAG production relative to that of the negative control ($p < 0.01$). Noteworthy, however, is the total amount of growth factor delivered to encapsulated cells in this study. In chondrogenic medium, TGFβ is added to the media at a concentration of 5 ng/mL (0.2 nM), which is a much lower concentration than used in the hydrogels (1 to 100 nM). However, over 22 days of culture with media exchanges every 2 days, the total amount of TGFβ delivered in the soluble form is 55 ng/gel. In contrast, incorporating tethered TGFβ at 100 nM required 100 ng/gel, while the 10 nM gels used only 10 ng growth factor/gel to achieve a similar level of chondrogenesis. Further, tethering growth factors or other proteins into an implantable biomaterial provides a method to signal cells delivered \textit{in-vivo}, and this approach could be readily used to present multiple protein signals to cells, even in various regions of a single material.
Figure 5.4. Glycosaminoglycan production by hMSCs encapsulated in tethered TGFβ hydrogels. A) ChSA production was quantified on a per gel basis via the DMMB assay, and is a measure of cellular GAG production, indicative of chondrogenesis. When TGFβ was tethered at 1 nM, GAG production was limited and similar in magnitude to that of hydrogels cultured in growth medium, which acts as a negative control condition for chondrogenesis. At higher concentrations (10 and 100 nM) of tethered TGFβ, GAG production exceeded that of the positive control (chondrogenic media), where TGFβ was dosed solubly. B-E) Safranin-O staining shows GAG distribution in the pericellular space for chondrogenic media culture (C), as well at 10 (E) and 100 nM (F) tethered TGFβ gels. Gels culture in growth media (B) and those with 1 nM tethered TGFβ (D) had negligible staining for GAGs. Scale bar = 100 µm.

Histological analyses of MSC cultured in tethered TGFβ hydrogels for extended time periods provide a spatial representation of GAG deposition (Figure 5.4B). Hydrogels were harvested at day 21, cryosectioned, and stained with Safranin-O, which stains GAGs red and counterstained with haematoxylin with stains nuclei blue/black. Gels cultured in growth medium
had negligible red color surrounding nuclei, confirming data obtained from the DMMB assay. Likewise, gels incorporating 1 nM tethered TGFβ have a low level of proteoglycan content, observed by faint staining. In contrast, hydrogels cultured in chondrogenic media had significant GAG deposition in the pericellular region and beyond, as did gels formed with either 10 or 100 nM tethered TGFβ. Since this hydrogel platform has no degradable crosslinks, there is limited interstitial space for deposition of secreted ECM and this leads to some of the observed spatial heterogeneity. However, this data agrees with previously published work on GAG deposition for primary chondrocytes encapsulated in similar PEG-based materials\textsuperscript{34, 35}.

**Collagen II production in tethered TGFβ hydrogels**

Collagen type II secretion is another hallmark of chondrogenic differentiation of hMSCs, while expression of the CD-105 cell surface marker is often used as one of the characteristic markers for un-differentiated hMSC\textsuperscript{21, 32, 36}. To further characterize the role that tethered TGFβ plays in promoting later stages of chondrogenesis, cell-laden gels were harvested after 21 days of culture for immunostaining. Samples were stained for type II collagen, CD-105, and DAPI to visualize cell nuclei. Representative images are presented in Figure 5. Growth samples express the CD-105 marker to a greater degree than any samples cultured in chondrogenic conditions (green in Figure 5.5), and only 2.2% ± 0.6% were positive for collagen-II. Interestingly, while 1 nM tethered TGFβ hydrogels did not produce appreciable amounts of GAGs (Figure 5.4), cells encapsulated in this gel formulation were negative for CD-105 staining, while 5.5% ± 0.7% of cells counted were positive for collagen-II, suggesting that the MSCs may be differentiating down other pathways. Cells encapsulated in hydrogels formed with either 10 (84.0% ± 13.9%) or 100 nM (91.0% ± 0.2%) tethered TGFβ were largely positive for collagen-II production (red
in Figure 5.5), similar to levels observed with the positive control (89.3% ± 7.2%), chondrogenic medium with soluble TGFβ.

Figure 5.5. Immunostaining shows collagen II (red) or CD-105 (green) production by hMSCs encapsulated in PEG hydrogels. Nuclei are counterstained with DAPI (blue). Growth samples were positive for CD-105, indicating non-differentiated cells. Gels cultured in chondrogenic media with soluble TGFβ expressed little CD-105, but were positive for collagen-II, a hallmark of chondrogenic differentiation. Cells cultured in hydrogels with tethered TGFβ at 1 nM, 10 nM, or 100 nM were all positive for collagen-II and negative for CD-105. Scale bar = 200 µm.

5.5 Conclusions

Thiolated TGFβ was shown to maintain its bioactivity, and a surface ELISA method was developed to confirm that TGFβ was detectable following covalent incorporation in PEGDA hydrogels. PE.25 cells encapsulated in such tethered TGFβ hydrogels were used to confirm that tethered TGFβ maintained its bioactivity when presented in this fashion. When hMSCs were
encapsulated in tethered TGFβ hydrogels, the tethered growth factor promoted chondrogenic differentiation at levels equal to or exceeding that of positive controls, where TGFβ was dosed solubly via culture medium. Additionally, tethered TGFβ hydrogels utilized a lower total dosage to promote differentiation. Collectively, these results demonstrate the feasibility of delivering bioactive protein signals in a three-dimensional culture platform to control stem cell fate, which may have further implications in the design of delivery vehicles for hMSCs to promote chondrogenesis and cartilage regeneration in vivo.

5.6 Acknowledgements

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5.7 References

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6.1 Abstract

Valvular interstitial cells (VICs) comprise the primary population of cells found in heart valve tissue, and are responsible for tissue homeostasis and wound repair. These cells can be activated into a myofibroblastic phenotype by changes in substrate elasticity or through transforming growth factor β (TGFβ) signaling, both of which can occur in response to injury or pathology. While the role of these factors is commonly studied individually, less is understood about the competing or synergistic role they can play in VIC activation. In this work, we form thiol-ene hydrogels with tunable elasticity (E = 3.5 – 23 kPa) to create activating and non-activating substrates, and covalently tether TGFβ onto these gel surfaces using a secondary thiol-ene photocoupling reaction. TGFβ surface density is controlled by varying the thiolated TGFβ concentration in the patterning solution, across a range from 1 to 100 nM, and the resulting gel surfaces are characterized with both a surface ELISA and a cellular bioactivity assay. VICs cultured on tethered TGFβ surfaces have increased levels of αSMA fiber formation relative to cultures on gels with no TGFβ. Additionally, VICs cultured on tethered TGFβ gels with elastic moduli greater than ~12 kPa formed multicellular nodules, further indicating an increased level
of myofibroblastic cells. These results demonstrate the utility of tethered TGFβ hydrogels to study the interplay between biochemical and biomechanical cues in regulating VIC phenotype.

6.2 Introduction.

Valvular interstitial cells (VICs) are the main population of cells in the aortic heart valve, and are essential in the maintenance and repair of the heart valve leaflets' extracellular matrix (ECM)\textsuperscript{1-6}. Upon valve injury, VICs can undergo transition from a quiescent fibroblast state into an activated, secretory myofibroblastic phenotype, which is able to proliferate\textsuperscript{6-8}, produces increased levels of cytokines, including transforming growth factor β (TGFβ)\textsuperscript{6, 8, 9}, and ECM\textsuperscript{3, 6-8, 10, 11}, and expresses the myofibroblast marker α-smooth muscle actin (αSMA)\textsuperscript{5, 6, 8, 11}. Following repair, this myofibroblast population is believed to then transition back to a quiescent state\textsuperscript{12}. Prolonged activation of these cells can lead to pathological VIC behavior (i.e. osteogenic-like gene and collagen expression) pervading the entire leaflet, which can eventually lead to valve stenosis with subsequent loss of function\textsuperscript{5, 6, 13}.

Multiple factors from the cellular microenvironment can play a role in regulating the activation of VICs. One such factor, the cytokine TGFβ, influences a host of cellular activities, including proliferation\textsuperscript{14-16}, differentiation\textsuperscript{14-16}, immune response\textsuperscript{17}, and ECM deposition\textsuperscript{18}, among others. While healthy heart valves maintain low levels of this protein, in stenotic valve samples, TGFβ is localized at high concentrations in calcific nodules\textsuperscript{19}. Alternatively, biomechanical properties of the valve tissue itself can also act as a trigger for a myofibroblast transition. Native, healthy heart valve tissue has an elastic modulus on the order of \(~7\) kPa\textsuperscript{20}, while stenotic valves are more stiff, with reports of calcified valve leaflets measured with \(E = 32\) kPa\textsuperscript{21}.  

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Hydrogel cell scaffolds are emerging as powerful tools for studying such biochemical and biomechanical cues and their influence on cellular behavior. PEG hydrogels can be synthesized via numerous cytocompatible chemistries to form culture platforms with highly tunable mechanical properties\textsuperscript{22-25}. As an example, our group has recently used these materials to explore the critical role that substrate stiffness plays in controlling the myofibroblast activation of VICs. A threshold of \( \sim 15 \) kPa (E) has been demonstrated for VIC activation to \( \alpha \)SMA fiber positive myofibroblasts\textsuperscript{26}. However, less understood is the competing or cooperative roles that biomechanical and biochemical cues play in VIC activation.

In this work, we utilize a thiol-ene “click” reaction to form PEG hydrogels with tunable elasticity as a platform for studying the complex interplay between biochemical and biomechanical factors influencing the activation of VICs to a myofibroblast phenotype. Here, we use a 4-armed PEG (\( M_n = 10,000 \) Da) norbornene (PEG-4-NB) copolymerized with linear PEG bis(thiol) (\( M_n = 2,000 \) Da) to form a step-growth PEG hydrogel for VIC culture. By varying the stoichiometric ratio of bis(thiol) crosslinker to PEG-4-NB, this platform can be readily tuned to provide gels with a range of crosslinking densities and resulting elasticities. Gels formed with a 0.67 thiol: norbornene ratio provide a soft substrate (\( \sim 3.5 \) kPa) with minimal myofibroblast activation. Increasing this ratio to 0.90 results in a substrate stiffness (\( \sim 23 \) kPa) above the reported 15 kPa activation threshold for VIC activation\textsuperscript{26}, while a thiol: ene ratio of 0.75 produces an intermediate modulus (\( \sim 11.5 \) kPa).

TGF\( \beta \) is thiolated via reaction with 2-iminothiolane and subsequently tethered to hydrogel surfaces using a second photocoupling reaction. The TGF\( \beta \)-modified surfaces are characterized for relative TGF\( \beta \) density using a modified surface ELISA approach\textsuperscript{17}, and bioactivity of the surfaces are confirmed by culture with the PE.25 SMAD2 reporter cell line\textsuperscript{27}. 
When VICs are cultured on tethered TGFβ surfaces, myofibroblast populations are increased in a concentration dependent manner, for soft, intermediate, and stiff substrate elasticity hydrogels. We further demonstrate the utility of this protein tethering approach by photolithographically patterning surface features onto PEG hydrogels, and using these features to investigate modes of calcific nodule formation and multicellular responses on stiff substrates. This platform provides a powerful tool to study, in-vitro, effects of the cellular microenvironment on VIC activation.

6.3 Materials and Methods.

Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise noted.

Monomer synthesis

4-arm PEG-OH (Mₐ=10,000) was purchased from JenKem USA and functionalized with terminal norbornene groups as previously described²⁸. Briefly, 5-norbornene 2-carboxylic acid (5 equivalents: hydroxyls) was dissolved in dichloromethane, to which was added 5x dicyclohexylcarbodiimide. The solution was mixed at room temp for not less than ten minutes. 4-arm PEG-OH was dissolved in DCM with 5x pyridine, 0.5x 4-(dimethylamino)pyridine. The PEG solution was then added to the norbornene/DCC vessel, and the reaction proceeded overnight under argon and mixing. The resulting product was precipitated into ice-cold ethyl ether and washed via soxhlet extraction into ether.
Thiol-ene PEG hydrogel synthesis

Recombinant TGFβ isoform 1 (Peprotech) was used throughout this study. TGFβ was thiolated by reaction with 2-iminothiolane (Pierce) for 1 hour at room temperature, and the product was diluted in 2 mg/mL BSA for storage at -20° C. 4-arm PEG norbornene (PEG-4-NB) was crosslinked with PEG di(thiol), $M_n=2,000$ at 10-wt% PEG-4-NB, and all gels were formulated with 1 mM of the CRGDS adhesion peptide. The photopolymerization was initiated with lithium acylphosphinate (LAP)$^{29}$, present at a concentration of 1 mM, at an intensity of $I_o=10$ mW/cm$^2$ ($\lambda=365$ nm). Hydrogels were formed of varying elasticity by changing the ratio of (thiol: norbornene) in the initial monomer formulation.

PEG hydrogels were functionalized with tethered TGFβ through a secondary thiol-ene photocoupling. All hydrogels were formed with excess norbornene functional group concentration. Prior to tethering, hydrogels were swollen in a solution of 0.05% Irgacure-2959 for not less than 15 minutes. Thiolated TGFβ (0, 5, or 50 nM) was added to the gel surface, and the gel was photopolymerized ($I_o=3.5$ mW/cm$^2$, $\lambda=365$ nm) for approximately 30 seconds. Relative surface density of TGFβ was analyzed via a modified surface ELISA, as reported elsewhere$^{17}$. Bioactivity of the TGFβ functionalized hydrogels was confirmed with the PE.25 cell line$^{27}$. PE.25 cells were seeded onto tethered TGFβ hydrogels in a 24-well plate (100,000 cells/well) and incubated overnight in serum-free Dulbecco’s modified Eagle medium. Cells were incubated for 10 minutes in Glo-lysis buffer (Promega), frozen for not less than 2 hours at -70°C, and incubated with luciferase substrate (Promega). Lysate luciferase activity was measured using a Biotek H1 spectrophotometer.
Valvular interstitial cell isolation

Primary VICs were isolated from aortic leaflets, which were excised from freshly slaughtered porcine hearts (Hormel) within 24 hours. The leaflets were subjected to a sequential collagenase digestion as previously described by Filip et al.\textsuperscript{30}, aliquoted and stored in liquid nitrogen. Briefly, the leaflets were incubated in Earle’s balanced salt solution containing 250 U/mL collagenase for 30 minute to remove the endothelial cells, followed by a 60-minute incubation in fresh collagenase solution and vortexing to remove the VICs. The VIC suspension was filtered using a 100µm strainer to remove the degraded leaflets and centrifuged at 1000rpm for 10 minutes. The VIC pellet was resuspended in Media 199 (Gibco) supplemented with 15 v/v% fetal bovine serum (FBS), 0.4 v/v% amphotecerin, and 2 v/v% penicillin/streptomycin (i.e., growth media), plated on TCPS dishes, and cultured to 70% confluency at 37 °C and 5 % CO\textsubscript{2}. The isolated VICs were then trypsinized, pelleted, and resuspended in 50 v/v% FBS, 45 v/v% M199 media, and 5 v/v% dimethyl-sulfoxide (DMSO) to 1,000,000 cells/mL. This VIC suspension was transferred to cryovials in 1 mL fractions, which were then placed in a slow temperature gradient freezing box at -70°C overnight, then stored in liquid nitrogen until needed for culture.

Valvular interstitial cell culture

PEG hydrogels were prepared as described above, and then sterilized by incubating with 70% ethanol for ten minutes, followed by incubation overnight in sterile PBS augmented with 2 v/v% penicillin/streptomycin, 0.4 v/v% amphotecerin, and 0.4 v/v% gentamicin. VICs were seeded onto PEG hydrogels at 10,000 cells/cm\textsuperscript{2} or 20,000 cells/cm\textsuperscript{2} for activation or nodule formation studies, respectively. The VICs were cultured in 1 v/v% FBS M199 media to prevent
proliferation during the experiment and cultured for 3 or 5 days for activation or nodule studies, respectively. Media was changed every 3 days.

Analysis of VIC activation

After 72 hours culture on PEG hydrogels, cell/hydrogel constructs were fixed in 10 % formalin overnight at 4°C. Samples were washed with 0.05 wt% Tween-20 PBS (PBST) 3 times for 5 minutes each, blocked in 1 % BSA for 1 hour, and then incubated for 1 hour in mouse anti α-smooth muscle actin (αSMA) (abcam) diluted 1:200 in 1 % BSA, both at room temperature. Following washing, samples were incubated in goat anti-mouse Alexa488 (invitrogen) diluted 1:300 in 1% BSA for 1 hour at room temperature. Samples were then incubated for 5 minutes at room temperature with DAPI (invitrogen) diluted 1:1000 in diH2O and washed prior to imaging. Images were collected using a Zeiss LSM model 710 confocal microscope. The percentage of activated VICs was determined by manually counting VICs with fibrous αSMA and normalizing to the number of cell nuclei, using ImageJ software.

Analysis of calcific nodule formation

Calcific nodules were stained by fixing samples in 10% formalin solution overnight at 4°C and incubated in 1% alizarin red S for 2-4 minutes at room temperature. Brightfield images were collected on a Nikon inverted microscope and analyzed using ImageJ software for the number of nodules present.
6.4 Results.

**PEG hydrogel elasticity regulates VIC activation**

Thiol-ene hydrogels were formed with tunable substrate elasticity as seen in Figure 6.1. The four-arm PEG norbornene (PEG-4-NB) was held at a constant concentration (40 mM norbornene) in the initial monomer solutions, while the relative ratio of thiol:ene was varied from 0.5 to 1.0. The lowest thiol:ene ratio of 0.5 formed gels with an equilibrium modulus of 0.4 ± 0.1 kPa (E), while increasing the ratio to 0.67 resulted in a gel of 3.4 ± 0.1 kPa. Increasing the ratio of thiol to ene increased the gel elasticity; at a thiol:ene ratio of 0.75, gels had a compressive modulus of 11.6 ± 0.9 kPa, and the highest ratio (0.90) formed gels with a modulus of 23.1 ± 0.6 kPa. Collectively, these formulations spanned a range of elasticities of interest for VIC cultures\(^1,13,26\).

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Figure 6.1 PEG hydrogel elasticity was tuned by varying the ratio of thiol to ene functional groups in the initial monomer solution. Specifically, PEG-4-NB (M\(_n\)=10,000) was crosslinked with varying concentrations of PEG bis(thiol) (M\(_n\)=2,000), allowed to equilibrate, and characterized using rheometrical analysis. Results are presented as the monomer functional group ratio, and the dashed line demarks the 15 kPa threshold for VIC activation.
VICs were seeded onto thiol-ene hydrogels formed at three thiol:ene ratios: soft (0.67), intermediate (0.75), and stiff (0.90), and cultured for three days in 1% FBS media to minimize proliferation. At day three, cells were fixed and stained for αSMA production, as seen in Figure 6.2. While VICs cultured on soft PEG hydrogels exhibited little αSMA expression (Figure 6.2A), production was increased on intermediate and stiff substrates (Figure 6.2B, C). Quantification of activation level on each substrate was calculated as the number of αSMA positive cells divided by the number of nuclei; results are presented in Figure 6.3. These findings agree with previously published work showing a 15 kPa VIC activation threshold, defined as greater than 50% of cells positive for αSMA\textsuperscript{26}.

Figure 6.2 VIC activation on PEG hydrogels of varying elasticities. αSMA is stained green, while nuclei are blue. A) VICs on soft PEG gels had minimal levels of αSMA fibers, but cells seeded on B) intermediate or C) stiff gels had higher levels of organized αSMA fibers. Scale bar is 100 µm.
Figure 6.3 VIC activation as a function of thiol:ene ratio. VICs were seeded on thiol:ene hydrogels formed at 0.67, 0.75, or 0.90 thiol:ene ratio. At the lowest elasticity (0.67 thiol:ene), 36% of the cells were positive for αSMA fibers, an indicator of activation, while on intermediate substrates (0.75), 50% of the VICs were activated. At the stiffest composition of 0.90 thiol:ene ratio, 56% of the cells were positive for αSMA fibers.

**Characterization of bioactive tethered TGFβ hydrogels**

Next, thiol-ene PEG hydrogels were formed with surface-tethered TGFβ by using a secondary thiol-ene photocoupling reaction. Preformed thiol-ene gels were incubated with thiolated TGFβ (0, 12.5, 25, 50, or 100 nM) and exposed to light (I_o=3.5 mW/cm^2, λ = 365 nm) for 30 seconds, then swollen to remove unconjugated growth factor. TGFβ surface density was then characterized using a surface ELISA (Figure 6.4A), as well as by seeding gels with the PE.25 TGFβ reporter cell line (Figure 6.4B).
Figure 6.4 PEG hydrogels formed with tunable concentrations of bioactive tethered TGFβ. Thiolated TGFβ was tethered to PEG hydrogels formed with excess norbornene concentration using a photoinitiated thiol-ene reaction. TGFβ surface density was tuned by changing the concentration of thiolated TGFβ in the patterning solution. A) Surface ELISA results confirm a tunable detectable TGFβ surface, while B) bioactivity was confirmed by seeding the gels with the PE.25 reporter cell line. In each plot, error bars represent s.e.m. (n=4). Gels were formed at 0.9 thiol:ene ratio and equilibrated in 0.05 wt% I-2959 initiator prior to photocoupling thiolated TGFβ (I₀ = 3.5 mW/cm², λ=365 nm, t = 15 sec).

Results demonstrate that the photocoupling approach is viable for forming gel surfaces with tunable concentrations of TGFβ, as characterized via antibody detection and that these gel surfaces present a bioactive signal to cells.

**VIC activation on tethered TGFβ surfaces**

Thiol-ene PEG hydrogels were formed using the 0.67, 0.75, or 0.9 thiol:ene ratios as previously characterized with respect to levels of VIC activation, and then photopatterned with either 5 or 50 nM thiolated TGFβ. The gels were swollen to remove any non-coupled growth factor, then seeded with VICs and cultured for 3 days. Following culture, cells were fixed and stained for αSMA production. Results are included in Figure 6.5. The softest gels (lowest elasticity) had few cells that were positive for αSMA fibers, regardless of the TGFβ concentration (Figure 6.5 A, D). In contrast, higher stiffness substrates exhibited high levels of
activated cells, and this trend was only increased in the presence of the TGFβ signal (B, C, E, and F in Figure 6.5). In addition, at both 5 and 50 nM tethered TGFβ, multicellular nodule-like formations were also observed, as represented in Figure 6.5. Previous studies have shown the importance of persistent αSMA expression as a precursor to the nucleation of nodules\textsuperscript{1,31}.

Figure 6.5. VIC activation on tethered-TGFβ hydrogel surfaces. Thiol-ene hydrogels of varying elasticity were photopatterned with thiolated TGFβ, at either 5 nM (A, B, and C) or 50 nM (D, E, and F), then seeded with VICs and cultured for three days. Gels were formed at soft (A,D), intermediate (B, E), or stiff (C, F) elasticity. Soft gels had the least level of organized αSMA staining, regardless of TGFβ surface density. Intermediate and stiff gels exhibited much higher levels of αSMA, relative to hydrogels with no surface conjugated TGFβ (Figure 6.2). Tethered TGFβ gels at intermediate and stiff elasticity also exhibited multi-cellular nodules, as seen in panels B, C, E, and F. Scale bar is 100 µm.
6.5 Discussion.

VIC activation on PEG hydrogels

As substrate elasticity is one factor that contributes to the activation of VICs, it was important to characterize the role of monomer choice and functionality on equilibrium modulus for these photopolymerized thiol-ene PEG hydrogels. Varying the ratio of functional groups from 0.5 to 0.9 (thiol:ene) resulted in hydrogels with elasticity ranging from ~0.5 to 23 kPa (E). Since previous studies have reported a threshold modulus of 15 kPa for VIC activation\textsuperscript{26}, this approach confirmed that such a thiol-ene hydrogel platform could be used to synthesize materials with a range of elasticities of interest for VIC activation. In addition, each gel formed contained an excess ene concentration, providing a reactive handle for subsequent patterning of thiol-containing moieties, and particularly thiolated proteins, of interest.

When VICs were seeded on soft gels (0.67 thiol:ene, E = 3.4 kPa), the cells expressed low levels of organized $\alpha$SMA, indicating minimal activation levels as expected for this soft substrate. Increasing the ratio of thiol:ene to 0.75 produced an intermediate stiffness material (E=11.6 kPa), and VICs cultured on intermediate gels exhibited a higher level of fibrous $\alpha$SMA. The stiffest hydrogel substrate was formed at a 0.9 thiol:ene ratio (E = 23 kPa), and VICs on this surface expressed the highest levels of $\alpha$SMA fibers, as seen in Figure 6.2. VIC activation was quantified by counting the number of cells that were positive for $\alpha$SMA fibers, and normalizing to the number of DAPI-stained nuclei. Results (Figure 3) confirm the trend of increasing VIC activation as a function of increased hydrogel elasticity and support previously published work with other gel systems\textsuperscript{1,26}.
**TGFβ-modified hydrogel surfaces**

Hydrogels were formed with an excess concentration of ene relative to the total thiol concentration, in order to form hydrogels with pendant norbornene functional groups. This approach provided a simple method to subsequently modify the PEG gel chemistry using the photoinitiated thiol-ene reaction to conjugate thiolated biological signals. Here, TGFβ was thiolated via reaction with 2-iminothiolane, and this thiolated TGFβ was subsequently photoconjugated onto PEG hydrogel surfaces using this approach. Because the reaction is photochemically controlled, the reaction rate can be controlled by the light dose, and the reaction can be confined to specific spatial regions, allowing photopatterning. In this contribution, TGFβ surface density on the gels was tuned by changing the concentration of thiolated TGFβ in the patterning solution, as characterized by a surface ELISA (Figure 6.4A). In order to more fully characterize the activity of the tethered TGFβ surfaces, gels were seeded with PE.25 cells, an epithelial line permanently transfected with a luciferase reporter gene for SMAD2 activity. PE.25 cell culture confirmed the bioavailability of TGFβ tethered onto thiol-ene hydrogel surfaces, with a similar dose response to that observed via our surface ELISA.

**VIC activation on photopatterned TGFβ hydrogels**

VICs were seeded on thiol-ene hydrogels formed with 0.67, 0.75, or 0.90 monomer thiol-ene ratio, as described previously. Following gelation, the hydrogels were surface modified with thiolated TGFβ (5 or 50 nM) using a secondary thiol-ene reaction to create surfaces with varying densities of this cytokine. VICs were seeded onto the tethered TGFβ hydrogels and cultured for three days, and then fixed and stained for αSMA. Cells on the lowest elasticity (E ~ 3.5 kPa) had low levels of αSMA-positive cells, similar to that on soft gels with no tethered TGFβ. This
result was quite interesting as TGFβ is a potent activator of myofibroblasts, including VICs, and thus, alludes to the influence of the context of the microenvironment in determining the response of VICs to TGFβ. Interestingly, TGFβ tethered to intermediate (~12 kPa) or stiff (~23 kPa) hydrogels promoted VIC activation at levels higher than that of non-patterned substrates. Additionally, for both tethered TGFβ concentrations tested, VICs seeded on intermediate and stiff surfaces also formed multicellular nodules, another indicator of a myofibroblast-like phenotype. Benton et al. showed that αSMA production is necessary for VICs to form calcific nodules, and that TGFβ treatment promoted nodule formation on TCPS surfaces with very high (on the order of gPa) elasticity.

Our observation of nodule formation on softer substrates suggests the importance of the context of TGFβ dosing, and we plan to expand the current body of work by utilizing a spatial patterning approach for tethering TGFβ. We have synthesized a thiolated-fluorophor tracer molecule that can be mixed with thiolated TGFβ solutions prior to photocoupling, in order to visualize areas of a gel surface that have been exposed to a photopolymerization. We plan to use a photolithographic approach to pattern circular “islands” of tethered TGFβ with various diameters ranging from 50 to 500 µm. VICs will be seeded onto these spatially patterned gel surfaces, and cultured for 4 days in order to allow nodule formation, following the protocol of Benton et al. This approach should provide insight into the role of exogenous TGFβ delivery (i.e. surface tethered) versus paracrine signaling that may occur as cells on the tethered TGFβ islands in turn upregulate TGFβ secretion after activation.

Alternatively, this spatial patterning technique may also be used to investigate competing protein signals. Cushing et al. detailed the role that basic fibroblast growth factor (bFGF) could play in downregulating VIC activation. Using thiolated bFGF in conjunction with tethered
TGFβ would provide more insight into the competing roles of these two growth factors, and how interplay between the two, both of which are commonly ECM-bound in heart valves and other tissues.

6.6 Conclusions.

Thiol-ene hydrogels were formed using 4-armed PEG norbornene crosslinked with PEG bis(thiol) while varying the ratio of thiol:ene functional groups to provide facile control of the final equilibrium substrate elasticity. VICs cultured for three days on hydrogels formed at 0.67 thiol:ene ratio (soft) gels exhibited low activation levels, as characterized by αSMA fiber formation, while greater than 50% of cells on gels formed at 0.75 (intermediate) or 0.90 (stiff) thiol:ene ratios were activated. TGFβ was thiolated and tethered onto surfaces of thiol:ene hydrogels, utilizing a secondary thiol-ene photocoupling reaction to create tunable surface densities of bioactive proteins. When VICs were seeded on gels patterned with 5 or 50 nM tethered TGFβ, there was a minimal increase in activation for cells cultured on soft gels. VICs on tethered TGFβ gels formed at intermediate or stiff substrate elasticity, in contrast, had higher levels of activated cells and formed multicellular nodules indicative of a population of myofibroblast-like cells. These results suggest that VIC activation depends strongly on microenvironmental context and its response to TGFβ signaling depends on the local mechanical environment; thus, thiol-ene hydrogels might be used as a robust platform to investigate the cross-talk between these two important factors.
6.7 References.


Chapter 7

Conclusions and Recommendations

The cellular microenvironment plays a crucial role in governing cellular behavior and function. A more complete understanding of this microenvironment is needed in order to design tissue-engineered solutions for a host of applications in regenerative medicine. However, decoupling individual contributions of factors like tissue elasticity or soluble protein concentration presents a challenge in vivo, due to the complexity of signals presented to cells in the native extracellular matrix. One powerful method for studying how cells receive cues from their microenvironment is through encapsulation in a synthetic matrix that can be tuned to incorporate one or more of these biological cues.

In chapter 3, methods to photoencapsulate the cytokine transforming growth factor beta (TGFβ) in PEG diacrylate hydrogels were developed. TGFβ was encapsulated in 10-wt% PEGDA hydrogels, using various molecular weights of PEG as the crosslinking molecule. While each formulation resulted in hydrogels with mesh sizes adequate for diffusion of TGFβ, only the highest molecular weight PEG (M_n=10,000) released an appreciable amount of the encapsulated protein, suggesting inadvertent protein-polymer conjugation. As the acrylate concentration was different for each hydrogel tested, TGFβ recovery from non-gelling photoinitiated monoacrylate reactions was characterized over a wide range of [acrylate]. While high monomer PEG-acrylate concentration increased the amount of TGFβ recovered, as detected via ELISA, bioactivity was maximized at intermediate PEG-acrylate concentrations.
Collectively, these results suggested PEGylation of the protein, thereby influencing its solubility, diffusivity, and activity during encapsulation in PEG hydrogels. To circumvent some of these issues, the protective effects of affinity peptides were next investigated. Affinity peptides WSHW and KRIWFIPRSSWY were tested via SPR techniques to confirm binding affinity for TGFβ. When incubated with affinity peptides, no decrease in TGFβ bioavailability was detected. When affinity peptides were included in PEGDA monomer solutions, increased amounts of bioactive, TGFβ were detected, via ELISA and cellular bioactivity assays. Further, affinity peptides increased the amount of TGFβ release and recovery in a dose-dependent manner.

Having identified photopolymerization conditions that allow the release of soluble, bioactive TGFβ from PEGDA hydrogels, this platform might be next utilized as a controlled-release material. Affinity peptides, synthesized with an appropriate spacer and terminated with cysteine residues, could be easily incorporated into PEG hydrogels using a thiol-acrylate polymerization. Additionally, our finding that affinity peptides helped protect TGFβ during photopolymerization could be enhanced by further analysis of the mode of protein inactivation. Gel electrophoresis or MALDI mass spectrometry might be used to confirm that TGFβ is PEGylated during acryl chain-growth reactions, and the inhibition of such PEGylation by affinity peptides could then be shown directly. Further, knowledge of the specific binding site for each peptide sequence could be obtained, through an ELISA where the affinity peptides were used for sequestration, in place of antibodies. If each peptide interacts with a unique binding site, synergy between the two sequences is expected, and could enhance tunability of protein-peptide interactions for controlled release applications.
In Chapter 4, protein inactivation following exposure to photoinitiated step- and chain-growth reactions was characterized and compared. In-situ photorheometry was used to quantify the light dosages necessary to form hydrogels via acryl chain-growth and thiol-ene step growth mechanisms, using the photoinitiator LAP. When solutions of lysozyme and LAP were exposed to similar ranges of light doses, radically mediated loss of protein function was rapid. Next, model systems based on non-gelling monomer systems (PEG monoacrylate and PEG-4-norbornene/cysteine) were photopolymerized with lysozyme and TGFβ, at light doses required to fully form hydrogels. The acrylate chain-growth polymerization mitigated some measure of lysozyme destruction, as 50% of pre-polymerized activity was retained, but TGFβ was completely inactivated. In contrast, 100% bioactivity was maintained following thiol-ene polymerization, for both lysozyme and TGFβ. To more fully understand the thiol-ene protective effect, solution polymerizations of that monomer system were performed at multiple light intensities and LAP concentrations. For a constant exposure time, only the highest LAP concentration tested (10 mM) exhibited significant loss of lysozyme activity. Lysozyme destruction in the presence of thiol-ene monomer was then tested for two LAP concentrations, 0.1 and 10 mM, \((I_o=10 \text{ mW/cm}^2)\) where exposure time was varied from 5 to 120 seconds. When protein bioactivity was plotted versus total photogenerated radicals, protein activity was
maintained up to a much higher radical concentration than in solutions with no monomer present.

Figure 7.1. Data adapted from chapter 4 showing protein destruction as a function of photogenerated radicals, plotted over identical radical concentration ranges. A) lysozyme and LAP, and B) lysozyme, LAP, and thiol-ene monomers.

These results confirmed that the rapid polymerization kinetics of the thiol-ene reaction provide a facile method for gel formation with minimal impact on the bioactivity of an encapsulated protein payload. We theorize that this may be due, in part, to the fact that thiol-ene reactions are not oxygen inhibited, in contrast to the acrylate chain-growth reaction. This means thiol-ene polymerizations require lower light dosage and total radicals generated for hydrogel formation. Other possibilities for this thiol-ene protection include differences in the radical lifetime for the species involved, or the nature of the radical species itself (i.e. a propagating acryl radical in chain-growth vs thyl or norbornene radicals in the step-growth reaction). Although we demonstrated protein protection with lysozyme and TGFβ in this thesis, these findings would be enhanced by a more precise understanding of the mode of protein radical protection, in order to make this knowledge more universally applicable.

With knowledge of how to maintain protein bioactivity during in-situ polymerization, via both chain- and step-growth mechanisms, we then applied this approach to form hydrogels that incorporated covalently tethered bioactive proteins. Transforming growth factor beta, a cytokine
known to induce numerous cellular processes, was thiolated to provide a facile handle for incorporation into PEG hydrogels. By utilizing either thiol-acrylate or thiol-ene reactions, thiolated TGFβ was covalently tethered into hydrogels. As a first demonstration, we selected the PEG diacrylate platform as it has been successfully used to promote chondrogenic differentiation of human mesenchymal stem cells (hMSCs), a process in which TGFβ plays a role.

In Chapter 5, tethered TGFβ was utilized to promote chondrogenic differentiation of encapsulated hMSCs. TGFβ was thiolated with 2-iminothiolane and shown to retain bioactivity, as compared to native growth factor. Further, the [TGFβ] in PEGDA hydrogels was easily tuned by varying the monomer concentration of thiolated protein. When the PE.25 reporter cell line was encapsulated in tethered TGFβ hydrogels, bioactivity of the protein and its ability to signal encapsulated cells were confirmed in this 3D culture system. hMSCs were then encapsulated in such tethered growth factor hydrogels to characterize the potential of the functionalized material to promote chondrogenic differentiation. Culture in growth media served as a negative control, and chondrogenic media augmented with soluble TGFβ was used as a positive control for chondrogenesis. Over a 21-day culture, cells cultured in gels with either 10 or 100 nM tethered TGFβ were positive for chondrogenesis markers, including GAG deposition and collagen-II secretion. Cells in gels with 1 nM tethered TGFβ were positive for collagen-II production, but produced GAGs at low levels similar to production of the negative control. Soluble growth factor delivery via media exchange is limited in practical application, while this tethered growth factor approach offers promise for materials-based hMSC delivery and cartilage regeneration therapies.
Figure 7.2. Persistence of TGFβ signaling in 2- and 3D systems. PE.25 cells were plated (A) or encapsulated in PEGDA hydrogels (B) with native and thiolated TGFβ. Luciferase activity was normalized to day 0 signal, and each day was normalized to double-stranded DNA content.

As proteins are covalently linked into the 3D encapsulating hydrogel in this model, TGFβ cannot be endocytosed, as would occur in soluble culture experiments. Thus, this tethered protein platform might be used as a tool to study persistent growth factor presentation and protein signaling dynamics in a novel 3D presentation. This work could also be complemented by characterization of the persistence of a tethered protein on cell signaling, both in-vitro and in-vivo. If the thioester bond that links thiolated proteins to the acryl-PEG hydrogel was suitably stable, i.e. for a time period of weeks or months, this approach could have application as a method for cell recruitment and differentiation. For instance, if a cell-invasive tethered TGFβ hydrogel could be implanted into a cartilage defect, the stem cells released by microfracture surgery might be retained and differentiated in the area of need, improving patient outcome.

TGFβ also plays a role in numerous other cellular functions, including chemotaxis, apoptosis, and immunological responses including fibrosis and dendritic cell activation. TGFβ can also induce the activation of valvular interstitial cells, promoting a fibroblast to myofibroblast transition. This myofibroblast phenotype is indicative of a diseased state, with
increased production of α-smooth muscle actin fibrils and calcific nodule formation in-vivo. These activated VICs then play a role in upregulation of ECM implicated in causing valve stiffening, ultimately leading to loss of function.

In Chapter 6, thiol-ene hydrogels were used to probe the role that cellular microenvironmental factors play in promoting the fibroblast to myofibroblast transition. A hydrogel platform with 1) tunable mechanical stiffness and 2) unreacted norbornene functionality were synthesized and characterized. By controlling the relative ratio of norbornene: thiol (ene:thiol) in the initial monomer formulation, the resulting hydrogel elasticity was tuned. Previous work from our group has shown that VICs cultured on hydrogels with elasticity below a 15 kPa (E) threshold maintain a quiescent phenotype, but are activated upon cultured on gels with stiffnesses above this threshold. Using an ene:thiol ratio of 0.67 provided a culture substrate of ~3.5 kPa (E), while a stiffer hydrogel was formed using a ratio of 0.9, for a swollen modulus of ~23 kPa (E). To further characterize this system, an intermediate substrate elasticity of ~12 kPa (E) was obtained using an ene:thiol ratio of 0.75. To confirm these ranges for activation of VICs, cells were cultured on the low, intermediate, and stiff modulus gels and stained for αSMA fiber formation.

This thiol-ene protein patterning approach has exciting utility for studying complex cell-protein interactions. For the VICs-TGFβ system, we are currently conducting studies whereby photolithographic techniques are used to pattern various “islands” of TGFβ on an otherwise inert surface. This approach for spatial TGFβ patterning will allow us to answer questions about the myofibroblast activation cascade, and whether small regions of initial TGFβ activation can influence disease progression across larger cell numbers. Alternatively, the growth factor basic fibroblast growth factor (bFGF) can prevent TGFβ-induced activation of VICs. Patterning
surfaces with thiolated bFGF could provide a negative control for activation via TGFβ delivered solubly, or the two factors could be patterned concurrently to investigate the dynamic interaction between the two signals and implications of each on valve tissue homeostasis.

This excess-[ene] hydrogel platform has application for other cell-protein systems, especially for cell functions like migration and others where protein gradients are important. As an example, TGFβ is implicated in metastasis of melanoma cells, an event that results in greatly increased mortality. Culturing melanoma cells in or on tethered TGFβ hydrogel may provide insight into physiologically relevant TGFβ concentrations required to cause metastasis. Further, using the “moving platform” approach for the photopatterning thiol-ene reaction, as others have demonstrated to create 3D gradients of thiolated peptides, would allow patterning of complex spatial gradients in TGFβ or other proteins. Beyond melanoma metastasis, such a platform would also have utility for the development of materials that guide axonal outgrowth using tethered glial derived neurotrophic factor (GDNF), promote ingrowth of hMSCs via tethered stromal differentiation factor 1 (SDF-1), and even Noggin or sonic hedgehog (ShH) for control of embryonic stem cell differentiation.

In summary, this thesis presents a framework for maximizing the stability of proteins present during photoinitiated radical polymerizations, and makes use of that framework in order to develop biomaterial platforms capable of presenting bioactive protein cues to surrounding cells. These methods provide for facile incorporation of bioactive proteins into synthetic hydrogel materials, with tunable spatially controlled concentrations. Such 3D cell scaffolds can be used to study and control complex interactions between cells, signaling proteins, and culture substrates in a physiologically meaningful context.
Chapter 8

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