A Biomimetic and Biodegradable Hydrogel and the Impact of Macrophages for Bone Tissue Engineering

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A Biomimetic and Biodegradable Hydrogel and the Impact of Macrophages for Bone Tissue Engineering

by

MARIA CARLES CARNER

B.S., Universitat Autònoma de Barcelona, 2015

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 Master of Science
Department of Chemical and Biological Engineering

2017
This thesis entitled:
A Biomimetic and Biodegradable Hydrogel and the Impact of Macrophages for Bone Tissue Engineering
written by MARIA CARLES CARNER
has been approved for the Department of Chemical and Biological Engineering

(Stephanie J Bryant)

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Date____________

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.
This thesis investigates the incorporation of hydroxyapatite (HA) nanoparticles into poly(ethylene glycol) (PEG) hydrogels as a scaffold to enhance osteogenic differentiation of the encapsulated murine pre-osteoblast MC3T3-E1 cells for applications in tissue engineering.

Specifically, poly(ethylene-glycol) (PEG) hydrogels have been chosen because they offer several advantages that make them promising scaffolds to investigate. They provide an adequate 3D matrix that mimics the ECM and helps cell growth and proliferation maintaining an elevated cell viability and improve the formation of new bone. They are also highly tunable for a number cell types easing the incorporation of biological moieties which make possible to regulate the degradation rate in order to match the rate of tissue formation. Hydroxyapatite, which is a bioactive mineral, was incorporated into the synthetic PEG hydrogels because it is known that it helps providing a biomimetic environment for the hydrogels which enhance their osteoconductive and osteoinductive capabilities.

However, it is known that, in vivo, synthetic materials elicit a foreign body reaction (FBR) when they are implanted. The FBR, which is an immune response that is regulated by activated macrophages, is characterized by the formation of a fibrous capsule around the hydrogels that limits their performance.

Therefore, the purpose of this thesis is first, to characterize an 8-arm thiol-norbornene PEG hydrogel with added HA nanoparticles to elucidate how they affect the differentiation into osteoblasts of the encapsulated MC3T3-E1 cells. After understanding the effects of adding HA nanoparticles to the synthetic scaffolds, and showing that they enhanced bone tissue deposition, the hydrogels containing 1% (w/w) of hydroxyapatite were used to study how the foreign body response impacts the performance of this MMP-sensitive PEG hydrogels. This was done by
characterizing the influence that the macrophages have over encapsulated pre-osteoblast MC3T3-E1 cells *in vitro* when they are activated in a co-culture system.
Dedication

I would first like to thank my advisor Stephanie J. Bryant. Her door was always open whenever I ran into a trouble or had questions. She consistently allowed this paper to be my own work, but steered me in the right direction whenever she thought I needed it.

Also, thanks to all of those who have shared the D355 laboratory with me: Sadhana Sharma, Ph.D., Luke Amer, Ph.D., Elizabeth Aisenbrey, Alex Anderson, Aaron Aziz, Camila Uzcategui, Stanley Chu, Kristen Eller, Archish Muralidharan, Leila Saleh, Margaret Schneider and Michael Trombold. All of them are part of my success, too. I do also recognize the support I have received from the rest of ChBE students and staff. Without them, the goals accomplished by the department would not be possible.

A very special gratitude goes to the Balsells Foundation because they have been the ones that made that financially possible and without their help I would have not been able to have my Master’s Degree. Special thanks to Pete Balsells, who started the Fellowship and to Robert H. Davis, former Dean of the College of Engineering and Applied Science at the University of Colorado at Boulder first for trusting me and selecting me amongst a very competitive group of engineers and also for his guidance during these two very special years of my life.

Finally, I want to express my very profound gratitude to my parents, Mercè Carner and Joan Carles, for their unconditional emotional support. Their advices have guided me and given me the strength whenever I have needed them. To my sister, Anna Carles, for her cheerfulness. To my grandmother, Maria Teresa, for her optimism, modesty and understanding. It was not until I got to live on my own that I realized how important the education I have received from my family and from all the people I have crossed paths with during my life was. All the members from my family have been such an example to me and I feel that the distance have brought all of us closer. I also want to give aa special thank you to my boyfriend, Sebastian, for supporting me continuously and encouraging me throughout this journey and
through the process of researching and writing this thesis, that has not been easy for me. He made me feel loved at every moment. I feel very proud to have all of them in my life. This accomplishment would not have been possible without them.

Thank you.
Acknowledgments

The research reported in this publication has been supported by the Balsells Fellowship to Maria Carles Carner. Leila S. Saleh was supported by NIH Grant #R21AR064436, a Department of Education GAANN fellowship. The author would also like to thank Eric Ellison, Sr. Professional Research Assistant at Alexis Templeton research group for his assistance with the Raman Microspectroscopy and analysis of the obtained data.
CONTENTS

Chapter 1: The Effect of Hydroxyapatite Particles on MC3T3-E1 Pre-Osteoblast in a Biomimetic and Biodegradable Poly(Ethylene Glycol) Hydrogel for Bone Tissue Engineering..................................................................................................................1

1. Introduction..................................................................................................................2

2. Materials and Methods................................................................................................5
   2.1 Cell Culture..............................................................................................................5
   2.2 Macromer Synthesis and Preparation of the Hydrogel Precursor Solution..................5
   2.3 Osteoblast Induction...............................................................................................6
   2.4 Raman Spectroscopy.............................................................................................7
   2.5 Viability and Biochemical Assays...........................................................................7
   2.6 Immunochemistry Analysis...................................................................................8
   2.7 Statistical Analysis...............................................................................................8

3. Results.........................................................................................................................10
   3.1 Formation and Characterization of the MMP-Sensitive PEG Hydrogels....................10
   3.2 Viability and Cell Morphology Assessment ..............................................................13
   3.3 Mechanical Properties of the Hydrogels with Encapsulated MC3T3-E1 Cells...............15
   3.4 Mineralization and Alkaline Phosphatase Assessment................................................16
   3.5 Collagen Type I Formation and Total Collagen Assessment......................................19

4. Discussion....................................................................................................................21

5. Conclusions..................................................................................................................25
Chapter 2: The *In Vitro* Effects of Macrophages on the Osteogenic Capabilities of MC3T3-E1 Cells Encapsulated in a Biomimetic Poly(Ethylene Glycol) Hydrogel

1. Introduction..............................................................................................................27

2. Materials and Methods. ..........................................................................................30
   2.1 Macromer Synthesis and Hydrogel Formation.................................................30
   2.2 MC3T3-E1 Cell Culture and Encapsulation.....................................................31
   2.3 RAW 264.7 Cell Culture..................................................................................31
   2.4 Primary Monocyte Isolation, Differentiation and Culture...............................32
   2.5 Assessment of MC3T3-E1 Pre-Osteoblast Morphology....................................32
   2.6 Immunohistochemical and Histological Analysis..............................................33
   2.7 DNA, Biochemical and Cytokine Assays..........................................................34
   2.8 Statistical Analysis...........................................................................................34
   2.9 IACUC Approval...............................................................................................34

3. Results....................................................................................................................36
   3.1 The Effect of Macrophages on MC3T3-E1 Cell Apoptosis..................................36
   3.2 The Effect of Macrophages on MC3T3-E1 Morphology......................................39
   3.3 The Effects of Macrophages on Collagen I Deposition by MC3T3-E1 Cells........40
   3.4 The Effects of Macrophages on ALP Activity and Mineralization in MC3T3-E1-Laden Biomimetic Hydrogels.................................................................43
   3.5 Cytokine Secretion from the *In Vitro* Co-Culture Model.................................45

4. Discussion...............................................................................................................47

5. Conclusions.............................................................................................................53

6. References..............................................................................................................54
   6.1 First Chapter......................................................................................................54
   6.2 Second Chapter.................................................................................................59
TABLES

Table

1. P-values calculated with a 3-way ANOVA Interaction between 3 independent variables: time, % HA, type of media..............................................................9

2. Table-1. P-values calculated with a 2-way ANOVA Interaction between 2 independent variables: time and treatment.........................................................35
FIGURES

Figure

1. Hydrogel formation and experimental design........................................11

2. Characterization of the effects of hydroxyapatite particles on the properties on acellular hydrogels.................................................................12

3. Cell viability, morphology and cell number as a function of experimental treatment and culture time..............................................................14

4. Compressive modulus (in kPa) of the hydrogels with encapsulated MC3T3-E1 cells for different concentrations of HA in the hydrogel after 28 days of culture in osteogenic medium.................................................................15

5. Mineralization assessment of encapsulated MC3T3-E1 cells in the PEG hydrogels and cultured for 28 days..........................................................17

6. Collagen type I assessment........................................................................20

7. Representative PEG-NB hydrogel formulation. Schematic of the experimental co-culture setup in this study, wherein macrophages were refreshed weekly and LPS in the media was refreshed every 48 hours........................................36

8. The effects of macrophages on MC3T3-E1 cell apoptosis............................38

9. The effects of macrophages on MC3T3-E1 cell morphology........................40

10. The effect of macrophages on collagen I deposition by MC3T3-E1 cells ....42
11. The effects of macrophages on ALP activity and mineralization in MC3T3-E1-laden PEG hydrogels

12. Cytokine secretion from the in vitro co-culture model
CHAPTER 1

The Effect of Hydroxyapatite Particles on MC3T3-E1 Pre-Osteoblast in a Biomimetic and Biodegradable PEG Hydrogel for Bone Tissue Engineering

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1. INTRODUCTION

*In situ* forming hydrogels are promising platforms to deliver cells in a three-dimensional (3D) environment and through minimally invasive methods. This process enables filling any irregularly shaped defect without *a priori* knowledge. Moreover, the 3D environment of the hydrogel can be tailored with biochemical cues to support differentiation and promote deposition of newly synthesized extracellular matrix (ECM) molecules that lead to tissue regeneration. In particular, synthetic hydrogels have several advantages over naturally derived hydrogels (e.g., collagen gels) including reproducible properties (e.g., mechanical and swelling)\(^1\), precision over the introduction of biochemical cues\(^2\), and tunable rates of degradation\(^3\).

Synthetic hydrogels formed from poly(ethylene glycol) or PEG are one of the most widely investigated hydrogels used for cell encapsulation\(^4,5\). This observation is in part due to the hydrophilic nature of PEG as well as the ability to functionalize each PEG chain with two or more polymerizable groups that react to form a crosslinked polymer. Moreover, since cells do not directly interact with PEG, the chemistry of the hydrogel can be further tuned to create biomimetic environments that are highly specific to a particular tissue engineering application. To this end, PEG hydrogels have been investigated for the delivery of stem cells and osteoblasts for bone tissue engineering\(^6\).

The incorporation of ECM analogs, such as peptides, into synthetic hydrogels can create environments that emulate aspects of the native ECM. Two ECM analogs that have been studied in hydrogels for bone tissue engineering are cell adhesion peptides, via tethered peptides, and peptide crosslinks that are sensitive to cell secreted enzymes\(^7-9\). The cell adhesion peptide, RGD, has been widely studied for bone tissue engineering due its recognition by the \(\alpha_5\beta_1\) integrin\(^10,11\), which has been shown to be important in osteogenesis\(^12,13\).

The incorporation of peptide crosslinks that are sensitive to matrix metalloproteinase (MMP) enzymes have shown promise in bone tissue engineering\(^14\). Through slight changes in a single peptide, the kinetics of
degradation can be controlled enabling the tuning of degradation to match the rate of tissue formation\textsuperscript{15,16}.

In bone, the major inorganic component is hydroxyapatite, which gives rise to the high stiffness of bone. Since hydrogels on their own do not have an intrinsic capability to mineralize, several strategies have been investigated to induce mineralization\textsuperscript{17}. One of the most common strategies is to introduce inorganic particles into the hydrogel prior to gelation\textsuperscript{18}. Specifically, hydroxyapatite is known for its osteoconductivity and osteoinductivity\textsuperscript{19} properties and thus can serve as an ECM cue that promotes osteogenesis of the encapsulated cells. The incorporation of hydroxyapatite into PEG-hydrogel that has shown promising bone integration abilities, tunable degradation rate, and improved biocompatibility\textsuperscript{20}. Moreover, the introduction of inorganic particles, such as hydroxyapatite can serve as a nucleation site for further mineralization. This is particularly important as adding in high amounts of mineral (e.g., bone is \textasciitilde60-70\% mineral) is difficult if not impossible in a hydrogel setting\textsuperscript{21}.

The overall objective of this study was to investigate the effects of adding hydroxyapatite particles into a PEG hydrogel containing the cell adhesion peptide, RGD and MMP-sensitive crosslinks. PEG hydrogels formed from a photoclick chemistry\textsuperscript{22,23} were chosen where thiol and norbornene functionalized monomers are reacted off-stoichiometry such that thiolated biological molecules (i.e., peptide tethers and peptide crosslinks) are readily introduced\textsuperscript{24,25}. Specifically, this research tests the hypothesis that the addition of hydroxyapatite, in a concentration dependent manner, improves differentiation and bone-like ECM deposition by MC3T3-E1 pre-osteoblastic cells when encapsulated in a biomimetic and biodegradable PEG hydrogel. To test this hypothesis, the response of MC3T3-E1 cells were investigated as a function of three hydroxyapatite concentrations (0\%, 0.1\% and 1\% w/w) and two culture media (growth medium and osteogenic medium) over the course of 28 days. Collectively our findings support our hypothesis that introducing hydroxyapatite particles improves the osteogenic response of MC3T3-
E1 cells, which is observed in the absence of differentiation factors, but which is even greater in the presence of differentiation factors.
2. MATERIALS AND METHODS

2.1 Cell Culture

In this study, the murine pre-osteoblast-like cell line MC3T3-E1 (ATCC, CRL-2593) were encapsulated into the hydrogels. The cells have the capacity to differentiate into osteoblasts and osteocytes and have been demonstrated to form calcified bone tissue in vitro.

MC3T3-E1 cells were seeded in 225cm² flasks and they were cultured in the growth media formed by Minimum Essential Medium (α-MEM, from Gibco) supplemented with 10% of fetal bovine serum (FBS, from Atlanta Biologicals) and 1% antibiotics (5,000 Units/mL Penicillin, 5,000 µg/mL Streptomycin from Corning) at a humidified atmosphere at 37°C and 5% CO₂. When cells reached ~80-90% confluency, they were treated with with 0.25% Trypsin-EDTA (from Gibco) for 7 minutes at 37°C. The cell suspension was washed with equal parts of growth media, centrifuged to recover the cells, counted with a hemocytometer and resuspended to the desired concentration to either passage them or encapsulate into the hydrogels (20 million cells/mL). The culture media was changed every other day and the cells used were passaged less than 12 times.

2.2 Macromer Synthesis and Preparation of the Hydrogel Precursor Solution

The 8-arm Poly(ethylene glycol) (PEG, 20kDa) polymer was synthesized previously following the determined procedures and functionalized with norbornene. The functionalization of PEG with norbornene was considered to be ~100%. Hydrogels of 30 µL of volume (approximately 4.5mm diameter and 2 mm height) were made in cylindrical molds by polymerizing, under UV light (352 nm light at 6mW/cm²) for ~6-7 minutes, a sterile filtered solution (filter was 0.22µm) formed by 8% (w/w) 8-arm-PEG with MMP-2/9 degradable peptide crosslinker
(CVPLSLYSGC) at a 0.83 thiol:ene ratio and 2.5 mM of cell adhesion peptide (CRGDS, Genscript), 0.05% (w/w) of photoinitiator (Irgacure 2959, BASF) and hydroxyapatite (HA, from Sigma) nanoparticles (nanopowder, <200nm particle size) which were first sonicated for ~20 min and brought to the corresponding concentration (0%, 0.1% or 1% w/w of HA) dissolved in phosphate buffered saline (PBS, Cellgro). Finally, the cells were mixed with the hydrogel precursor solution at 20 million cells/mL before the polymerization. All the hydrogel formation procedure was performed inside a biosafety cabinet using previously sterilized (or autoclaved) materials and instruments.

The hydrogels made without cells (acellular hydrogels) had the same formulation. However, they did not have cell adhesion peptide or MC3T3-E1 cells. They were made following the same protocol, but they did not need to be made under sterile conditions inside the biosafety cabinets using sterilized instrumentation.

2.3 Osteoblast Induction

The cellular hydrogels were incubated at 37°C and 5% CO₂ and they were seeded every 2 days with two different types of media.

The hydrogels cultured under osteogenic differentiation media, were seeded with MC3T3-E1 cells growth media with the presence of 10 mM β-glycerophosphate (Sigma, St. Louis, MO), 50 mM L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma) and 0.1 mM Dexamethasone (Sigma) which induce osteogenic differentiation of the cells. The other media condition in which the MC3T3-E1 do not differentiate, the hydrogels were cultured with the same growth media used for the MC3T3-E1 cells (α-MEM supplemented with 10% FBS, 1% penicillin/streptomycin).
2.4 Raman Spectroscopy

Raman spectra were collected using a Horiba LabRAM HR Evolution Raman spectrometer at the Raman Microspectroscopy Laboratory, University of Colorado-Boulder. The 532 nm (green) laser beam was focused through a 50x LWD (0.75 NA) objective lens, yielding a spatial resolution of ~2 µm and power at the sample surface of 29 mW. A 600 lines/mm grating and a 100 µm confocal pinhole were used to give a spectral resolution of 4.5 cm\(^{-1}\) full width at half maximum. The spectrometer was calibrated using the 520 cm\(^{-1}\) Raman peak of Si prior to analysis. Spectra were collected by averaging 15 accumulated spectra collected with a 2 sec counting time. Spectral data were corrected for instrumental artifacts and a polynomial baseline was subtracted in LabSpec 6 (Horiba Scientific).

2.5 Viability and Biochemical Assays

At 3 different time points (Day-0, Day-14 and Day-28), hydrogels were removed from culture and analyzed for viability and cell morphology by Life/Dead Cell Viability Assay staining kit (Life Technologies, Thermo Fisher Scientific), where the calcein stains live cells in green and ethidium stains dead cells in red. A group of hydrogels was transferred DNAse free water and disrupted with a tissue lyser (Qiagen). Hydrogel lysates were assessed for total DNA content with the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific), Alkaline Phosphatase Activity by enzyme cleavage of p-nitrophenol phosphate and total Collagen content by digesting the samples with pepsin powder (Worthington) in 0.5M acetic acid overnight and incubated with Sirius Red (Sigma). All assays were performed following the instructions and protocols provided by the manufacturer's and using a spectrophotometer to measure either the fluorescence or the absorbance.
2.6 Immunohistochemistry Analysis

For immunohistochemical and histological analysis the hydrogels were collected after 28 days of culture and fixed in neutral-buffered formalin for 30 minutes (at room temperature inside the fumes hood). They were then dehydrated and embedded in paraffin wax. The samples were sectioned in 10 µm slices and processed for histological staining.

The sections were stained for Collagen I and for mineralization by Von Kossa following standard protocols. Collagen I stained samples were imaged by laser scanning confocal microscopy (Zeiss LSM5 Pascal) and Von Kossa samples were imaged with brightfield illumination (Zeiss Axioimager M1).

2.7 Statistical Analysis

Data presented in this paper is expressed as the mean of n=3 replicates and presented with ± standard deviations represented as error bars in all the figures.

The statistical analysis (Table 1) of the data was performed using the Mac version of Real Statistics add-in for Excel and analyzed through a three-way ANOVA. Factors analyzed were culture time, % of HA and media type (osteogenic differentiation or growth media). A p-value of <0.05 was used to determine statistical significance.
Table 1. P-values calculated with a 3-way ANOVA Interaction between 3 independent variables: time, % HA, type of media.

<table>
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<th>Assay</th>
<th>Statistical analysis</th>
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<td></td>
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<td>Acellular</td>
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<td></td>
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<td>Cellular</td>
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<td></td>
<td>Wet weight</td>
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<td></td>
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<td></td>
<td>Collagen</td>
<td>p &lt; 0.001</td>
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3. RESULTS

3.1 Characterization of Acellular Biomimetic PEG Hydrogels Containing Hydroxyapatite Particles

The biomimetic hydrogels were fabricated as shown in Figure 1 with varied concentrations of incorporated hydroxyapatite particles from 0, 0.1 to 1 wt%. The hydrogels were initially characterized in the absence of cells (Figure 2). Immediately after hydrogel formation, but after reaching equilibrium (referred to as day 0), the hydrogels were also characterized by Raman spectroscopy (Figure 1C) and compared to hydroxyapatite particles and a PEG hydrogel. The characteristic peak associated with hydroxyapatite at 960 cm⁻¹, which represents the calcium phosphate group and correlates with hydroxyapatite, was present in the hydrogel sample with hydroxyapatite, but not in the PEG hydrogel lacking hydroxyapatite. The acellular hydrogels were also characterized as a function of time when cultured in growth medium for compressive modulus (Figure 2A) and wet weight (Figure 2B). Both time and hydroxyapatite concentration were factors in the wet weight.
Figure 1. Hydrogel formation and experimental design. A) The monomers, along with their chemical structures, MC3T3-E1 pre-osteoblasts, and hydroxyapatite particles that were combined prior to hydrogel formation. B)
Hydrogels were formed by photopolymerization to create three experimental treatments with 0, 0.1 and 1wt% hydroxyapatite. C) Raman spectra for hydrogels with 1 wt% of hydroxyapatite (green), hydroxyapatite particles alone (red) and PEG hydrogel (purple).

Figure-2. Characterization of the effects of hydroxyapatite particles on the properties on acellular hydrogels: A) Compressive modulus (kPa) as a function of culture time in growth medium. B) Wet weight (mg) as a function of culture time in growth medium. Data are presented as the mean with standard deviation as error bars for n=3 replicates. Statistical analysis from a two-way ANOVA with time and hydroxyapatite concentration as the factors are reported in Table 1.
3.2 Viability and Cell Morphology Assessment in Hydrogels with MC3T3-E1 cells

Representative confocal microscopy images assessing viability of encapsulated MC3T3-E1 cells as a function of hydroxyapatite concentration, culture time and culture medium are shown in Figure 3A. Qualitatively, viable MC3T3-E1 cells were present throughout the experiment and across all experimental treatments. Initially at day 0 (i.e., 24 hours post-encapsulation), the encapsulated MC3T3-E1 cells displayed a rounded morphology. There was evidence of a few cells spreading by day 14, which was maintained at day 28 in the growth media condition across all hydroxyapatite concentrations. However when cultured in differentiation media, cell spreading was much more prevalent by day 14 and even greater by day 28 with the formation of a connected cellular network. There was no apparent difference in morphology as a function of hydroxyapatite concentration. The total number of cells within the constructs was assessed quantitatively by dsDNA content (Figure 3B). Overall, the amount of dsDNA decreased (p<0.001) with time, but this decrease was ~≤25% for all treatments. The dsDNA content was minimally affected (p=0.064 and p=0.072, respectively) by hydroxyapatite concentration and culture medium.
Figure-3. Cell viability, morphology and cell number as a function of experimental treatment and culture time: A) Representative confocal microscopy images of Live/Dead Cell Viability Staining Assay for encapsulated MC3T3-E1 cells at day-0 and at 28 day as a function of hydroxyapatite concentration and culture medium. Live cells stained are green and nuclei of dead cells are stained red. Scale bar represents 100 µm. B) Cell number as measured by double stranded DNA (dsDNA) per construct. Data are presented as mean with standard deviation as error bars for n=3 replicates. Statistical analysis from a three-way ANOVA with
culture time, hydroxyapatite concentration and culture medium as the factors are reported in Table 1.

3.3 Mechanical Properties of the Hydrogels with Encapsulated MC3T3-E1 Cells

The compressive modulus of the cellular constructs was assessed as a function of culture time and experimental treatment (Figure 4). Time was (p=0.002) a factor, but not hydroxyapatite concentration or culture medium in the compressive modulus.

![Graph showing compressive modulus of hydrogels with encapsulated MC3T3-E1 cells for different concentrations of HA in the hydrogel after 28 days of culture in osteogenic medium. Data presented as the mean with standard deviation as error bars for n=3 replicates. P-values are reported by three-way ANOVA with time, concentration of HA (%HA) and type of media as the factors.]

Figure-4. Compressive modulus (in kPa) of the hydrogels with encapsulated MC3T3-E1 cells for different concentrations of HA in the hydrogel after 28 days of culture in osteogenic medium. Data presented as the mean with standard deviation as error bars for n=3 replicates. P-values are reported by three-way ANOVA with time, concentration of HA (%HA) and type of media as the factors.
3.4 Alkaline Phosphatase and Mineralization Assessment in Cellular Hydrogels

Alkaline phosphatase (ALP) activity was assessed as a measure osteoblast differentiation as a function of culture time, hydroxyapatite concentration, and culture medium (Figure 5A). ALP activity was affected ($p<0.001$, $p<0.001$, and $p=0.002$, respectively) by time, hydroxyapatite concentration, and culture medium. ALP activity increased with time and in general the greatest increase was observed between days 0 and 14. ALP activity was highest in the 1wt% hydroxyapatite condition compared to the 0% and 0.1 wt% hydroxyapatite conditions for both culture media. ALP activity was higher in differentiation medium for the treatment conditions with hydroxyapatite.

The hydrogels were stained by Von Kossa for mineralization at day 0 and at day 28 (Figure 5B). There was minimal positive staining at day 0. Qualitatively, there was an increase in mineral deposition with increasing hydroxyapatite concentration in both culture media. In differentiation medium, the amount of mineral deposits appeared to be much greater than in growth medium. This observation was most pronounced with 1wt% hydroxyapatite where mineral deposits were present throughout the hydrogel in the differentiation media condition, but were more localized in the growth media condition.

At 28 days, Raman spectra were collected for all experimental treatments (Figure 5C and 5D). The regions that stained positive for mineral presence by Von
Kossa correlated to the hydroxyapatite peak observed through Raman spectroscopy. All treatment conditions showed the well-defined characteristic peak of hydroxyapatite at 960 cm\(^{-1}\).
Figure 5. Mineralization assessment of encapsulated MC3T3-E1 cells in the PEG hydrogels and cultured for 28 days. A) Alkaline phosphatase activity assay. Data presented as the mean with standard deviation as error bars for n=3. P-values are reported by three-way ANOVA with time, concentration of HA (%HA) and type of media as the factors. B) Microscopy images of sections stained by Von Kossa showing the mineral deposition by MC3T3-E1 cells in the hydrogel. Mineral stained black and cell nuclei pink. Scale bar is 100 µm. C) and D) Characteristic Raman spectra for different concentrations of HA in the hydrogel after 28 days of culture in osteogenic medium.
3.5 Collagen Accumulation in Cellular Hydrogels

Total collagen content normalized to dsDNA was assessed as a function of culture time, hydroxyapatite concentration, and culture medium (Figure 6A). Total collagen content was affected ($p<0.001$, $p<0.001$, $p=0.001$, respectively) by culture time, hydroxyapatite concentration and culture medium. In general, total collagen content increased with culture time and was the highest with 1wt% hydroxyapatite and even higher with differentiation medium. The cellular hydrogels were also assessed qualitative for the presence and spatial distribution of collagen type I by immunohistochemistry (Figure 6B). There was no detectable collagen I staining at day 0 in hydrogels with 0wt% hydroxyapatite. However, with 1wt% hydroxyapatite, pericellular deposition of collagen I was evident. By day 28, collagen I was present in all experimental treatments and appeared to be the highest in the hydrogels with 1wt% hydroxyapatite, which corresponded with the total collagen content. Qualitatively, both media showed similar spatial deposition of collagen I.
Figure 6. Collagen type I deposition assessment. A) Total Collagen Content as function of time. Data presented as the mean with standard deviation as error bars for n=3. P-values are reported by three-way ANOVA with time, concentration of HA (%HA) and type of media as the factors. B) Immunohistochemical images of Collagen Type I deposition in the hydrogels cultured with time. Collagen type I stains green and DAPI (Blue) stains for cells. Scale bar is 50 µm.
4. DISCUSSION

This study demonstrates that the addition of hydroxyapatite particles into a MMP-sensitive PEG hydrogel with RGD enhances differentiation of MC3T3-E1 cells evident by increased alkaline phosphatase activity and increased deposition of ECM that was comprised of mineral deposits and collagen, the two main ECM components in bone. While hydroxyapatite on its own appeared to have some osteogenic potential within the PEG hydrogels, the combination of hydroxyapatite with differentiation cues in the culture medium led to the highest alkaline phosphatase activity and ECM deposition.

The live/dead viability staining results show the morphology of the MC3T3-E1 immediately after encapsulation, after 14 and 28 days (Figure 3A). Initially, the cells have a rounded shape, typical of normal MC3T3-E1 cells before being differentiated. Similar to the mechanical results, the cells become spread, acquiring a different morphology. However, their shape do not change when they are incubated in normal MC3T3-E1 growth media, since these changes are not seen when they are cultured without differentiation factors. Besides that, the concentration of hydroxyapatite doesn’t affect the level of differentiation of the cells since it doesn’t seem to be a difference in the morphology of them. The cellular DNA content showed a small decay in the number of cells with time. At the same time, the number of cells didn’t show significant differences between media types and concentration of HA. An elevated number of cells indicates cell survival, robust growth and proliferation within the hydrogels and that they did not migrate out of the scaffolds.

The addition of a small amount of hydroxyapatite (in form of nanoparticles powder), up to 1wt%, does not affect the initial mechanical properties. Mechanical properties of the acellular hydrogels were tested to characterize the scaffolds before encapsulating cells and starting the study (Figure 2). Compressive modulus and wet weights were obtained (Figure 2A and 2B). The hydrogels were stable evident by the mechanical properties and the weight (both of which do not show change).
Even though the addition of mineral is known to be challenging in some cases, the authors did not find any difficulties under the conditions studied in this paper.

Mechanical data of the hydrogels with encapsulated pre-osteoblast MC3T3-E1 cells, suggest ECM-like matrix formation, since compressive modulus increase over time (Figure 4).

Raman spectroscopy performed at the end of the study, by day-28 (Figure 5C and 5D), showed that hydroxyapatite was present in all hydrogels for all conditions (type of media and concentration of HA), confirming its presence after encapsulation and swelling, which implies that at some level, the MC3T3-E1 cells started to differentiate, even at the 0% HA concentration condition, since hydroxyapatite is a marker of cell differentiation into osteoblast and the main component of human bone, also known as bone mineral (70%w/w)\textsuperscript{30}. Raman was performed in order to characterize the spectrums of pure hydroxyapatite particles and was also measured for PEG hydrogel (which is the main component of the hydrogels when they are dried for measuring) as shown in Figure 1C.

The staining showed that this type of hydrogels support differentiation of MC3T3-E1 cells into osteoblasts. Mineralization was assessed through Von Kossa staining and ALP activity assay. MC3T3-E1 cells secreted and deposited minerals, mainly seen when they were cultured in osteogenic media. However, in this case, the Von Kossa staining showed a difference between concentrations of hydroxyapatite as well (i.e., black regions), which supported the stated hypothesis that a higher amount of HA induces a higher level of differentiation of the osteoblast phenotype. MC3T3-E1 pre-osteoblast cells are known to express elevated Alkaline Phosphatase activity, which is an osteoblast marker that indicates cell differentiation\textsuperscript{31,32}. The results for the ALP enzyme activity show that the osteogenic differentiation of the cells is enhanced by the addition of HA to the scaffolds, especially when they are incubated in the presence of ascorbic acid, β-glycerolphosphate and dexamethasone over the course of 28 days.
In osteogenic media, there is not a lot of mineral staining evident, but hydroxyapatite leads to widespread mineralization. This finding is really promising because a greater source of phosphate can help support mineralization. Also, an increased ALP shows that cells are more osteogenic, which could mean that the high mineral deposits are potentially due to a combination of hydroxyapatite serving as a nucleation site and the enhanced osteogenic state of cells caused by the differentiation factors supplemented in the medium.

Even in growth media, some mineral deposits were evident with increasing hydroxyapatite. That could happen because the medium in which the hydrogels were incubated (phosphate buffered saline, PBS) can provide a source of phosphate and calcium mineral to the scaffolds. Consequently, these minerals present in the medium can serve as nucleation sites that cause further mineralization together with the MC3T3-E1 cells that are differentiating and secreting molecules that contribute to mineralization as well.

Collagen type I deposition staining showed an increase over time, even though there wasn’t a big difference between the two types of medias, which means that the pre-osteoblast cells deposited collagen regardless, without being affected by the differentiation level.

Accordingly, the total collagen assay indicated an increase of collagen deposition with time, and even though the media where they were incubated was probed to be statistically significant, the obtained values of total collagen didn’t show a big difference, supporting the findings of the Collagen I staining. Both assessments showed that the concentration of HA plays an important role on calcium deposition.

The difference between 0% and 0.1% was minimal, but both the values and images showed a bigger difference displaying higher collagen deposition for the 1% HA concentration. Those findings confirm the hypothesis that higher concentrations of HA would enhance higher levels of cellular osteogenic differentiation. 


Furthermore, it also demonstrates that 0.1% HA helps differentiating cells but that 1% HA is a better option if the goal is to obtain a higher level of differentiated osteoblasts in a shorter period of time.
5. CONCLUSIONS

The findings of this paper will help in the design of synthetically formed scaffolds to improve the bone tissue engineering outcome. The addition of hydroxyapatite has been proved to support osteogenic differentiation of the encapsulated MC3T3-E1 cells while maintaining cell viability and proliferation throughout the study. This conclusion is consistent with previous reports showing that hydroxyapatite present in scaffolds formed by synthetic materials since the beginning of the culture, promoted higher osteogenic differentiation of the cells\textsuperscript{35-38, 24-26}. However, this is the first time that the effect of hydroxyapatite has been studied for this particular system of biodegradable MMP-sensitive PEG hydrogels showing positive results.
CHAPTER 2

The *In Vitro* Effects of Macrophages
on the Osteogenic Capabilities of MC3T3-E1 Cells Encapsulated in Biomimetic and Enzymatically Degradable Poly(Ethylene Glycol) Hydrogel

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1. INTRODUCTION

Synthetic hydrogels with their high water content are promising platforms to encapsulate cells in three-dimensional (3D) scaffolds for tissue engineering. Extracellular matrix (ECM) moieties and degradable crosslinks are readily incorporated into synthetic hydrogels creating highly tunable 3D environments that can be designed to direct cell fate and promote tissue growth [1–6]. Injection and/or implantation of cell-laden hydrogels has several benefits over ex vivo tissue engineering. These benefits include the presence of local cues that are native to the tissue environment, improved integration of the engineered tissue with the host tissue, and removing the need for long-term culture prior to implantation. However, the in vivo environment introduces additional complexities, which may affect the ability of cells to synthesize and deposit tissue. While numerous studies have focused on hydrogel designs to promote growth of a specific tissue, the impact of the in vivo environment is not well understood. This environment, however, will be critical to the translation of a successful in vivo tissue engineering strategy.

When any cell-laden scaffold is placed in vivo, the process of surgically implanting the scaffold injures the surrounding tissue and induces an acute inflammatory response. For non-biological scaffolds, this response will lead to a sustained foreign body response (FBR). The FBR is a localized innate immune response [7], which is characterized by the persistent presence of macrophages, chronic inflammation and fibrous encapsulation [8]. The FBR occurs ubiquitously to nearly all non-biological materials [9] and is considered a normal, biocompatible response to implanted scaffolds [10]. A number of studies has reported a FBR to implanted hydrogels marked by fibrosis and prolonged inflammation. These include crosslinked collagen hydrogels [11], dextran-based hydrogels [12], alginate hydrogels [13], poly(2-hydroxyethyl methacrylate) hydrogels [10], poly(ethylene glycol) hydrogels [14,15], poly(lactic acid-b-ethylene glycol-b-lactic acid) hydrogels [16], PEG/sebacic acid-based hydrogels [15], and poly(propylene fumarate-co-ethylene glycol) [17]. We have characterized the FBR to PEG hydrogels and
reported that \textit{in vitro} macrophages readily attach to PEG hydrogels through non-specific protein adsorption [14] and that \textit{in vivo} macrophages are recruited to the implant within two days post-implantation [18–21], and a fibrous capsule forms within four weeks [22]. While the FBR to hydrogels has been studied extensively, the impact of the FBR on cell-laden hydrogels has received less attention.

Although many implants (e.g., arterial stents, artificial joints) can function despite the FBR, tissue engineering strategies, where cells are delivered within the scaffold, require that the cells themselves function to synthesize and deposit their own tissue. It is well known that chronic inflammation inhibits tissue remodeling [23], which suggests that the events of the FBR may adversely affect the cells that reside within a scaffold. We previously reported that inflammatory macrophages seeded directly on top of a PEG hydrogel with encapsulated fibroblasts adversely affected the fibroblasts by reducing gene expression of ECM molecules and elevating gene expression for pro-inflammatory cytokines [24]. In another study, a distinct FBR was noted with increased macrophage presence concomitant with diminished cartilage regeneration when a cell-laden poly(l-lactic acid) scaffold was placed into a cartilage defect of a canine model [25]. In addition, the presence of a fibrous capsule created a barrier between an oligo(poly(ethylene glycol) fumarate) hydrogel that was implanted into a rabbit cranial bone defect and new bone that had formed at the perimeter of the defect [26]. Collectively, these studies and others demonstrate that the events associated with the FBR can impede tissue regeneration and integration for implanted scaffolds, thus warranting further study.

The overall goal for this study was to examine the effects of macrophages, the drivers of chronic inflammation in the FBR, on the long-term culture of a cell-laden hydrogel for bone tissue engineering. To achieve this goal, an \textit{in vitro} co-culture model system was used based on MC3T3-E1 pre-osteoblastic cells, which were encapsulated in a degradable bone mimetic hydrogel, and cultured in a transwell configuration in the presence of murine macrophages. A bone tissue engineering system with murine MC3T3-E1 cells was chosen because these well-characterized
cells produce a quantifiable amount of bone ECM. A photoclickable and degradable PEG hydrogel based on the thiol and norbornene click reaction was chosen for its promise as a scaffold for wide range of tissue engineering applications [27] including bone [28] and for its ease with which ECM moieties are introduced via thiol-ene click chemistry [29–32]. Herein, the MC3T3-E1 cells were encapsulated in a bone mimetic hydrogel containing the cell-adhesion peptide, RGD, matrix metalloproteinase (MMP)-sensitive peptide crosslinks, and hydroxyapatite particles. Moreover, we have previously confirmed the FBR to MMP-sensitive PEG hydrogels with the accumulation of inflammatory cells and fibrous encapsulation in a subcutaneous mouse model [20].

The specific objectives of this study were to a) evaluate the cell-laden hydrogels for cell viability, cellular morphology, osteogenic capabilities, and ECM deposition under in vitro simulated FBR conditions and b) evaluate how macrophage source (i.e. a murine macrophage cell line (RAW 264.7) and macrophages derived from murine bone marrow monocytes [33]), which has been shown to differ in their activation in vitro [34] affects the cell-laden hydrogels.

This study demonstrates that while MC3T3-E1 cells are able to secrete bone-like ECM molecules within the bone mimetic hydrogel, osteogenic capabilities and ECM accumulation are compromised under simulated FBR conditions, but in a manner that depends on the macrophage source. Findings from this study further support the idea that the FBR can impede tissue growth in cell-laden synthetic-based hydrogels.
2. MATERIALS AND METHODS

2.1 Macromer Synthesis and Hydrogel Formation

The macromolecular monomer (macromer), 8-arm poly(ethylene glycol) functionalized with norbornene, was synthesized following established protocols [29,31]. Briefly, 8-arm PEG-NH\textsubscript{2} (20kDa, JenKemUSA) was dissolved in dimethylformamide (Sigma) and reacted overnight with excess 5-norbornene-2-carboxylic acid (Sigma), 2-(1H-7-Azabenzotriazol-1-yl)1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (Sigma), and N,N-Di-isopropylethylamine (Sigma). The product was precipitated in cold diethyl ether, dialyzed against de-ionized water, and lyophilized. Functionalization of each arm of a PEG molecule with a norbornene was confirmed to be >95\% by \textsuperscript{1}H-NMR.

A precursor solution was prepared with 8\% (w/w) 8-arm PEG norbornene, 2.5 mM CRGDS (Genscript), the bis-cysteine MMP sensitive crosslinker GCVPLSLYSGCG [20] (Genscript) at a 0.83 [thiol]:[ene] molar ratio for the crosslinker to the 8-arm macromer, and 0.05\% (w/w) photoinitiator (Irgacure 2959, BASF) in phosphate buffered saline (PBS, pH 7.4). The precursor solution was sterile filtered (0.22µm). Hydroxyapatite nanoparticles (HA, Sigma) were sterilized by autoclave and then combined with the precursor solution at 1\% (w/w). This precursor solution was polymerized under 352 nm light at 6 mW/cm\textsuperscript{2} for 6 minutes in cylindrical molds of ~2 mm height and 4.5 mm diameter. All procedures were performed in a biosafety cabinet following aseptic techniques with sterilized instruments.
2.2 MC3T3-E1 Cell Culture and Encapsulation

A murine pre-osteoblast cell line MC3T3-E1 (ATCC, CRL-2593) was expanded in alpha-MEM media (Gibco) with 10% fetal bovine serum (FBS, Atlanta Biologicals) and 50 U/mL penicillin, 50 µg/mL streptomycin (1% P/S, Corning).

MC3T3-E1 cells were cultured to ~90% confluency and then collected using 0.05% trypsin/EDTA (Gibco). Cells were combined at 2x10^7 cells per mL precursor solution and polymerized as described above. Cell-laden hydrogels were cultured in 24-well plates as mono-cultures or in a 24-well transwell inserts with RAW 264.7 or primary murine macrophages (see below) seeded at the bottom of the well plates as co-cultures. The mono-culture and co-cultures were placed in osteogenic differentiation media containing alpha-MEM, 10% FBS, 1% P/S, supplemented with 10 mM β-glycerophosphate (Sigma), 0.1mM dexamethasone (Sigma), and 50 µg/ml L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma). The medium was supplemented or not with 1 µg/mL lipopolysaccharide from E. coli (LPS-EB O111:B4, standard purity, Invitrogen).

2.3 RAW 264.7 Cell Culture

A murine macrophage cell line RAW 264.7 (ATCC, TIB-71) was expanded in DMEM (Gibco) supplemented with 10% FBS (Atlanta Biologicals) and 1% P/S (Corning) with 0.05% Fungizone (Corning).

RAW 264.7 cells were cultured to ~85% confluency and then collected using a cell scraper. Macrophages were plated at 2,650 cells/cm² in the bottom of 24 well plates and allowed to adhere overnight. At which time, MC3T3-E1-laden hydrogels were placed into the wells containing macrophages using transwell inserts. The co-culture system was then cultured in osteogenic differentiation medium with or without LPS as described above and medium exchanged every two days.
Weekly, macrophages were refreshed by plating new macrophages in a 24 well plate following methods just described and transferring the MC3T3-E1-laden hydrogels along with the transwell inserts to the new 24 well plates.

2.4 Primary Monocyte Isolation, Differentiation and Culture

Bone marrow derived monocytes were isolated from the long bones of 6-8 week old C57BL/6 mice (Charles River Laboratories) by flushing and collecting the bone marrow with Iscove’s Modified Dulbecco’s Medium (IMDM, Gibco) containing 10% FBS, 1% P/S, and 0.5% Fungizone layered with Lympholyte M (CedarLane).

Mononuclear cells were plated on non-tissue culture treated polystyrene and differentiated for 10 days in media containing IMDM, 20% FBS, 1% P/S, 0.5% Fungizone, 2mM L-Glutamine, 1.5 ng/mL human macrophage colony stimulating factor (hMCSF, R&D Systems), and 100 ng/mL huFLT-3 (R&D Systems).

After differentiation, cells were collected by a cell scraper and plated at 2,650 cells/cm² in the bottom of 24 well plates and allowed to adhere overnight. At which time, MC3T3-E1-laden hydrogels were placed in transwell inserts and into the 24 well plates with macrophages.

Following the same methods described above for RAW 264.7 macrophages, the co-culture system was cultured in osteogenic differentiation medium with or without LPS. Freshly isolated and differentiated primary macrophages were refreshed weekly. Medium was refreshed every two days.

2.5 Assessment of MC3T3-E1 pre-osteoblast morphology

Cell morphology was visualized by live cellular staining with 4 nM calcein AM (Corning) immediately after encapsulation and after 10 days of culture in the monocultures and co-cultures.

Whole gels (n=3-4) were cut in half and the cross-section, interior side was imaged by laser scanning confocal microscopy (Zeiss LSM 150).
2.6 Immunohistochemical and Histological Analysis

For immunohistochemical and histological analysis, MC3T3-E1-laden hydrogels (n = 3-4) were collected at 0, 10, and 28 days and fixed in neutral-buffered formalin for four hours at room temperature, then dehydrated and embedded in paraffin following standard protocols. Sections (10 µm) were deparaffinized, hydrated, and stained for apoptosis, anti-collagen I, or mineralization.

Sections were stained for apoptotic cells using the DeadEnd Fluorometric TUNEL system (Promega) per manufacturer instructions. Sections were counterstained with DAPI for nuclei. Sections were imaged by laser scanning confocal microscopy where apoptotic cells were indicated by green fluorescence and nuclei indicated by blue fluorescence.

Four images per hydrogel were acquired per hydrogel and total nuclei and the number of positively stained apoptotic cells were counted per image using NIH Image J and the average percent apoptotic cells per hydrogel was determined.

For anti-collagen I, sections were pretreated with 1 mg/mL pepsin (Sigma) followed by antigen retrieval (Retrievagen, BD Biosciences). After permeabilization and blocking, sections were incubated with collagen I antibody (1:50, Abcam, ab34710) in blocking solution overnight at 4°C. Sections were then treated with a secondary antibody, AlexaFluor 546 goat anti-rabbit antibody (1:200, Invitrogen), for one hour at room temperature and nuclei counterstained with DAPI. Collagen type I is indicated by red fluorescence and nuclei are indicated by blue fluorescence.

Four images were acquired per hydrogel, and analyzed using NIH Image J for average total fluorescence (arbitrary units) normalized to the number of nuclei in each image to determine the fluorescence per nuclei per hydrogel.

For the TUNEL and collagen I stains, sections were stained at the same time and imaged, under the same settings, using a laser scanning confocal microscope.

Sections were stained for mineralization following standard protocols for von Kossa staining. Sections were treated with 1% (w/w) silver nitrate (Sigma) under ultraviolet light for 30 minutes. Unreacted silver was removed by 5% (w/w) sodium
thiosulfate (Sigma) for 5 minutes at room temperature. Sections were counterstained with nuclear fast red (RICCA Chemical Company). Sections were imaged using light microscopy (Axiovert 40 C Zeiss). Mineralization stains black and nuclei stain pink to red.

### 2.7 DNA, Biochemical and Cytokine Assays

Hydrogels (n = 2-4) were collected on days 0, 10, and 28, flash frozen with nuclease-free water in liquid nitrogen, and stored at -700°C. Gels were homogenized for 5 minutes at 30 Hz using a Qiagen TissueLyser II.

Homogenized samples were assessed for ALP activity using p-nitrophenol phosphate with p-nitrophenol used as a positive control and for Calcium content by using the Calcium (CPC) Liquicolor Assay kit (Stanbio).

DNA was quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen). Media from each sample were collected and flash frozen on day 10, which represents conditioned medium from day 8 to day 10, and on day 28, which represents conditioned medium from day 26 to day 28.

Media samples were assessed for cytokines interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) using standard enzyme-linked immunosorbent assay kits (ELISA, R&D Systems for TNF-α, eBioscience for IL-6).

### 2.8 Statistical Analysis

Data are presented as the mean of n = 2-4 hydrogel replicates with standard deviation as error bars in all plots and parenthetically in the text. Statistical analysis was performed using the XLSTAT add-in software for Microsoft Excel® with a two-way unbalanced ANOVA (Table 1).

Factors were culture time (Day 0, 10, 28) and treatment type (± RAW264.7, ± primary macrophage, ±LPS). Comparisons were made using a Tukey post-hoc
analysis with $\alpha = 0.05$. P-values are reported to indicate level of significance with a $p < 0.05$ considered to be statistically significant.

<table>
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<th></th>
<th>Time</th>
<th>Treatment</th>
<th>Time*Treatment</th>
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<td>TNF-α</td>
<td>$p = 0.023$</td>
<td>$p &lt; 0.0001$</td>
<td>$p &lt; 0.0001$</td>
</tr>
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Table-1. P-values calculated with a 2-way ANOVA Interaction between 2 independent variables: time and treatment.

### 2.9 IACUC Approval

All animal protocols were approved by the University of Colorado at Boulder Institutional Animal Care and Use Committee (IACUC) and follow the NIH guidelines for care and use of laboratory animals.
3. RESULTS

3.1 The Effect of Macrophages on MC3T3-E1 Cell Apoptosis

An in vitro model system was designed to study the effects of macrophages on MC3T3-E1 cells encapsulated in a 3D bone mimetic PEG hydrogel containing MMP-sensitive crosslinks, the cell adhesion peptide, RGD, and hydroxyapatite nanoparticles (Fig. 1A). The cell-laden hydrogel was cultured under six experimental treatment conditions to simulate the FBR in vitro, which included culture medium supplemented with or without LPS and mono-culture or co-culture with either RAW 264.7 macrophages or primary macrophages.

The experimental set-up is shown in Fig. 1B. RAW 264.7 macrophages and primary macrophages were refreshed weekly while LPS was refreshed every 48 hours.
Figure-1. A) Representative poly(ethylene glycol)-norbornene (PEG-NB) hydrogel formulation. B) Schematic of the experimental co-culture setup in this study, wherein macrophages were refreshed weekly and lipopolysaccharide (LPS) in the media was refreshed every 48 hours.

The effect of LPS, macrophages, and their combination on MC3T3-E1 apoptosis in the 3D biomimetic hydrogel was assessed with culture time by TUNEL staining (Fig 2). The percentage of apoptotic cells was affected by time (p < 0.0001) and treatment (p < 0.019) (Table 1). Immediately after encapsulation, fewer than 1% of cells stained positive for apoptosis. Culture with RAW 264.7 macrophages did not lead to differences in the percentage of apoptotic cells. Culture with primary macrophages increased apoptosis by 6% at 10 days (p = 0.04) and 12% by 28 days (p = 0.002). LPS stimulation did not significantly affect the percentage of apoptotic cells, whether alone or in co-culture with macrophages.
Figure 2 – The effects of macrophages on MC3T3-E1 cell apoptosis. A) Representative confocal microscopy images of hydrogels immediately after encapsulation and after 10 and 28 days of culture. Cells were stained for apoptosis (green) and counterstained with DAPI for cell nuclei (blue). Scale bar = 50 um. B) Semi-quantification of the percent of apoptotic cells, normalized to number of nuclei in hydrogels after 0, 10, or 28 days of culture. Data are shown as mean (n=2-4) with standard deviation as error bars. “#” denotes statistical significance over day 0, “&” denotes statistical significance over day 10.
3.2 The Effect of Macrophages on MC3T3-E1 Morphology

The morphology of the encapsulated MC3T3-E1 cells in the 3D biomimetic hydrogel was qualitatively assessed by confocal microscopy as a function of LPS, macrophages, and their combination (Fig. 3).

Immediately after encapsulation, the MC3T3-E1 cells displayed a round morphology. At 10 days of culture, the majority of the MC3T3-E1 cells within the hydrogels exhibited signs of cell spreading evident by extended cellular processes.

With the addition of LPS, cell spreading was still evident. However, the encapsulated MC3T3-E1 cells cultured in the presence of RAW 264.7 macrophages with or without LPS retained their round morphology with no observable signs of MC3T3-E1 cell spreading. In the presence of primary macrophages, there were cells that exhibited extended processes as well as round cells. When cultured with LPS and in the presence of primary macrophages, MC3T3-E1 cell spreading appeared to be reduced. These results indicate that while MC3T3-E1 cells are capable of spreading within the biomimetic hydrogel, the presence of RAW 264.7 macrophages and to a lesser extent primary macrophages inhibit MC3T3-E1 cell spreading. LPS stimulation appeared to have minimal effects.
Figure 3 – The effects of macrophages on MC3T3-E1 cell morphology. Representative confocal microscopy images of hydrogels immediately after encapsulation and after 10 days of culture. Cells were stained using calcein AM to visualize morphology. Scale bar = 100 um.

3.3 The Effects of Macrophages on Collagen I Deposition by MC3T3-E1 Cells

MC3T3-E1 cell-laden biomimetic hydrogels were assessed for collagen I deposition one day following encapsulation and after 10 and 28 days in culture as a function of LPS, macrophages, and their combination (Fig. 4). Representative confocal microscopy images are shown in Fig. 4A along with quantitative analysis in Fig. 4B.

Initially, the MC3T3-E1 cells stained for collagen I, but the staining was largely localized in what appeared intracellularly. At day 10, MC3T3-E1 cells deposited collagen I that formed an interconnected matrix within aggregates of cells. Similar results were observed at day 28. With the addition of LPS, aggregates of cells with an interconnected matrix of collagen I were evident, but the staining was not as pronounced. In the presence of RAW 264.7 macrophages, collagen I staining was evident, but it was localized pericellularly at day 10 and remained...
localized at day 28. Treatment with LPS did not appear to affect the spatial organization of collagen I. Similar results were observed with primary macrophages.

Fluorescence intensity of the collagen I stain per nuclei was quantified as a measure of collagen I content. Collagen I was affected by time (p < 0.0001) and treatment (p < 0.0001) (Table 1). In mono-culture, collagen I increased (p = 0.0004) from day 0 to day 10 and then remained constant at day 28 without LPS stimulation. With LPS stimulation, collagen I increased but not to the same extent as without LPS at day 10, but by day 28 was similar regardless of LPS. In co-culture with RAW 264.7 macrophages regardless of LPS stimulation, collagen I did not change with culture time with the exception of day 10 with LPS stimulation, which was higher than day 0. By day 28, collagen I was lower in the cultures with RAW 264.7 macrophages without (p < 0.0001) and with (p = 0.0004) LPS compared to unstimulated mono-cultures. In co-culture with primary macrophages, collagen I increased by day 28 without LPS, but was slightly lower (p = 0.053) than unstimulated mono-cultures. However, with LPS, collagen I was not different from day 0 and lower (p <0.001) when compared to unstimulated mono-cultures.

Overall, the presence of macrophages, whether RAW 264.7 or primary, stimulated with LPS inhibited collagen I at 28 day of the study.
Figure 4 – The effect of macrophages on collagen I deposition by MC3T3-E1 cells. A) Representative confocal microscopy images of hydrogels after 0, 10, and 28 days of culture. Sections were stained for anti-collagen I (red) and counterstained with DAPI for cell nuclei (blue). Scale bar = 50um. B) Semi-quantification of the fluorescence (arbitrary units) normalized to the number of nuclei per image. Data are shown as mean (n=3-4) with standard deviation as error bars. “#” denotes statistical significance as compared to day 0, “&” denotes statistical significance as compared to day 10.
3.4 The Effects of Macrophages on ALP Activity and Mineralization in MC3T3-E1-Laden Biomimetic Hydrogels

MC3T3-E1 cell-laden biomimetic hydrogels were assessed for alkaline phosphatase (ALP) activity (Fig. 5A), total calcium content (Fig. 5B), and spatial organization of mineral deposition (Fig. 5C) as a function of LPS, macrophages, and their combination.

ALP activity was affected by time \((p = 0.012)\) and treatment \((p < 0.0001)\) (Table 1). At day 10, the unstimulated mono-cultures of the MC3T3-E1 cells in the hydrogels had the highest \((p < 0.0001)\) ALP activity compared to all other treatments at the same timepoint, and was approximately threefold higher \((p < 0.0001)\) when compared to day 0. For all other treatment conditions at day 10, ALP activity was not different to day 0. At day 28, ALP activity in the mono-cultures returned to levels similar to day 0. In the co-culture with RAW 264.7 macrophages at day 28, stimulated with LPS or not, MC3T3-E1 cells had the higher \((p < 0.0001)\) ALP activity when compared to the mono-culture of the MC3T3-E1 cell-laden hydrogels without LPS. However, in co-culture with primary macrophages, stimulated with LPS or not, ALP activity was similar to that on day 0.

Mineralization was assessed by total calcium and von Kossa staining. Total calcium content per hydrogel was affected by time \((p < 0.0001)\), but not by treatment (Table 1).

Total calcium content increased \((p < 0.0001)\) from day 0 to day 10. There was no significant change in total calcium content from day 10 to day 28.

Spatial organization of mineral deposition was qualitatively assessed through von Kossa staining. At day 0, minimal mineral deposition was detected.

Qualitatively, mineral deposition was apparent by day 10 and present in all treatment conditions. At day 10, MC3T3-E1 cell-laden hydrogels cultured with RAW 264.7 macrophages, regardless of LPS, appeared to show more elaborate mineral deposition throughout the construct when compared to the mono-culture and the co-culture with primary macrophages. At day 28, mineral deposition was
pronounced throughout all of the hydrogels with no observable differences among treatment conditions.

Figure 5 – The effects of macrophages on ALP activity and mineralization in MC3T3-E1-laden PEG hydrogels. A) ALP activity after 0, 10, and 28 days of culture.
B) Total calcium content in hydrogels after 0, 10, and 28 days of culture. Data for A and B shown as mean (n = 2-4) with standard deviation as error bars. “#” denotes statistical significance as compared to day 0. C) Representative microscopy images of hydrogels fixed after 0, 10, and 28 days of culture and stained for von Kossa mineralization (black) and counterstained with nuclear fast red for nuclei (pink). Scale bar = 100um.

3.5 Cytokine Secretion from the In-Vitro Co-Culture Model

Secretion of interleukin-6 (IL-6) (Fig. 6A) and tumor necrosis factor-alpha (TNF-α) (Fig. 6B) into the culture medium was quantified by ELISA at select time points of day 10 and day 28, which corresponded to all of the other assessments of the hydrogel. The media at day 10 represents conditioned media from day 8-10, and the media at day 28 represents conditioned media from day 26-28.

IL-6 was not affected by time or treatment independently, but there was a crossover interaction (Table 1). At day 10 in the absence of LPS stimulation, IL-6 levels were undetectable in the mono-culture and co-culture with RAW 264.7 and primary macrophages. At day 10 with LPS stimulation, there was pronounced IL-6 levels in the mono-culture and co-culture with RAW 264.7 and primary macrophages. At day 28, IL-6 levels were detectable in the mono-culture and in the co-culture with primary macrophages without LPS. With LPS, IL-6 levels were detectable, but lower (p = 0.01 for mono-culture; p < 0.001 for co-culture) when compared to their respective treatment without LPS. The co-culture with RAW 264.7 macrophages did not have detectable levels of IL-6 at day 28.

TNF-α was affected by time (p = 0.023) and treatment (p < 0.0001) and there was a significant interaction between time and treatment (Table 1). Secretion of TNF-α was not detected in the mono-culture without LPS at day 10 or day 28, but was detectable at day 28 with LPS. In the co-culture with RAW 264.7 macrophages, TNF-α levels were ~60 pg/ml and increased (p = 0.002) by 10-fold with LPS at day
10. At day 28, TNF-α was not detectable in the co-culture with RAW 264.7 macrophages without LPS, but was detectable with LPS stimulation. In the co-culture with primary macrophages, TNF-α was not detectable in the culture without LPS at day 10, but was detected at ~40 pg/ml with LPS at day 10. At day 28, TNF-α levels in co-culture with primary macrophages had increased from day 10 levels and were higher with LPS.

Figure 6 – Cytokine secretion from the in vitro co-culture model. A) Interleukin-6 (IL-6) and B) tumor necrosis factor alpha (TNF-α) secretion in the media on day 10 and 28 as assessed by enzyme-linked immunosorbent assay (ELISA). Data are shown as mean (n=2-4) with standard deviation as error bars. Double dagger denotes undetectable levels.
4. DISCUSSION

Though PEG hydrogels are promising cell delivery vehicles for tissue engineering [35], their induction of a FBR in vivo, as with any non-biological scaffold, raises questions regarding the role of the FBR in the context of tissue regeneration. This study identified that macrophages led to elevated MC3T3-E1 cell apoptosis, reduced cell spreading, delayed or inhibited alkaline phosphatase activity, and decreased collagen elaboration with or without an exogenous inflammatory stimulant. The specific macrophage effect on MC3T3-E1 cells was dependent on whether the macrophages were from a cell line or derived from bone-marrow monocytes, although both sources had a negative effect on the MC3T3-E1 cells. Collectively, this study demonstrates that while MC3T3-E1 cells are capable of differentiating and depositing bone-like ECM in the presence of macrophages and LPS, this process is significantly impaired.

The biomimetic hydrogel system used in this study supported MC3T3-E1 differentiation and deposition of bone ECM in the absence of macrophages and LPS. Specifically, MC3T3-E1 cells were able to degrade the MMP-sensitive crosslinks, which is consistent with previous reports indicating that MMPs are upregulated during osteogenesis [36,37]. The MC3T3-E1 cells were able to undergo cell spreading in the hydrogel, which is enabled by the combined presence of RGD and MMP-sensitive crosslinks. We have previously shown that cell spreading in similar hydrogels is mediated by cell-secreted MMPs [20]. Osteogenic differentiation was confirmed by increased ALP activity, a known early marker of osteogenesis. Bone ECM deposition was evident by collagen I and mineralized matrix present throughout the hydrogel construct. The presence of the hydroxyapatite nanoparticles, which provide osteoinductive cues to the cells, can serve as nucleation sites for mineralization. Overall, these results confirm that the bone-mimetic hydrogel is a promising system for bone tissue engineering and thus is a suitable system to determine whether a simulated FBR would affect the encapsulated cells and their ability to synthesize ECM.
Chronic inflammation, which is a part of the FBR, is known to induce programmed cell death and contribute to tissue injury [38]. In the simulated FBR, the MC3T3-E1 cells were continuously exposed to macrophages and/or LPS. Overall the percentage apoptotic MC3T3-E1 cells remained below ~15% during the 28 day culture regardless of condition. Primary macrophages had the most significant effect on MC3T3-E1 apoptosis over the untreated hydrogels, which is likely through leukocyte secreted proteins [39]. LPS did not have any additional effect, suggesting that macrophages on their own are capable of inducing apoptosis. Interestingly, long-term exposure of the MC3T3-E1 cells to LPS did not induce apoptosis. LPS has been shown to induce apoptosis in several cell types [40], including osteoblasts in vitro [41,42]. However, other studies have reported an increased resistance to drug treatment when cells are cultured in 3D cultures when compared to 2D cultures [43]. Furthermore, the nature of the 3D culture may also serve as a barrier to transport especially as cells deposit their own ECM. These findings demonstrate that macrophages in the absence of LPS stimulation are able to induce MC3T3-E1 apoptosis, albeit at low levels, the in vitro co-culture model.

The biomimetic hydrogel created an environment whereby encapsulated MC3T3-E1 cells locally degraded the hydrogel enabling extension of their cellular processes and cell spreading. The presence of macrophages reduced or inhibited MC3T3-E1 cell spreading, which was most pronounced in the presence of RAW 264.7 macrophages. This observation suggests that MMP activity was inhibited either directly or indirectly by regulation of MMP activity in the MC3T3-E1 cells. MMPs have been shown to be upregulated during osteogenesis [36,37] and are necessary for bone formation in vivo [44,45]. Specifically, studies with osteogenically differentiating MC3T3-E1 cells have reported increased expression MMP-2 and MMP-9 [46]. Thus, it is possible that an inhibition in osteogenesis, due to the simulated FBR environment with macrophages, led to the reduction in MMP activity and subsequent reduction in cell spreading. A reduction in cell spreading may further slow differentiation [47–49] and subsequently slow ECM deposition and growth. Although chronic inflammation is generally associated with increased
levels of MMP activity [50,51]. MMP activity is tightly regulated. Indeed studies have shown that macrophages *in vitro* secrete MMPs (e.g., MMP-2 and MMP-9), but these are accompanied by TIMPs (e.g., TIMP-1) [52], where TIMP-1 closely regulates MMP-9 activity [53,54]. We have also reported that macrophages *in vitro* do not rapidly degrade the MMP-sensitive crosslinker in a PEG hydrogel nor does the hydrogel undergo rapid degradation in vivo [20]. While it is also possible that macrophage-secreted TIMPs may contribute to the reduction in MMP activity within the hydrogel, it seems more likely that macrophages influence MC3T3-E1 cells, which then affects their MMP activity. Additional studies are needed to identify the exact mechanism(s) that contributed to the observed reduction of cell spreading.

Osteogenic differentiation of the MC3T3-E1 cells, as measured by ALP activity, was affected by LPS, macrophages and their combination. An inflammatory environment has been shown to be detrimental to osteogenesis [55,56]. At 10 days, ALP activity was inhibited under all inflammatory conditions. LPS and pro-inflammatory cytokines (e.g., TNF-α) act by up-regulating NF-κB signaling [57], which has been shown to inhibit ALP activity in osteoblast-like cells [58–60]. In addition, many cell types including MC3T3-E1 [61] and macrophages [62] have toll-like receptor 4, which recognizes LPS [63]. Interestingly, an increase in ALP activity was observed by day 28 in MC3T3-E1 cells cultured with RAW 264.7 macrophages regardless of LPS. Because ALP activity generally peaks between ~7-14 days in culture [64], this result indicates a delay in osteogenic differentiation, which was not observed in the LPS or primary macrophage conditions. However, it is possible that a peak in ALP activity was not captured due to the selected time points. Nonetheless, these data indicate that the inflammatory environment, resulting from LPS alone or macrophages, has a significant effect on osteogenic differentiation within the bone-mimetic hydrogels.

Bone ECM is comprised predominantly of a collagen I and mineralized matrix. The *in vitro* simulated FBR differentially affected ECM deposition by the MC3T3-E3 cells in the bone-mimetic hydrogel. At day 10, collagen deposition was
present under all inflammatory conditions, but substantially reduced when compared to the untreated condition. Collagen synthesis has been shown to be highly susceptible to the presence of pro-inflammatory cytokines [65,66] and thus may be affected by LPS alone as well as by macrophages. However, long-term macrophages had the most significant effect on collagen I deposition, indicating that their secreted factors are more potent over LPS alone. Although the reduction in collagen I deposition may be due to an inhibition in collagen synthesis or due to degradation of the deposited collagen, we believe that the former is likely the dominant mechanism in this study. Since the hydrogel is susceptible to similar MMPs as collagen, rapid degradation of the hydrogel would likely occur if MMP activity was sufficiently high to breakdown the cell-secreted collagen. Moreover, the reduced cell spreading and ALP activity point to a delay in osteogenesis, which would also contribute to a delay in collagen synthesis.

On the other hand, mineralization in the bone-mimetic hydrogels was not sensitive to the in vitro simulated FBR. Mineralization requires mineral precursors and nucleation sites [67,68], which are typically released by osteoblasts in matrix vesicles, which are rich in hydroxyapatite, phosphatases, and calcium-binding materials [69]. Upon breakdown of matrix vesicle membranes, the HA particles are then exposed to ECM (e.g., collagen), where mineralization is regulated [70,71]. By incorporating HA particles directly into the bone-mimetic hydrogel, it is possible to “bypass” the phosphatase-controlled vesicle phase of mineralization, whereby hydroxyapatite particles can act on their own as nucleation sites for mineralization. Alternatively, it has been reported that pro-inflammatory cytokines induce a mineralizing phenotype in bone marrow-derived mesenchymal stem cells [72]. While this mineralization pathway still necessitates ALP activity, the threshold is much lower for mineralization to occur [72]. While we reported high levels of ALP only in the untreated hydrogels cultures, it is possible that since the MC3T3-E1 cells are initially preosteoblasts, the may display basal levels of ALP activity that are above the threshold to induce inflammation-mediated mineralization. Thus, it is possible that mineralization may occur even if differentiation is inhibited. The
presence of mineral, which is known to be osteoinductive, may help to overcome the initial negative effects of the inflammatory environment and thus could explain the observed delay in differentiation.

The release of pro-inflammatory cytokines is known to direct cross talk and differentiation between macrophages and other cells [73,74]. IL-6 is a multifunctional cytokine that regulates a diverse range of functions from inflammation to homeostasis [75]. In bone, the role of IL-6 is primarily understood as a promotor of osteoclastogenesis and important to bone regeneration, but studies on its role in osteoblast differentiation have been conflicting [76–78]. While some studies have implicated IL-6 as an inhibitor of osteoblast maturation [79], others have marked it as being crucial to osteoblast maturation [80]. The role of TNF-α in bone homeostasis, however, is more well-defined, where studies have reported inhibition of osteoblast differentiation and activity by TNF-α [81–85]. LPS stimulation leads to NF-kappa B induced expression of IL-6 and TNF-α, which has been shown in macrophages and mature osteoblasts [86,87]. At day 10, IL-6 was only detectable in the LPS stimulated conditions, which points to LPS-mediated induction of IL-6 by both MC3T3-E1 cells and macrophages. Co-culture with RAW 264.7 macrophages and LPS produced the highest levels of TNF-α of the time points investigated. Interestingly, co-culture with primary macrophages and LPS was the only condition that led to detectable amounts of both cytokines at the day 10 and day 28 time points that were investigated. This condition correlated with significant apoptosis, inhibition in ALP activity, and reduction in collagen deposition. Taken together, these findings confirm that with LPS and/or macrophages, TNF-α is secreted at some point during the culture period and thus may contribute to the adverse effects in the MC3T3-E1 cells in the bone mimetic hydrogel.

It is well known that macrophage phenotype differs with cell source [34,88]. In this study, we sought to investigate the differences in the effects of a murine macrophage cell line (RAW 264.7) and primary bone marrow-derived macrophages on osteogenically differentiating cells. Culture with either macrophage source induced marginal apoptosis and reduced collagen I deposition. Notably, osteoblasts
cultured with RAW 264.7 macrophages were able to recover ALP activity by day 28, while ALP activity remained low in cultures with primary derived macrophages. Macrophage cell lines have been noted to exhibit lower protein secretion than primary macrophages [89,90]. Additionally, IL-6 was measured in the absence of LPS stimulation in co-culture with primary macrophages, but not with RAW 264.7 cell lines. This general reduced activity could explain the recovery in ALP activity in co-culture with the macrophage cell line.

There are several limitations of this work. Crucially, the macrophages were not assessed alone. Therefore, we cannot say how co-culture with the MC3T3-E1 cells impacted macrophage activation. The results we observe in the co-cultures in the absence of LPS stimulation imply cross-talk between the two cell types, and future studies will assess macrophage activity in co-culture as well. Additionally, due to the nature of co-culture systems, the cytokine levels reported in this study cannot be directly attributed to either the macrophages or encapsulated pre-osteoblasts. Future studies will seek to establish an understanding of IL-6 and TNF-α expression in osteoblastogenesis through quantitative polymerase chain reaction (qPCR). Cytokine expression and ALP activity were measured at discrete time points and thus a true temporal change in their levels was not addressed.
5. CONCLUSIONS

Our in vitro studies show that inflammatory macrophages can impact osteoblastic cells, leading to decreased cell spreading within MMP-sensitive PEG hydrogels, reduced alkaline phosphatase activity, and lower collagen I deposition. Importantly, our findings implicate that the FBR, as shown here through in vitro simulated conditions, can delay osteogenesis and slow bone ECM deposition.

Future work will need to determine how the in vivo scenario and the FBR, which is more complex than the in vitro experiments, affects tissue growth. In summary, the FBR to non-biological scaffolds may have a negative effect on the ability of the embedded cells to synthesize and deposit new tissue. Thus, biomaterial designs that reduce the FBR may be critical to improving tissue regeneration capabilities of encapsulated cells.
6. REFERENCES

6.1 First Chapter


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6.2 Second Chapter


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