Characterization of the kinetics and mechanism of degradation of human mesenchymal stem cell-laden poly(ethylene glycol) hydrogels

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Characterization of the kinetics and mechanism of degradation of human mesenchymal stem cell-laden poly(ethylene glycol) hydrogels

by

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Contents

Acknowledgements iv

List of Figures vii

Abstract 1

1 Introduction 3

2 Materials and Methods 8
   2.1 hMSC culture ........................................... 8
   2.2 Device fabrication ....................................... 9
   2.3 Hydrogel fabrication .................................... 9
   2.4 Hydrogel degradation .................................. 10
   2.5 Bulk rheology ........................................... 11
   2.6 Cell viability ........................................... 12

3 Results and Discussion 13
   3.1 Hydrolytic scaffold degradation ......................... 13
   3.2 Non-cellular enzymatic scaffold degradation ............ 16
   3.3 Cell viability ........................................... 20
   3.4 Cell-mediated degradation ............................... 24

4 Conclusions 30

Bibliography 32
List of Figures

3.1 Hydrogel swelling, shown visually and quantified using bulk rheology. With swelling, hydrogels visibly increase in size and color as growth medium diffuses into the hydrogel. Note that the growth medium in our experiments is a reddish-pink color, and as growth medium diffuses into the hydrogel, the hydrogel also becomes a bright pink color. Hydrogel swelling is complete in approximately 4 hours and decreases the initial elastic moduli, $G'_0$, of the scaffold. .................................................. 14

3.2 Hydrolysis of PEG-norbornene hydrogel scaffolds without hMSCs. a) Bulk rheological measurements of the elastic moduli, $G'$, as a function of time throughout degradation. b) Normalized elastic moduli, $G'/G'_0$, as a function of time. This data is fit to Equation 3.1, resulting in a hydrolysis kinetic constant, $k_h = 7.5 \times 10^{-3} \pm 7.1 \times 10^{-4}$ hr$^{-1}$. ............................ 15

3.3 Normalized elastic moduli, $G'/G'_0$, as a function of time for hydrolysis of hydrogels without hMSCs. Hydrolysis follows first-order kinetics and the data for each experiment is fit to Equation 3.1. These graphs show the individual experiments and the resulting hydrolysis kinetic constant, $k_h$. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 16

3.4 Non-cellular enzymatic degradation, initiated by immersing hydrogels without hMSCs in a 0.3 mg/mL collagenase solution. a) Bulk rheology measures the elastic moduli, $G'$, as a function of time. b) Normalized elastic moduli, $G'/G'_0$, as a function of time. Enzymatic degradation is modeled using Equation 3.2, resulting in an enzymatic kinetic constant, $k^* = 86.7 \pm 0.71$ M$^{-1}$ s$^{-1}$. ................................................................. 17
3.5 Normalized elastic moduli, \( G'/G'_0 \), as a function of time for individual non-cellular enzymatic degradation experiments degraded by a 0.3 mg/mL collagenase solution. The data is fit to Equation 3.2, which is based on Michaelis-Menten kinetics. The collagenase first-order rate constant, \( k_d \), and initial concentration of collagenase, \([\text{collagenase}_0]\), equal 0.02 hr\(^{-1}\) and \(2.31 \times 10^{-6}\) M, respectively, as discussed in the main text. Using these constants and fitting the data to Equation 3.2, the enzymatic kinetic constant, \( k^* \), is determined for each experiment.

3.6 Normalized elastic moduli, \( G'/G'_0 \), measured with bulk rheology and multiple particle tracking microrheology, showing comparability between the characterization techniques over the entire non-cellular enzymatic degradation reaction [1,2].

3.7 Bulk rheology results for hydrogels without encapsulated hMSCs, comparing hydrolysis and non-cellular enzymatic degradation.

3.8 Incubation viability for hydrogels with encapsulated hMSCs quantified using a Live/Dead Assay. (Inset) Fluorescent images of live hMSCs at 4, 24, 48 and 120 hours after hMSC encapsulation. Data and images show high viability and increased motility with time. Scale bars are 250 \(\mu\)m.

3.9 Exposure viability of hydrogels with encapsulated hMSCs exposed to atmospheric conditions without growth medium for 0 to 30 minutes. Fluorescent images of live cells at (a) 0, (b) 10, (c) 20 and (d) 30 minutes. (e) Quantification of percent viability over the same time period. There is no significant change in viability over 30 minutes, indicating this exposure does not cause significant cell death.

3.10 hMSC viability as a function of time (shown as percent of viable cells), comparing sheared viability, where hMSC-laden hydrogels are sheared on the rheometer, to incubation viability, where hMSC-laden hydrogels do not experience shear.
3.11 Individual experiments of sheared viability for hMSC-laden hydrogels. These graphs show hMSC viability as a function of time after hMSC-laden hydrogels are sheared on the rheometer.

3.12 hMSC viability as a function of time at 0, 24 and 48 hours of incubation following shearing on the rheometer. Note that for each of these experiments, shearing is completed 48 hours after hydrogel formation, so viability is completed on hydrogels at 48, 72 and 96 hours, respectively, in real time.

3.13 Cell-mediated enzymatic degradation. a) Elastic moduli, $G'$, as a function of time, characterized with bulk rheology throughout the degradation reaction. b) Normalized elastic moduli, $G'/G'_0$ as a function of time. This data is fit to Equation 3.3, where $k_d$ and $k^*$ are the first-order rate and enzymatic kinetic constants, respectively. This fit resulted in an initial MMP concentration, $[MMP_0]$, of $1.52 \times 10^{-7} \pm 2.05 \times 10^{-8}$ M for a hMSC encapsulation concentration of $2 \times 10^5$ cells/mL.

3.14 Normalized elastic moduli, $G'/G'_0$, as a function of time for individual cell-mediated degradation experiments. The data is fit to Equation 3.3, which is based on Michaelis-Menten kinetics. The first-order rate constant, $k_d$, and enzymatic kinetic constant, $k^*$, are determined in previous non-cellular degradation experiments, and are $0.02 \text{ hr}^{-1}$ and $86.7 \text{ M}^{-1}\text{s}^{-1}$, respectively. Using these constants and fitting to Equation 3.3, the initial concentration of MMPs secreted by hMSCs, $[MMP_0]$, is determined for each measurement of hydrogels with an encapsulated hMSC concentration of $2 \times 10^5$ cells/mL.

3.15 Initial concentration of MMPs secreted by hMSCs as a function of hMSC encapsulation concentration. When more hMSCs are encapsulated in these hydrogels, more MMPs are secreted by the cells, resulting in a higher MMP concentration in the hydrogel.

3.16 Comparison of hydrolytic (gray line), non-cellular enzymatic (black, solid line) and cell-mediated (dashed line) scaffold degradation.
Abstract

Human mesenchymal stem cells (hMSCs) are motile cells that migrate from their native niche to wounded sites where they regulate inflammation during healing. New materials are being developed as hMSC delivery platforms to enhance wound healing. To act as an effective wound healing material, the hydrogel must degrade at the same rate as tissue regeneration while maintaining high cell viability. This work determines the kinetics and mechanism of cell-mediated degradation in hMSC-laden poly(ethylene glycol) (PEG) hydrogels. We use a well-established hydrogel scaffold that is composed of a backbone of four-arm star PEG functionalized with norbornene that is cross-linked with a matrix metalloproteinase (MMP) degradable peptide. This peptide sequence is cleaved by cell-secreted MMPs, which allow hMSCs to actively degrade the hydrogel during motility. Three mechanisms of degradation are characterized: hydrolytic, non-cellular enzymatic and cell-mediated degradation. We use bulk rheology to characterize hydrogel material properties and quantify degradation throughout the entire reaction. Hydrolysis and non-cellular enzymatic degradation are first characterized in hydrogels without hMSCs, and follow first-order and Michaelis-Menten kinetics, respectively. High cell viability is measured in hMSC-laden hydrogels, even after shearing on the rheometer. After confirming hMSC viability, bulk rheology characterizes cell-mediated degradation. When comparing cell-mediated degradation to non-cellular degradation mechanisms, cell-mediated degradation is dominated by enzymatic degradation. This indicates hydrogels with hMSCs are degraded primarily due to cell-secreted MMPs and very little network structure is lost due to hydrolysis. Modeling cell-mediated degradation provides an estimate of the initial concentration of MMPs secreted by hMSCs. By changing the concentration of hMSCs, we
determine the initial MMP concentration increases with increasing hMSC concentration. This work characterizes the rate and mechanism of scaffold degradation, giving new insight into the design of these materials as implantable scaffolds.
Chapter 1

Introduction

Hydrogel scaffolds have become an important class of biomaterials with applications in tissue regeneration, wound healing and 3D cell culture [1–26]. These hydrogels can be composed of biologic or synthetic materials and are being designed as implantable scaffolds to enhance wound healing and tissue regeneration [3–8,27–31]. Within wound healing and regenerative medicine, these hydrogels are used to deliver drugs, proteins or cells in vivo to specified locations within the body [3–7,25,27,28,32,33]. The encapsulation of human mesenchymal stem cells (hMSCs) is of particular interest because they are motile cells that naturally migrate from their native niche to wound sites to regulate inflammation, the immune response and healing [25,28,32,34–36]. In order for hMSCs to be motile, they secrete enzymes called matrix metalloproteinases (MMPs), which enable hMSCs to degrade the extracellular matrix and move through their microenvironment [1,6,32,37,38]. Their ability to differentiate into various cell types, including bone, cartilage, muscle and adipose tissue, also plays an important role in tissue regeneration [34,36]. If implantable and degradable hydrogels can deliver additional hMSCs to a wound, the rate of wound healing and tissue regeneration can be increased. To do this, hydrogels must enable high cell viability after encapsulation, provide structure to the tissue of interest and degrade at the same rate as tissue regeneration. Our work determines the kinetics and mechanism of cell-mediated degradation for a well-defined poly(ethylene glycol) (PEG)-peptide hydrogel. This work provides critical information that can be optimized in the design of new
implantable tissue engineering or wound healing scaffolds.

PEG-based hydrogels have been used extensively as synthetic scaffolds for 3D cell encapsulation to determine the feasibility of these materials as implantable scaffolds [1,3,4,11,16–18,33]. PEG hydrogels are biocompatible and can be designed to mimic the stiffness of soft tissues, providing an environment that promotes basic cellular processes [3,7,10]. Alternative materials include biologic hydrogels, which are scaffolds that incorporate components such as alginate, gelatin and proteins, such as collagen. These materials are biodegradable and biocompatible [3,7,13,29]. However, there are advantages to using synthetic PEG hydrogels over biologic hydrogels. First, biologic hydrogels are weaker mechanically and more prone to contamination [7]. Synthetic hydrogels have more controllable mechanical and chemical properties. They can also be tailored to cross-link with a wide range of chemistries and reaction schemes to form hydrogels within a wound site [8,11]. Unlike biologic hydrogels, synthetic PEG hydrogels can be designed to controllably present biological or physical cues to encapsulated hMSCs. Biological scaffolds mimic the native extracellular matrix, including presentation of native physical and chemical cues that cannot be engineered [19]. Additionally, PEG hydrogels are hydrophilic, enabling them to absorb relatively large amounts of water-based media to maintain high cell viability [17]. Their high mass transport capabilities and minimal protein adsorption makes PEG hydrogels an excellent material for cell encapsulation [4].

Due to the advantages of synthetic PEG hydrogels for cell encapsulation applications, we use PEG as the backbone for our scaffolds. In our hydrogel scaffold, multi-arm PEG is functionalized with norbornene anhydride to create end-terminated PEG-norbornene molecules [10,24,33]. PEG is cross-linked with a MMP degradable cross-linker, KCGPQG ↓ IWGCK. This cross-linker is chosen because it is degraded by hMSC-secreted MMPs during basic cellular processes. Additionally, this cross-linker is degraded at a faster rate when compared to other MMP degradable peptide cross-linkers that better mimic collagen, because this sequence contains tryptophan instead of alanine [14]. This cross-linker is important for biomedical applications, since hydrogels that degrade more rapidly in vivo can lead to improved healing [14]. This scaffold is cross-linked using a photopolymerized
step-growth reaction of thiols in the MMP degradable cross-linker and -enes in the nor-
bornene. This reaction is used because it provides relatively high mechanical integrity,
occurs at neutral pH and allows for better spatial and temporal control of the hydrogel
formed when compared to other step-growth reactions [10, 24, 33].

After encapsulating hMSCs in our degradable PEG-norbornene (PEG-N) hydrogel,
cell-mediated degradation is quantitatively characterized. In our work, bulk rheology is
used to measure the change in the material properties of hMSC-laden hydrogels. These
measurements determine the rate and mechanism of cell-mediated degradation. Rheology
measures the deformation and flow of a material [39]. In our work, small amplitude oscilla-
tory shear is used to measure the scaffold material properties. Bulk rheology characterizes
the complex modulus of the material, $G^*$ ($\omega$), using

$$G^*(\omega) = G'(\omega) + iG''(\omega)$$  \hspace{1cm} (1.1)

where $\omega$ is frequency, $i$ is the unit imaginary number, $G'$ is the storage or elastic modulus and $G''$ is the viscous or loss modulus. Focusing primarily on $G'$, these measurements character-
ize the material’s elastic response and can be used to calculate gel stiffness [40–44].

Elastic modulus and stiffness are important parameters in cell motility and hydrogel degra-
dation [20, 21, 29, 45, 46]. In addition to bulk rheology, microrheology can also be used
to characterize microenvironmental evolution around encapsulated cells [1, 20, 45, 47–52].
Microrheological measurements are in the low moduli and large dynamic frequency range,
making them a complimentary measurement technique to bulk rheology [1,20,47–50,52,53].
Specifically, multiple particle tracking microrheology (MPT), a passive microrheological
technique, has been used to measure temporal and spatial pericellular material properties
in cell-laden hydrogels. Using microrheology, previous work has determined important
information about cell-material interactions and how cells shape and degrade their hy-
drogel microenvironment. However, minimal work has used bulk rheology to determine
the material properties and degradation mechanisms during cell-laden hydrogel degrada-
tion [1, 2, 19, 51, 54–57]. Therefore, our work focuses on the rheological properties and
degradation mechanisms of PEG-N hMSC-laden hydrogels by measuring their evolving
elastic modulus, $G'$, during scaffold degradation. These results can be combined with micro-rheological measurements to provide a more complete picture at the macroscopic and microscopic length scales.

Using bulk rheology, degradation mechanisms for hydrogels with hMSCs are characterized. Cell-mediated scaffold degradation is necessary for the survival of cells in the scaffold and can be manipulated by physical cues in the scaffold to enhance cell delivery during wound healing and tissue regeneration. During motility, cells stretch, adhere and degrade pathways through the scaffold. This cell-mediated degradation changes the material properties, scaffold structure and kinetics of degradation [2,12,19,46,58–61]. Our hydrogel scaffold has two mechanisms of degradation: hydrolytic and enzymatic degradation. In order to understand the cell-mediated degradation rate, we must first characterize scaffold degradation by both mechanisms without cells [1,14,15,22,29].

We use bulk rheology to characterize hydrogel degradation with and without hMSCs. The goal is to determine the kinetics and mechanism of hMSC-mediated hydrogel degradation. Hydrolytic and enzymatic degradation of the PEG-N scaffold without encapsulated hMSCs is characterized. Models are developed to describe both degradation mechanisms. Hydrolysis is modeled using first-order kinetics. Michaelis-Menten kinetics are used to model enzymatic degradation. Hydrolytic and enzymatic kinetic constants are determined. Cell-mediated scaffold degradation is then characterized. High hMSC viability is measured in these scaffolds prior to and after shear is applied during bulk rheological characterization. Hydrolytic and enzymatic models are fit to experimental data of cell-mediated degradation for hMSC-laden hydrogels. This determines the contributions of hydrolytic and cell-secreted enzymatic degradation in the overall hMSC-laden hydrogel degradation. Our work finds that cell-mediated degradation is dominated by cell-secreted enzymatic degradation and there is minimal hydrolytic degradation. By understanding how hMSCs degrade the pericellular region, hydrogels can be designed to more accurately manipulate cellular responses and the rate of scaffold degradation [9,12,19,25,29,58]. With knowledge of the hydrogel macroscopic material properties, combined with an understanding of how encapsulated hMSCs degrade hydrogels, these materials can be optimized for applications.
including implantable wound healing and tissue regeneration scaffolds.
Chapter 2

Materials and Methods

2.1 hMSC culture

hMSCs are purchased from the Lonza Group in passage 2. Frozen hMSCs are resuspended in 4 mL of growth medium, which consists of 1 g/L D-glucose DMEM (Thermo Fisher Scientific), 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 50 µmol mL$^{-1}$ penicillin/streptomycin (Thermo Fisher Scientific), 0.5 µg mL$^{-1}$ fungizone (Thermo Fisher Scientific) and 1 ng mL$^{-1}$ recombinant human fibroblast growth factor (FGF, Pепrotech, Inc.). This growth medium will be referred to as growth medium with FGF. hMSCs are pelleted and resuspended in 200 to 400 µL of growth medium with FGF, and are then added to a cell culture plate (150 × 25 mm Style Treated Cell Culture Dish, Corning Inc.) containing 40 – 60 mL of growth medium with FGF. This cell culture plate is incubated at 37°C and 5% CO$_2$ (Galaxy 48R, New Brunswick Scientific Co., Inc.) [19, 25]. Growth medium with FGF is replenished after 3 days, which washes away any non-adherent hMSCs. After achieving 70 – 80% confluency, cells are either frozen in 95% 1× Dulbecco’s Phosphate Buffered Saline (PBS, VWR Life Science) and 5% dimethyl sulfoxide (DMSO, Sigma-Aldrich Corporation) and returned to the −130°C freezer, are passaged or remain in incubation for encapsulation. For passaging, 8 mL of a 0.25% trypsin-EDTA solution (Thermo Fischer Scientific) is added to the cell culture plate for 10 to 15 minutes to remove hMSCs from the bottom of the plate. 4 mL of growth medium with FGF is added to the
cell culture plate, and all liquid is centrifuged for 5 minutes at 2600 RPM, resulting in a pellet of hMSCs. The liquid is decanted and hMSCs are resuspended in 200 to 400 µL of PBS. hMSCs are then counted to determine the cell concentration from this passage. Cells are resuspended to the desired cell concentration during hydrogel fabrication, as discussed below. For all experiments, hMSCs are used in passage 2 – 6, and after passage 6, hMSCs are discarded.

2.2 Device fabrication

Hydrogels are made in custom sample chambers in a glass-bottomed petri dish \((d = 35 \text{ mm, no. 1.5 glass coverslip, MatTek Corporation})\). A polydimethylsiloxane (PDMS, Sylgard) cylindrical chamber with a 10 mm outer diameter and 8 mm inner diameter is made using biopsy punches (Integra Biosciences). The PDMS chamber is then loosely attached to the bottom of the glass-bottomed petri dish using ultraviolet (UV) curing adhesive (Norland Optical Adhesive 81, Norland Products Inc.) which is cured with UV light at 365 nm for 3 minutes. This adhesive allows for sufficient attachment to the petri dish but also allows for removal of the PDMS chamber. The PDMS chamber is used to hold the polymer precursor solution during the gelation process and is removed following gelation to enable equal swelling (in growth medium) in the axial and radial directions.

2.3 Hydrogel fabrication

Our hydrogel scaffold is composed of a four-arm star PEG-norbornene backbone \((M_n 20,000 \text{ g/mol, } 3 \text{ mM, Sigma-Aldrich Corporation})\) cross-linked by a MMP degradable peptide, KCGPQGIWGCK \((M_n 1,305 \text{ g/mol, } 3.9 \text{ mM, Bachem})\). This scaffold is well-established for 3D cell encapsulation [4, 14, 25]. Hydrogels are photopolymerized with a thiol:ene stoichiometric ratio of 0.65 [1, 9, 10, 22, 33]. A 0.65 thiol:ene ratio is a low cross-linking density, which results in a low modulus material. In previous studies, this scaffold and cross-linking density has been shown to enable a high percentage of cells migrating within the hydrogel [25]. As discussed previously, since PEG-N scaffolds provide no physical
or chemical cues in the environment, an adhesion ligand CRGDS (\(M_n \approx 594 \text{ g/mol}\), 1 mM, American Peptide Company), is included to facilitate cell adhesion to the hydrogel [22]. Lithium phenyl-2,4,6-trimethylbenzoylphosphate, a photoinitiator synthesized using previously published protocols (LAP, 1.7 mM), is included to initiate the reaction [62].

For scaffolds without hMSCs, the polymer precursor solution is described above. For hMSC-laden hydrogels, hMSCs are added to the polymer precursor solution prior to gelation and are encapsulated at a final concentration of 2 \(\times\) 10^5 cells/mL. Note that this low cell concentration is used to limit cell-cell interactions within the hydrogel, so that we measure only cell-material interactions. For all scaffolds, 100 \(\mu\)L of polymer precursor solution is pipetted into the 8 mm sample chamber described above. The solution is exposed to UV light (365 nm, Analytik Jena US) for 3 minutes to initiate gelation. A cross-linked network forms through a radically mediated thiol:ene step-growth mechanism. Previous work has shown that UV exposure leads to successful 3D cell encapsulation and does not damage or kill the majority of hMSCs [10, 62]. After gelation, the PDMS chamber is removed from the sample chamber to allow the gel to swell isotropically. The hydrogel is incubated in 3 mL of growth medium without FGF at 37°C and 5% CO₂.

### 2.4 Hydrogel degradation

Three types of hydrogel degradation are characterized: hydrolytic, non-cellular enzymatic and cell-mediated degradation. In hydrolysis, the ester linkage in PEG molecules is hydrolyzed. This occurs when the scaffold is incubated in water-based growth medium, and results in degradation of hydrogels over the course of approximately two weeks. For these experiments, hydrogels are incubated in growth medium without FGF at 37°C and 5% CO₂ throughout the entire degradation reaction. They are only removed immediately before characterization on the bulk rheometer. On the rheometer, hydrogels are incubated in an immersion cup (TA Instruments) filled with 6 mL of growth medium without FGF at 37°C.

Non-cellular enzymatic degradation is initiated by incubating hydrogels without hMSCs in a solution of collagenase, which is a solution of enzymes including MMPs. The enzymes
2.5. BULK RHEOLOGY

cleave the MMP degradable cross-linker in the hydrogel, resulting in degradation. In our experiments, we use a 0.3 mg/mL collagenase solution (Fisher BioReagents). This solution degrades the hydrogel over several hours, on a much faster time scale than hydrolytic degradation. For these experiments, hydrogels are incubated in an immersion cup filled with 6 mL of collagenase at 37°C on the rheometer. The elastic moduli is measured at timed intervals over the entire degradation reaction.

Cell-mediated degradation is measured in hMSC-laden hydrogel scaffolds. For these experiments, hMSCs are encapsulated in the hydrogel, which are incubated in growth medium without FGF. Following encapsulation, hMSCs secrete MMPs that degrade the MMP degradable cross-linker within the gel. This degradation allows hMSCs to shape their environment in order to move through the hydrogel. For these experiments, hydrogels are kept in the incubator until running the scaffold on the rheometer. On the rheometer, hydrogels are incubated in an immersion cup filled with 6 mL of growth medium without FGF at 37°C. Rheological measurements characterize hydrogel degradation over the five day degradation period.

2.5 Bulk rheology

Prior to bulk rheology measurements, petri dish sample chambers are removed from the incubator, and growth medium without FGF is removed from the inside of the petri dish. The hydrogel is removed from the sample chamber. The hydrogel is then cut using an 8 mm biopsy punch to ensure the hydrogel diameter matches the diameter of the geometry. Rheological properties are measured with a bulk rheometer (Ares G2, TA Instruments) using an 8 mm parallel plate, which is sandblasted to minimize slip. All hydrogels are incubated during measurements. An immersion cup is fixed to the Peltier plate and filled with 6 mL of solution. For hydrolysis and cell-mediated degradation, the hydrogels are incubated in growth medium without FGF at 37°C. This provides hMSC-laden hydrogels with growth medium during bulk rheology experiments. Non-cellular enzymatic hydrogels are incubated in 6 mL of collagenase during bulk rheological measurements. A frequency sweep from 0.1 to 40 Hz at 1% strain is used to measure the moduli in each hydrogel.
2.6 Cell viability

To assess hMSC viability, a Live/Dead Viability Assay Kit (Thermo Fisher Scientific) is used to differentiate live and dead cells based on the integrity of the cell membranes [4]. The green-fluorescent calcein-AM highlights intracellular esterase activity found in live cells, while the red-fluorescent ethidium homodimer-1 enters the degraded plasma membrane found in dead cells [63]. Viability tests are completed by removing all growth medium from the sample chamber and pipetting 1 mL of the Live/Dead Viability Assay solution directly onto the hydrogel. The solution is left on the hydrogel for 30 to 60 minutes. The scaffold is then imaged on an inverted fluorescent microscope (Axio Observer Z1, Zeiss) using a 10× objective to capture images of red and green staining. Quantitative analysis is completed in ImageJ (NIH Image) by counting live and dead cells.
Chapter 3

Results and Discussion

This thesis has been submitted for publication: Michelle S. Mazzeo, Tiffanie Chai and Kelly M. Schultz. Characterization of the kinetics and mechanism of degradation of human mesenchymal stem cell-laden poly(ethylene glycol) hydrogels. Submitted, 2018.

The overall goal of this work is to characterize scaffold degradation to determine the cell-mediated degradation rate and mechanism. By identifying the cell-mediated degradation mechanism, hydrogels can be optimized for future use as wound healing and tissue regeneration scaffolds. In order to determine the overall mechanism of cell-mediated degradation, we first characterize degradation of hydrogels without hMSCs by hydrolytic and enzymatic degradation. These degradation mechanisms are compared to cell-mediated degradation to determine how both degradation mechanisms contribute to cell-mediated degradation.

In addition to characterizing cell-mediated degradation, our experiments show hMSCs are viable within these hydrogels, even after experiencing stress. Our measurements determine enzymatic degradation dominates the cell-mediated degradation mechanism, indicating cell-secreted enzymes are primarily responsible for hMSC-laden hydrogel degradation.

3.1 Hydrolytic scaffold degradation

Hydrogels without hMSCs are first characterized during hydrolytic degradation. This work is done without hMSCs to establish the kinetic constants for a homogenous hydrolytic degradation reaction. Prior to characterization of scaffold degradation, the key parameters
3.1. HYDROLYTIC SCAFFOLD DEGRADATION

in bulk rheological measurements are determined. Briefly, the percent strain applied by the rheometer is optimal at 1% strain to measure the linear viscoelastic region.

Figure 3.1: Hydrogel swelling, shown visually and quantified using bulk rheology. With swelling, hydrogels visibly increase in size and color as growth medium diffuses into the hydrogel. Note that the growth medium in our experiments is a reddish-pink color, and as growth medium diffuses into the hydrogel, the hydrogel also becomes a bright pink color. Hydrogel swelling is complete in approximately 4 hours and decreases the initial elastic moduli, $G'_0$, of the scaffold.

Another key parameter to determine is the time for complete hydrogel swelling. As seen in Figure 3.1, when hydrogels swell during incubation in growth medium, there is a significant change in appearance, as the hydrogel increases in size and decreases in stiffness as growth medium diffuses into the scaffold. The effect of hydrogel swelling is quantified using bulk rheology, which measures a decrease in the elastic moduli over the swelling period, the first four hours of incubation in growth medium, until the moduli becomes constant and the scaffold is fully swollen. Since measuring the elastic moduli is also the primary method for tracking hydrogel degradation, it is important to use the swollen modulus as the initial modulus, $G'_0$. Therefore, a four hour time point, after swelling is complete, is included in all experiments and used as the initial elastic modulus.

Bulk rheological measurements characterize hydrolytic hydrogel degradation, quantifying the change in elastic moduli, $G'$. Hydrogels without cells degrade by hydrolysis of the ester linkage on PEG molecules, which occurs during incubation in water-based growth medium. As seen in Figure 3.2a, the elastic moduli is measured as a function of time
3.1. HYDROLYTIC SCAFFOLD DEGRADATION

Figure 3.2: Hydrolysis of PEG-norbornene hydrogel scaffolds without hMSCs. a) Bulk rheological measurements of the elastic moduli, $G'$, as a function of time throughout degradation. b) Normalized elastic moduli, $G'/G_0'$, as a function of time. This data is fit to Equation 3.1, resulting in a hydrolysis kinetic constant, $k_h = 7.5 \times 10^{-3} \pm 7.1 \times 10^{-4} \text{ hr}^{-1}$.

over the course of the hydrogel degradation reaction. Complete degradation takes approximately two weeks. The decrease in the elastic moduli quantifies degradation of the hydrogel. As the ester linkages on the PEG molecules hydrolyze, there is less scaffold connectivity, which results in an overall decrease in scaffold stiffness and loss of the hydrogel structure. In Figure 3.2b, the elastic moduli is normalized by the initial elastic moduli, $G'_0$, and modeled. Hydrolysis follows first-order reaction kinetics, and is modeled by

$$\frac{G'}{G'_0} = e^{-k_h t}$$

(3.1)

where $k_h$ is the hydrolysis kinetic constant and $t$ is time. This results in a hydrolysis kinetic constant of $7.5 \times 10^{-3} \pm 7.1 \times 10^{-4} \text{ hr}^{-1}$. This value agrees with literature values, which range
from $2.1 \times 10^{-2}$ hr$^{-1}$ to $4.2 \times 10^{-4}$ for similar hydrogel chemistries [16,64–66]. Individual hydrolysis replicate experiments, fits and kinetic constants are included for reference in Figure 3.3.

Figure 3.3: Normalized elastic moduli, $G'/G'_0$, as a function of time for hydrolysis of hydrogels without hMSCs. Hydrolysis follows first-order kinetics and the data for each experiment is fit to Equation 3.1. These graphs show the individual experiments and the resulting hydrolysis kinetic constant, $k_h$.

### 3.2 Non-cellular enzymatic scaffold degradation

Prior to measuring cell-mediated scaffold degradation, enzymatic degradation of our hydrogel scaffold is characterized. Degradation is initiated by immersing the hydrogel in a 0.3 mg/mL collagenase solution. Collagenase is a mixture of enzymes that include MMPs, making this a method to characterize homogeneous enzymatic hydrogel degradation prior to hMSC encapsulation. Figure 3.4a is bulk rheology of non-cellular enzymatic scaffold degradation. The elastic moduli decreases with time, which occurs on a much faster time scale when compared to hydrolytic degradation. Note that since the time of degradation is a function of the collagenase concentration, changing the enzyme concentration can be used to vary the time of degradation. While lowering the enzyme concentration can lengthen the reaction, it is unlikely that it would lengthen the experiment to the two week period of hydrolysis. We have also designed this experiment to happen on a fast time scale so that hydrolysis does not significantly contribute to the degradation reaction, and the experimental results characterize only enzymatic degradation.

Non-cellular enzymatic degradation is modeled by taking into account cleavage of the MMP degradable cross-linker, deactivation of collagenase and using material balances and
3.2. NON-CELLULAR ENZYMATIC SCAFFOLD DEGRADATION

Figure 3.4: Non-cellular enzymatic degradation, initiated by immersing hydrogels without hMSCs in a 0.3 mg/mL collagenase solution. a) Bulk rheology measures the elastic moduli, \( G' \), as a function of time. b) Normalized elastic moduli, \( G'/G'_0 \), as a function of time. Enzymatic degradation is modeled using Equation 3.2, resulting in an enzymatic kinetic constant, \( k^* = 86.7 \pm 0.71 \text{ M}^{-1} \text{s}^{-1} \).

Michaelis-Menten kinetics. For the cleavage of the MMP degradable cross-linker, this considers the probability, \( P \), of a given cross-link being cleaved. The probability is quantified in Equation 3.2, where \( \frac{N_{\text{cross-link}}}{N_{\text{cross-link}_0}} \) is the normalized number of cross-links within the hydrogel, and \( G'/G'_0 \) is the normalized elastic moduli, at a given time. In this model, the normalized number of cross-links is equivalent to the normalized elastic moduli, \( G'/G'_0 \). The enzymatic kinetic constant, \( k^* \) is then determined from this model,

\[
P = \frac{N_{\text{cross-link}}}{N_{\text{cross-link}_0}} = \frac{G'}{G'_0} = e^{k^*[\text{collagenase}]_0/k_d(e^{-k_d t} - 1)} \tag{3.2}
\]

where \([\text{collagenase}]_0 \) is the initial concentration of collagenase and \( k_d \) is the first-order rate constant \([1, 15, 65]\). The initial concentration of collagenase is equal to \( 2.31 \times 10^{-6} \text{ M for} \)
our experiments. The deactivation of collagenase follows a first-order decay. Using the previously measured half-life of collagenase, \( \approx 48 \) hr, the first-order rate constant \( k_d \) is 0.02 hr\(^{-1} \) [1,15,31]. Equation 3.2 is fit to the normalized elastic moduli data, Figure 3.4b, and results in an enzymatic kinetic constant of \( 86.7 \pm 0.71 \) M\(^{-1} \) s\(^{-1} \). Our enzymatic kinetic constant falls within the literature range of 50 to 11,000 M\(^{-1} \) s\(^{-1} \) [14]. The wide range of values in the literature is because previous work measured the cleavage of this specific MMP degradable cross-linker sequence by single MMPs (MMP-1, MMP-2, MMP-3, MMP-8 or MMP-9) [14]. Individual non-cellular enzymatic degradation replicate experiments, fits and kinetic constants are included for reference in Figure 3.5.

Figure 3.5: Normalized elastic moduli, \( G'/G'_0 \), as a function of time for individual non-cellular enzymatic degradation experiments degraded by a 0.3 mg/mL collagenase solution. The data is fit to Equation 3.2, which is based on Michaelis-Menten kinetics. The collagenase first-order rate constant, \( k_d \), and initial concentration of collagenase, \([\text{collagenase}]_0\), equal 0.02 hr\(^{-1} \) and \( 2.31 \times 10^{-6} \) M, respectively, as discussed in the main text. Using these constants and fitting the data to Equation 3.2, the enzymatic kinetic constant, \( k^* \), is determined for each experiment.

Bulk rheological measurements of non-cellular enzymatic degradation are compared to previous microrheology measurements of non-cellular enzymatic degradation in this material [1,2]. Previous multiple particle tracking microrheological characterization of this scaffold determines a value of \( k^* \) that falls within the range of enzymatic kinetic constants in the literature, \( 2,100 \) M\(^{-1} \) s\(^{-1} \) [1,14]. In this work, a different collagenase concentration is used to degrade the hydrogel, possibly accounting for the variability of the rate constant [1]. To account for the differences in these experiments, both bulk rheological and previous microrheological measurements are normalized and plotted together in Figure 3.6. The results are comparable over the entire degradation reaction regardless of measurement.
3.2. NON-CELLULAR ENZYMATIC SCAFFOLD DEGRADATION

technique. Additionally, Equation 3.2 accurately describes both sets of data.

![Graph showing normalized elastic moduli comparison]

Figure 3.6: Normalized elastic moduli, $G'/G'_0$, measured with bulk rheology and multiple particle tracking microrheology, showing comparability between the characterization techniques over the entire non-cellular enzymatic degradation reaction [1, 2].

Bulk rheological characterization of non-cellular enzymatic degradation is also compared to hydrolytic degradation results. Time is normalized by the final time of degradation, $t_f$, as these experiments occur over different time scales. Hydrolysis takes approximately two weeks while non-cellular enzymatic degradation occurs over several hours. Comparing the measurements and resulting fits, Figure 3.7, the shape of the curves are essentially the same. This is a result of the moduli during both degradation reactions having an exponential decay which is described in the models, Equations 3.1 and 3.2. However, there are differences between the two sets of data, including a slower rate of change in the moduli during hydrolysis, even after normalization. This indicates that the rate of loss of cross-links by enzymatic degradation is more efficient than during hydrolytic degradation. This will result in not only an overall faster degradation reaction, but more substantial loss of structure in the initial stages of degradation when the scaffold is degraded enzymatically.

Characterizing hydrolytic and enzymatic degradation in hydrogel scaffolds without hMSCs results in accurate measurements of the kinetics of scaffold degradation and the reaction constants. These experimental results and reaction constants are then compared to measurements of cell-mediated degradation. This will determine the mechanism of scaffold degradation, i.e. hydrolysis versus enzymatic degradation. From these initial experiments,
3.3. CELL VIABILITY

Figure 3.7: Bulk rheology results for hydrogels without encapsulated hMSCs, comparing hydrolysis and non-cellular enzymatic degradation.

we can use the measured reaction kinetics and constants to determine the contributions of both hydrolytic and enzymatic degradation in cell-mediated hydrogel degradation.

3.3 Cell viability

Prior to bulk rheological characterization of cell-mediated degradation of hMSC-laden hydrogels, we must confirm that hMSCs remain viable during and after the measurement. Hydrogels with hMSCs are swollen in growth medium, and hydrogels are stained with a Live/Dead assay to determine temporal changes in cell viability. Live cells are stained green due to intracellular esterase activity and dead cells are stained red due to lack of membrane integrity [63]. By counting the number of live and dead cells, we quantify hMSC viability for each hydrogel at various times throughout our experiments. Viability is first measured after only incubating cell-laden hydrogels at 37°C and 5% CO₂ with no external forces applied. These experiments will be referred to as “incubation viability”. The resulting data, Figure 3.8, shows that incubation viability remains high with only a slight decrease in viability over time. Additionally, cell imaging shows that cells are motile within the hydrogel. Figure 3.8 also shows hMSC stretching increases over time. This motility is indicative of hMSC viability within the hydrogel, as hMSCs are motile cells and naturally migrate in their environment [25, 28, 35, 36].
3.3. CELL VIABILITY

Figure 3.8: Incubation viability for hydrogels with encapsulated hMSCs quantified using a Live/Dead Assay. (Inset) Fluorescent images of live hMSCs at 4, 24, 48 and 120 hours after hMSC encapsulation. Data and images show high viability and increased motility with time. Scale bars are 250 µm.

Since hMSCs encapsulated in the hydrogels are exposed to ambient conditions during bulk rheological characterization, additional viability testing is done to confirm hMSC viability does not decrease during these experiments. During incubation, CO$_2$ is absorbed into the growth medium and is expected to have a relatively quick desorption from the hydrogels. Our hydrogels are water-based, and previous work has determined that CO$_2$ desorption in water at physiological temperatures occurs in five to ten minutes [67]. Therefore, it can be assumed that there will be CO$_2$ desorption from hydrogels before the completion of a bulk rheology experiment.

Due to this, we measure viability in hydrogels with encapsulated hMSCs that are exposed to ambient conditions without growth medium for up to thirty minutes. These experiments are done without growth medium to create the harshest environmental change a hydrogel will experience. All bulk rheological measurements are taken in growth medium. This is referred to as “exposure viability”. As seen in Figure 3.9, exposure viability results show no notable change in hMSC viability over a thirty minute time period. Since each bulk rheology experiment only exposes the hMSC-laden hydrogels for about fifteen minutes, it can be concluded that exposure to natural CO$_2$ conditions should not impact viability during bulk rheology experiments. These experiments show that any changes in
3.3. CELL VIABILITY

Figure 3.9: Exposure viability of hydrogels with encapsulated hMSCs exposed to atmospheric conditions without growth medium for 0 to 30 minutes. Fluorescent images of live cells at (a) 0, (b) 10, (c) 20 and (d) 30 minutes. (e) Quantification of percent viability over the same time period. There is no significant change in viability over 30 minutes, indicating this exposure does not cause significant cell death.

hMSC viability is a result of the shear applied during bulk rheology experiments, rather than exposure to ambient conditions.

After confirming both incubation and exposure viability remains high, cell viability is determined after hMSC-laden hydrogels are sheared on the rheometer, which is referred to as “sheared viability”. For these experiments, hMSC-laden hydrogels are incubated immediately after gelation until they are measured on the rheometer, where they experience shear, and then viability is completed immediately after the bulk rheology experiment. Figure 3.10 is a quantification of cell viability, where time is the incubation time following gelation and prior to bulk rheological characterization. In Figure 3.10, there is a slight decrease in viable cells after hMSC-laden hydrogels are sheared, but the sheared viability results are comparable and remain within error of incubation viability values. This indicates that hMSCs encapsulated in these hydrogels can survive bulk rheological measurements, and, therefore, hMSCs can withstand stresses when encapsulated within these hydrogels. Individual sheared viability replicate experiments are shown in Figure 3.11 for reference.

Additionally, hMSC viability is monitored at several time points following bulk rheology measurements. These experiments determine if additional hMSC death occurs at later time points following the initial application of stress. In this experiment, hMSC-laden hydrogels
3.3. CELL VIABILITY

Figure 3.10: hMSC viability as a function of time (shown as percent of viable cells), comparing sheared viability, where hMSC-laden hydrogels are sheared on the rheometer, to incubation viability, where hMSC-laden hydrogels do not experience shear.

Figure 3.11: Individual experiments of sheared viability for hMSC-laden hydrogels. These graphs show hMSC viability as a function of time after hMSC-laden hydrogels are sheared on the rheometer.

have viability measured at 0, 24 or 48 hours after shearing on the rheometer. Note that in real time, all shearing occurs at 48 hours and viability measurements are completed at 48, 72 or 96 hours after hydrogel gelation, respectively. As seen in Figure 3.12, 91 ± 10 % of hMSCs are viable immediately (zero hours) after shearing. hMSC viability remains constant 24 and 48 hours after shearing, with values of 87 ± 9.0% and 85 ± 8.0% respectively. This indicates that minimal to no additional hMSC death occurs hours after hMSCs experience stress.
Figure 3.12: hMSC viability as a function of time at 0, 24 and 48 hours of incubation following shearing on the rheometer. Note that for each of these experiments, shearing is completed 48 hours after hydrogel formation, so viability is completed on hydrogels at 48, 72 and 96 hours, respectively, in real time.

3.4 Cell-mediated degradation

After confirming hMSC viability, bulk rheology characterizes cell-mediated hydrogel degradation. Figure 3.13a shows the decrease in modulus over time. In Figure 3.13a, the initial elastic moduli of hMSC-laden hydrogels is $5 - 6 \times$ less than hydrogels without hMSCs. The initial elastic moduli of hydrogels with hMSCs is likely lower due to encapsulated hMSCs taking up additional space within the hydrogel and preventing cross-linking. hMSCs are on the micrometer scale and cross-links are on the nanometer scale, so the encapsulation of hMSCs prevents additional cross-links from forming. Therefore, the hydrogel will have fewer cross-links, resulting in a decrease in stiffness characterized by a lower initial elastic moduli. This can be verified by comparing the estimated number of cross-links lost in a hydrogel to the elastic moduli data for hydrogels with and without hMSCs. In an ideal system where 100 % of cross-links form, the maximum dimension of a pore in a hydrogel without hMSCs is approximately 10 nm, while hMSC-laden hydrogels have an average maximum pore length of about 17 nm. When factoring in that cross-linking efficiency is 30 % (determined from bulk rheological measurements), 80 % of cross-links are lost when hMSCs are encapsulated in a hydrogel. This is compared to bulk rheology data, where swollen hydrogels with hMSCs are about 100 Pa, whereas hydrogels without hMSCs are
550 Pa, which also measures about 80 % of cross-links are lost.

Figure 3.13: Cell-mediated enzymatic degradation. a) Elastic moduli, $G'$, as a function of time, characterized with bulk rheology throughout the degradation reaction. b) Normalized elastic moduli, $G'/G'_0$ as a function of time. This data is fit to Equation 3.3, where $k_d$ and $k^*$ are the first-order rate and enzymatic kinetic constants, respectively. This fit resulted in an initial MMP concentration, $[MMP_0]$, of $1.52 \times 10^{-7} \pm 2.05 \times 10^{-8}$ M for a hMSC encapsulation concentration of $2 \times 10^5$ cells/mL.

The normalized elastic moduli, $G'/G'_0$, is modeled to determine the degradation mechanism for hydrogels with encapsulated hMSCs. Data sets are fit to an enzymatic degradation model based on Michaelis-Menten kinetics that described the collagenase experiments. These experimental results were initially fit with hydrolytic, enzymatic and a combination of hydrolytic and enzymatic degradation kinetic models. The only model that fit the data is an enzymatic degradation model, indicating that hMSC-mediated scaffold degradation is due to MMP degradation within the scaffold and hydrolysis accounts for minimal scaffold degradation. During these measurements, the hydrogel is degraded enzymatically by cell-secreted MMPs, rather than by a collagenase solution. Due to this, modifications are
made, and the resulting equation describes cell-mediated degradation

\[
\frac{G'}{G_0} = e^{-\frac{t}{kd}} \left( e^{-k^*t} - 1 \right)
\]  

(3.3)

where the initial concentration of MMPs, \([MMP]_0\), is incorporated into the equation. Since collagenase is a solution of enzymes that includes MMPs, the first-order rate constant, \(k_d\), used in the previous enzymatic model, is used, and equals 0.02 hr\(^{-1}\). Additionally, the previously determined enzymatic kinetic constant, \(k^*\), is used to account for enzymatic degradation, which equals 86.7 \(\pm\) 0.71 M\(^{-1}\)s\(^{-1}\).

![Figure 3.14: Normalized elastic moduli, \(G'/G_0\), as a function of time for individual cell-mediated degradation experiments. The data is fit to Equation 3.3, which is based on Michaelis-Menten kinetics. The first-order rate constant, \(k_d\), and enzymatic kinetic constant, \(k^*\), are determined in previous non-cellular degradation experiments, and are 0.02 hr\(^{-1}\) and 86.7 M\(^{-1}\)s\(^{-1}\), respectively. Using these constants and fitting to Equation 3.3, the initial concentration of MMPs secreted by hMSCs, \([MMP]_0\), is determined for each measurement of hydrogels with an encapsulated hMSC concentration of 2 \(\times\) 10\(^5\) cells/mL.]

Since the initial concentration of secreted MMPs is unknown, we use Equation 3.3 to fit for \([MMP]_0\), which provides an approximation of the initial MMP concentration secreted by encapsulated hMSCs. This value is 1.52 \(\times\) 10\(^{-7}\) \(\pm\) 2.05 \(\times\) 10\(^{-8}\) M in a hydrogel with 2 \(\times\) 10\(^5\) cell/mL. Literature has confirmed the presence of specific MMPs and studies how MMPs can be used to regulate cell behavior, particularly involved with inflammation, tissue regeneration and cancer cell applications [57,68–70]. However, no estimate of a cell-secreted MMP concentration appears in these works, especially when encapsulated in hydrogels [57,68–70]. This model gives the first quantitative estimate of MMPs secreted by hMSCs encapsulated within these hydrogels, which can be used to target a specific degradation rate when designing these materials. Therefore, additional knowledge
3.4. CELL-MEDIATED DEGRADATION

about hMSC-secreted MMPs helps to increase the tailorability of these hydrogels in specific wound healing applications, by matching the rate of hydrogel degradation to the rate of tissue regeneration within a specific wound. Individual cell-mediated degradation replicate experiments, fits and corresponding initial MMP concentrations are included in Figure 3.14 for reference.

![Figure 3.15: Initial concentration of MMPs secreted by hMSCs as a function of hMSC encapsulation concentration. When more hMSCs are encapsulated in these hydrogels, more MMPs are secreted by the cells, resulting in a higher MMP concentration in the hydrogel.

hMSC-laden hydrogels can also be tailored by altering the hMSC concentration within the hydrogel. All previous experiments encapsulated hMSCs in hydrogels at a concentration of $2 \times 10^5$ cells/mL. In order to determine how hMSC concentration affects cell-mediated degradation in hMSC-laden hydrogels, bulk rheology experiments also characterize hydrogels with encapsulated hMSCs at concentrations of $0.5 \times 10^5$ and $1 \times 10^5$ cells/mL. Elastic moduli data at each concentration is fit to Equation 3.3, which fits for $[MMP]_0$, the initial concentration of MMPs secreted by hMSCs. From this, $[MMP]_0$ is found to be $1.04 \times 10^{-7} \pm 1.4 \times 10^{-8}$ M and $1.15 \times 10^{-7} \pm 1.3 \times 10^{-8}$ M for hMSC concentrations of $0.5 \times 10^5$ and $1 \times 10^5$ cells/mL, respectively. From this study, as seen in Figure 3.15, we find that as the concentration of hMSCs encapsulated within the hydrogels increases, the concentration of MMPs secreted by the cells also increases. This is an expected result, when more hMSCs are present, more MMPs are secreted that actively degrade the hydrogel to enable cell motility, which changes the rate of hydrogel degradation. The change in $[MMP_0]$ is
statistically significant between $1 \times 10^5$ and $2 \times 10^5$ cell/mL, but is within error for $0.5 \times 10^5$ and $1 \times 10^5$ cells/mL. The values being within error for the lower concentrations is not unexpected. In this low cell concentration, we expect lower overall MMP secretion. Additionally, with the smaller change between the two lower hMSC concentrations, a smaller decrease in the change of $[MMP_0]$ is expected. This shows that altering the hMSC concentration provides another method to optimize cell-mediated hydrogel degradation rates for different wound healing and tissue regeneration applications.

![Figure 3.16: Comparison of hydrolytic (gray line), non-cellular enzymatic (black, solid line) and cell-mediated (dashed line) scaffold degradation.](image)

In Figure 3.16, all degradation reactions are plotted together. Since all of the previously discussed types of degradation occur on different time scales, all elastic moduli data are normalized, which enables direct comparison of all the degradation mechanisms. The hydrolytic degradation rate is slower than the non-cellular enzymatic and cell-mediated degradation rates. When comparing hydrolytic degradation to cell-mediated degradation without normalizing time, degradation of hydrogels with encapsulated hMSCs occurs at a rate approximately $4 \times$ faster than hydrolytic degradation. This indicates hydrolysis plays a minimal role in the overall cell-mediated degradation mechanism. As cells secrete MMPs, the MMP degradable cross-linker is cleaved, thus degrading the hydrogel at a faster rate when compared to hydrolysis alone. On the other hand, when comparing cell-mediated degradation to non-cellular enzymatic degradation, these fits are closely aligned, and nearly
overlap. The initial collagenase and MMP concentration are an order of magnitude different, \([\text{collagenase}_0] \approx 2.3 \times 10^{-6}\) and \([\text{MMP}_0] \approx 1 \times 10^{-7}\) M, which accounts for deviations in degradation data. This indicates that cell-mediated degradation is dominated by an enzymatic degradation mechanism. This confirms that as cells are actively secreting MMPs, these enzymes degrade the hydrogel on a time scale much faster than hydrolysis, thereby minimizing effects from hydrolytic degradation. Therefore, cell-mediated degradation is dominated by enzymatic degradation mechanism.
Chapter 4

Conclusions

PEG-N hydrogel degradation is characterized to determine the mechanism of cell-mediated degradation. Bulk rheology is used to determine the kinetics of three types of degradation: hydrolytic, non-cellular enzymatic and cell-mediated degradation. Hydrolytic degradation occurs due to hydrolysis of the ester linkage in the PEG molecules when hydrogels are incubated in growth medium. These experiments result in a hydrolysis kinetic constant, \( k_h = 7.5 \times 10^{-3} \pm 7.1 \times 10^{-4} \text{ hr}^{-1} \). Non-cellular enzymatic degradation is initiated by incubating hydrogels in a collagenase solution. This cleaves the MMP degradable cross-linker in the hydrogel, and results in an enzymatic kinetic constant, \( k^* = 86.7 \pm 0.71 \text{ M}^{-1} \text{ s}^{-1} \).

Prior to measuring cell-mediated scaffold degradation, hMSC viability measurements show that these hydrogels maintain high hMSC viability and provide an environment where hMSCs survive in the presence of stress from bulk rheological characterization. Once high hMSC viability is confirmed, bulk rheology characterizes cell-mediated hydrogel degradation. In these experiments, we determine that cell-mediated degradation is due to MMP-secretion and hydrolytic degradation is minimal. To model this reaction, the enzymatic degradation model developed for non-cellular enzymatic degradation is modified. Constants from hydrolytic and non-cellular enzymatic degradation are used to determine the initial concentration of MMPs in the scaffold. By changing the concentration of encapsulated hMSCs, we measure that the amount of MMPs in the hydrogel increases with increasing
hMSC concentration. This work provides important new information about cell-mediated degradation. Namely, the mechanism of scaffold degradation has a minimal contribution from hydrolysis and is due to enzymatic degradation by cell-secreted MMPs. This work also provides a method to estimate the initial concentration of MMPs present in a cell-laden hydrogel scaffold. These results characterize the evolving material properties of these hydrogels throughout degradation, which can be used to optimize cell-laden hydrogels in future regenerative medicine and wound healing applications.
Bibliography


[63] Live/dead viability/cytotoxicity kit, for mammalian cells.


Michelle S. Mazzeo completed her undergraduate studies at Lehigh University, getting a B.S. in Chemical Engineering and a Biotechnology minor in 2017. While a Lehigh undergraduate, she was awarded with the Robert C. Hick’s Prize in 2016 for scholastic achievement in Chemical engineering and graduated with Highest Honors (Summa Cum Laude) for a 3.9 GPA. She participated in undergraduate research in Professor Kelly M. Schultz’s lab, working with PEG hydrogels concentrated above their overlap concentration. Her research was included in the paper: Matthew D. Wehrman, Andrew Leduc, Holly E. Callahan, Michelle S. Mazzeo, Mark Schumm and Kelly M. Schultz. Rheological properties and structure of step and chain growth gels concentrated above the overlap concentration. *AIChE Journal*, 64(8): 3168-3176, 2018. Her professional experience outside of Lehigh includes several summer internships. Her first summer internship was at Cincinnati Children’s Hospital, where she treated adult mouse fibroblasts with experimental drugs to inhibit MLK3 and decrease inflammation and fibrosis in the heart. Her next internship was at Regeneron in the Purification Development Analytics department, where she optimized a multiplex qPCR assay for improved DNA detection. Her third internship was at Regeneron in the Formulation Development department, where she optimized a free fatty acid particulate counting technique to assess the sensitivities of particulate counting. Michelle completed her Master’s at Lehigh University, graduating with a Master of Science in Bioengineering in 2018. Funding for this program came from the Lehigh Presidential Scholarship program, which is awarded to Lehigh undergraduates with a GPA of 3.75 and above. During her Master’s, she attended the 2017 Society of Rheology (SOR) Conference and was awarded with second place in the SOR Conference Graduate Poster Competition.
She also attended and gave a talk at the 2018 ACS Colloids Conference.