

# Bone Marrow Microenvironment Culturing System for Mesenchymal Stem Cells

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# I. Introduction and Background

## Motivation

Degenerative musculoskeletal diseases, such as osteoarthritis (OA), affect the majority of adults ages 65 and over [1]. OA occurs as a progressive breakdown of cartilage that results in debilitating pain, stiffness, and swelling in the joints [2]. In extreme cases, OA is addressed by total joint replacement surgery [3]. While a joint replacement can provide the patient with pain relief and restored function, this option is complicated by the risk of surgical mortality, an undesirable recovery period, and a high cost associated with the procedure [4]. Although there are less invasive treatments for early-to-moderate-stage disease, such as pharmacological approaches, patient education, and physical therapy, these approaches are aimed at symptom relief and do not address the joint degeneration that underlies the disease process [5]. In response to the lack of therapies that target the actual disease mechanism, a regenerative medicine approach employing human mesenchymal stem cells (hMSCs) has emerged.

## Human Mesenchymal Stem Cell Biology

hMSCs are multipotent and self-renewing, with the capability of differentiation into osteocytes, adipocytes, and chondrocytes [6]. Beyond their multipotency, hMSCs exhibit immunoregulatory properties, homing abilities, and secretion of multiple growth factors and cytokines that make these cells desirable for use in regenerative medicine applications [6, 7]. Thus, introduction of hMSCs to the joint space could modulate the chemical environment of the joint and promote the regeneration of cartilage, combating the inflammatory and degenerative mechanisms of OA [8]. However, the study of hMSCs for therapeutic application is currently hindered by loss of potency and inability to achieve quiescence during *in vitro* culture [9].

*In vivo*, undifferentiated hMSCs reside in several microenvironments including bone marrow, adipose tissue, and placenta [7]. Bone marrow is considered the most accessible and enriched source of hMSCs for medical research and application, with a microenvironment that is characterized by a low stiffness extracellular matrix, low oxygen tension, and a milieu of secreted factors, all of which modulate the state of hMSC behavior [7, 10]. Previous studies suggest that hMSC fate is modulated by substrate stiffness, as hMSCs differentiate into the cells composing the tissue of similar elastic modulus as the culture substrate [11]. hMSCs have also been found to maintain quiescence *in vitro* when cultured on scaffolds of 250 Pa [9]. Under hypoxic conditions, hMSCs have been shown to proliferate and can be maintained in a multipotent state [12]. However, current research has not evaluated the effects of co-variation of substrate stiffness, oxygen tension, and soluble factor milieu in an attempt to allow modulatory control over stem cell activity.

## Client Information

Our client, Professor Wan-Ju Li, is an associate professor of Biomedical Engineering at UW-Madison. His research is focused on musculoskeletal disease and stem cell-based regenerative medicine. Specifically, Professor Li is interested in improved culture methods for hMSCs in order to maintain their quiescence *in vitro*, which can advance the study of potential treatments for musculoskeletal disease.

## Problem Statement

Current culture methods are variable in their ability to maintain mesenchymal stem cells (MSCs) in a multipotent state, and do not attempt to recreate the physiological conditions that prevent differentiation and maintain quiescence.

For this reason, there is a need for a culture system that sustains cell quiescence by mimicking the bone marrow niche through substrate stiffness and oxygen tension.

## Existing Designs

Current biomaterial research focuses on recapitulating the bone marrow microenvironment using various biomaterials, such as derivatives of poly(ethylene glycol), fibrin, and polycaprolactone (PCL). Previously, Raic et al. created macroporous poly(ethylene glycol) diacrylate (PEGDA) hydrogels, which mimic spongy trabecular bone, to support hMSC proliferation and differentiation [13]. PEGDA is chemically-defined and easily-modified, making its use for standardized recapitulation of the bone marrow microenvironment a feasible undertaking.

There are various systems on the market that allow for hypoxic cell culture. For example, the StemCell Technologies Hypoxia Incubator Chamber allows cell culture in a small container that fits inside existing incubators. The BioSpherix XVIVO System allows programmable regulation of oxygen tension in a complete hood and incubator system, eliminating unregulated conditions during cell transfer between locations in the laboratory. Unfortunately, these systems are costly; for example, the Hypoxia Incubator Chamber is \$605 for one unit. Thus, these systems are difficult to implement in a budget-conscious research setting.

One system exists that attempts to recapitulate the bone marrow microenvironment. The “Bone Marrow-On-A-Chip” model is a culture of living bone marrow that retains hematopoietic stem cells and progenitor cells for one week [14]. Drawbacks to this system are that it requires bone marrow generation in a mouse, and does not consider oxygen tension or mechanical properties of bone marrow.

## **Design Specifications**

This project consists of two different components: a biomaterial with modular stiffness and a bioreactor with modulatable oxygen tension.

The scaffold must be a natural or synthetic material that permits formation of a gel. By varying factors in the fabrication process, such as polymer concentration, chain length, or crosslinking density, multiple biorelevant stiffnesses ranging from 0.1 - 0.2 kPa [15] can be produced. The scaffold must be compatible with cell culture; it must be sterilizable and non-cytotoxic. Additionally, the scaffold should mimic the properties of the natural extracellular matrix components found in bone marrow.

The bioreactor must deliver a user-set oxygen tension to a cell culture chamber. Biorelevant oxygen tensions range from 1-5% [16], but the chamber should allow regulation of any oxygen tension between 0 and 21% to permit adaptive experimentation. The bioreactor must also deliver 5% carbon dioxide as well as nitrogen to make up the remainder of the gas mixture. Thus, nitrogen tension will vary based on the user-set oxygen tension. The bioreactor must provide humidity and a temperature of 37°C for appropriate cell culture. The chamber containing cells should be sterilizable and prevent introduction of outside contaminants. Overall, the system should be easily cleaned, maintained, and operated by research staff.

Additional details regarding scaffold and bioreactor design specifications can be found in the PDS in Appendix A.

## **II. Design Process**

### **Scaffold**

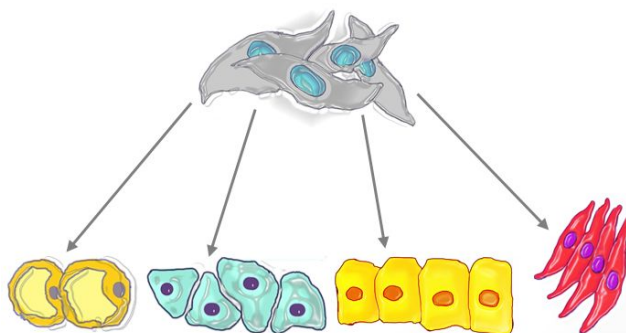
#### **Preliminary Design**

The extracellular matrix and mechanical cues provided by protein-cell and cell-cell interactions are considered key components of the stem cell niche. Substrate or matrix stiffness specifically has been shown to induce lineage-specific differentiation [17, 18]. Some of these lineages are shown in Figure 1. For example, substrates mimicking the physiological stiffnesses of neurogenic, myogenic, and osteogenic environments prompt hMSCs to develop a phenotype which corresponds to the respective tissue.

Current methods to regulate and maintain hMSC quiescence and stemness in culture, specifically when seeded on standard tissue culture plastic with a high Young's modulus, largely involve manipulation of the chemical components in the media. By creating a mechanical environment which resembles the substrate stiffness of human bone marrow, it may be possible

to reduce the dependence of hMSC culture on chemical cues, such as particular types or components of media.

To address this need, we created scaffolds with varying stiffnesses relevant to those of bone marrow, in order to experimentally determine the optimal stiffness for maintenance of hMSC quiescence during *in vitro* culture. The range of stiffness values we attained in the final product was ascertained through a literature search for the Young's modulus of bone marrow. A broad range of stiffnesses was reported, although most reports focused on a range of 0.1-0.2 kPa [19, 20, 15].



**Figure 1:** hMSC differentiation into (left to right) adipocytes, osteocytes, chondrocytes and myocytes.

Initial steps of the following experimental approach focus on obtaining the appropriate mechanical properties of a poly(ethylene glycol) (PEG)-based biomaterial, and the resulting optimized gel fabrication procedure was repeated in conjunction with functionalization for adhesion. PEG gels were crosslinked with a physiologically-relevant Arg-Gly-Asp (RGD) sequence, and later, the full fibronectin protein was covalently linked to PEG scaffolding to engineer a chemically-defined mimic of bone marrow extracellular matrix.

In this project, we fabricated a RGD-conjugated PEG hydrogels with varied elastic moduli to study the effect of substrate stiffness on hMSC quiescence. Previously, Raic et al. created macroporous PEG hydrogels, which mimic spongy trabecular bone, to support hMSC proliferation and differentiation [13]. We used standard salt-leaching methods for 3D pore creation and acrylation of our peptide to conjugate RGD to the gels. PEG is chemically-defined and can be modified with several chemical moieties, allowing us to reduce confounding variables in our experimental process.

## Fabrication

The full fabrication protocol can be found in Appendix A.

## PEGDA Scaffold Fabrication

The first step in scaffold fabrication was to prepare PEGDA gels with stiffness values that correspond to the results of bone marrow characterization, 0.1-0.2 kPa. Initial experiments used PEGDA polymers to facilitate initial RGD-sequence and protein attachment. To control degradation and mechanical properties, PEGDA molecular weight and PEGDA concentration in w/v% can be manipulated.  $M_n$  700 PEGDA (Sigma-Aldrich) at a concentration of 1.12g/mL was used for initial experimentation. The PEGDA can be diluted to the appropriate (determined by experimental testing) w/v% concentration using PBS and curing in UV light at an intensity of 2500  $\mu\text{W}/\text{cm}^2$  for 20 min. Just before curing, the photoinitiator, Irgacure 2959 (Sigma-Aldrich), is added to the hydrogel precursor at 10%w/v of the final solution.

## Acrylation of RGD

Preliminary experiments were performed to determine the effect of varied PEGDA stiffness values on hMSC quiescence. The peptide sequence Arg-Gly-Asp (RGD) was conjugated to PEGDA to permit cell attachment to the biomaterial.

Following the methods of Hern et al., RGD was conjugated to PEGDA by synthesizing mono-acrylamidoyl RGD (RGD-MA). The N-terminal amines of RGD peptides were functionalized with N-hydroxyl succinimide (NHS) ester of acrylic acid (AA-NHS) to produce RGD-MA [21]. First, the RGD peptide was dissolved to a final aqueous concentration of 1 mg/mL in 50mM of sodium bicarbonate buffer. The N-acryloxysuccinimide was dissolved in dimethylformamide (DMF, anhydrous) and added dropwise to the aqueous peptide solution (prepared at molar ratio of 10:1, ester:peptide) to compose 33% of the final volume (final volume of 1.5mL in reaction vial). The reaction vial was agitated continuously at room temperature for 24 hours. The peptides were separated from acrylic acid and hydroxyl succinimide byproducts by dialysis in deionized water for 48 hours with bath changes. This step is done to remove the acrylic acid and prevent the addition of carboxylic acid to the hydrogel during polymerization.

Once the RGD-MA was prepared, it was crosslinked into the hydrogel network by reacting the methacrylate group with acrylate groups on PEGDA through photoinitiation with Irgacure 2959 and UV during the as described above.

## Acrylation of GRGDS

After the gels fabricated with PEG-RGD failed to support cell adhesion, a second peptide, GRGDS was acrylated while using DMSO as the solvent for AA-NHS, dissolving the ester at 5X concentration. The peptide GRGDS was chosen because of its glycine spacer, which is included to better allow cell accessibility to the adhesion sequence. GRGDS was then added to the peptide solution to comprise less than 20% of the final reaction volume while maintaining the 10:1 ester:peptide ratio. The small volume of DMSO would present fewer barriers to fabrication during the dialysis step. 0.1 mg GRGDS was dissolved in 1mL sodium bicarbonate buffer, as before. This time, however, we made a stock of N-acryloxysuccinimide that was at 5X the



desired final concentration (10mg in 2mL DMSO). After consultation with colleagues, we determined that DMSO is as effective as DMF for this step of the methacrylation. By raising the stock concentration, we would be able to add only 200uL of stock to the final reaction mixture. Because the DMSO then comprised less than 20% of the final v/v%, it would be highly unlikely that it would interfere with our cellulose ester dialysis membrane. Additionally, the peptide itself, when the acrylate group was added for functionalization, is greater than 500 Da.

Again, once GRGDS-MA was prepared, it was crosslinked into the PEG hydrogel network using UV and photoinitiator to react acrylate groups.

### PEG-Gelatin Scaffold Fabrication

We first prepared 5.0% and 7.5% w/v% PEGDA Mn 700 dilutions. To make a 2.5% Gel-MA dilution, we dissolved 112.5 mg Gel-MA in 4.5 mL PBS, stirring continuously on a hot plate set to 50°C. 1.5 mL gelMA was added to 1.5 mL of each PEGDA dilution, and then 450 uL Irgacure was combined and mixed well. Polymerization occurred under UV, reacting the methacrylate groups on gelatin with the acrylate groups on PEG.

### Gelatin Scaffold Fabrication

To complete initial testing of the effect of substrate stiffness on quiescence, 5 w/v%, 10 w/v% and 20 w/v% gelatin gels were fabricated, since gelatin is an adhesive material. First, deionized water was warmed to 60-70°C on a hot plate. 0.5g, 1g, and 2g gelatin were each dissolved in 10 ml warm water. Each gelatin preparation was sterile filtered, and then 300 ul of sterile gelatin solution was added to each well of a 48-well plate. The plates were then placed at 4°C for at least two hours to allow gelation.

### Validation

#### Cell Adhesion Testing

For all scaffolds fabricated above (PEG-RGD, PEG-GRGDS, PEG-gelatin, and gelatin), we validated their efficacy in promoting cell adhesion by seeding hMSCs onto the scaffolds after sterilization of the scaffolds with UV and ethanol washes. To evaluate adhesion, the morphology of cells seeded on scaffold conditions was compared to that of cells seeded on tissue culture plastic.

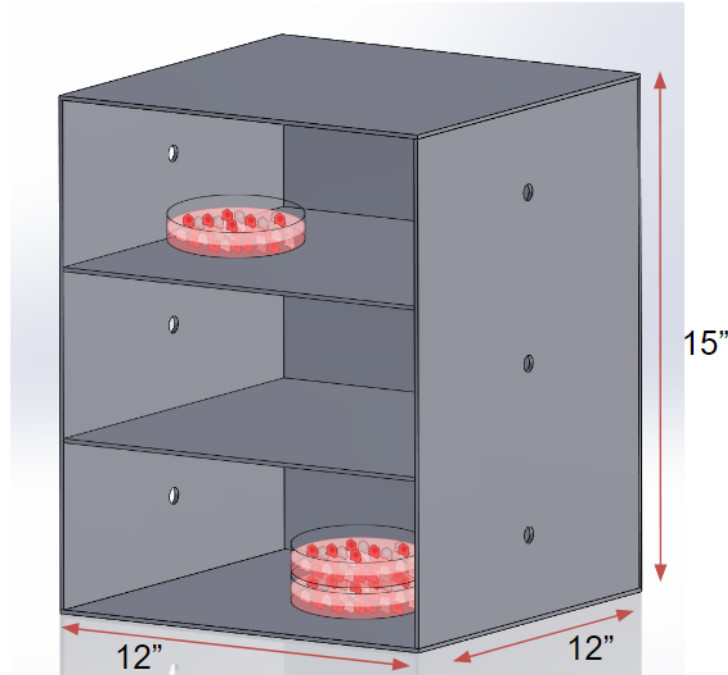
## **Bioreactor**

### Preliminary Designs

Preliminary design ideas for the development of a hypoxia-condition bioreactor included modifying an existing cell culture incubator to include three different hypoxic chambers,

developing a stand-alone chamber that is placed inside an incubator, and purchasing an existing hypoxic incubator.

The first potential design idea was to start with an existing cell culture incubator, and add dividers to create three separate compartments. Gas lines would then be run into each compartment (see holes on left and right walls of chamber in Figure 2) so that the user could set a distinct oxygen tension in each compartment.



**Figure 2:** Preliminary design 1: Modification of existing cell culture incubator.

While this design was simplified by the incubator starting point, which was already designed to provide gas flow and temperature regulation, it requires permanent modification to the incubator, which would prevent its use in different contexts in the future. Additionally, it would have been difficult to fabricate the shelves to prevent gas leakage between compartments.

The second potential design idea was to create a free-standing unit that could be inserted into an existing cell culture incubator. This device would regulate gas concentrations as well as humidity, but could be placed inside the incubator for temperature regulation. By placing three of these devices inside an incubator, experiments could be simultaneously conducted in multiple oxygen tensions. This option was similar to a product on the market, called the StemCell Hypoxia Incubator Chamber (Figure 3). While this device sells for around \$600, we could likely produce a similar product for less money.



**Figure 3:** The StemCell Hypoxia Incubator Chamber, which promoted the second potential design idea.

Drawbacks to a free-standing unit are that this system requires more moving parts, each of which require sterilization and arrangement in the incubator. Additionally, since this device is commercially available, intellectual property considerations should be taken.

The third potential design idea was to purchase a commercially available hypoxia workstation (Figure 4). This system allows complete culture of cells within the hypoxic environment; the cells are not exposed to ambient oxygen concentrations during media changes or cell passages.



**Figure 4:** Baker Ruskin InvivoO2 Hypoxia Workstation.

Although a commercially available hypoxia workstation allows complete cell exposure to the desired oxygen concentration, these units are expensive. Additionally, purchasing a unit would eliminate our experience of designing and building one ourselves: a process which could be replicated by other members of the laboratory if another hypoxia chamber was desired.

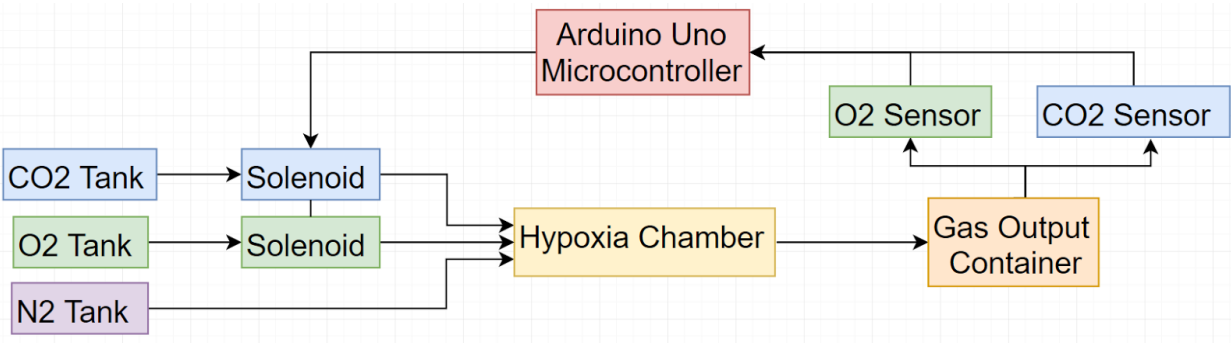
## Final Design

We based our final design off of the second potential design: a free-standing unit that is placed inside an existing cell culture incubator. An Arduino Uno microcontroller interfaces with a computer, where the user can set the desired carbon dioxide (typically 5%) and oxygen tensions (Figure 5). The Arduino then regulates signals sent to two solenoid valves, each of which controls either CO<sub>2</sub> or O<sub>2</sub> gas flow from storage tanks. The oxygen, carbon dioxide, and nitrogen are delivered into an airtight hypoxia chamber, which is housed inside a cell culture incubator.

The incubator provides appropriate temperature regulation, and a dish of water placed inside the chamber provides humidity. A block diagram of this system can be seen in Figure 6.

```
//set O2/CO2 tension desired in chamber
float setO2tension = 14;
float setCO2tension = 5;
```

**Figure 5:** Excerpt of Arduino code allowing the user to specify the desired oxygen and carbon dioxide tensions. In this image, O<sub>2</sub> is set to 14% and CO<sub>2</sub> is set to 5%.



**Figure 6:** Block diagram of bioreactor system.

Air from the hypoxia chamber flows out to a gas output container, which contains O<sub>2</sub> and CO<sub>2</sub> sensors. These sensors report the gas concentrations back to the Arduino. When the concentrations are below those set by the user, the Arduino signals to the appropriate solenoid valve to open and allow gas flow into the hypoxia chamber for two seconds. The pressure of each gas flowing from the storage tanks can be adjusted to fine tune the regulation of gas concentrations in the hypoxia chamber.

## Fabrication

### Materials

Commercial polypropylene containers made up the two core components of the hypoxia system: the gas sensing chamber and the cell culture chamber. Since we wanted a container with low cost and high manufacturability for our first prototype, Rubbermaid Tupperware was chosen. Polypropylene also allowed researchers to have the flexibility to autoclave or wipe down the container with ethanol for sterilization [22]. Other key components of the bioreactor system included an Arduino Uno microcontroller, O<sub>2</sub> sensor, CO<sub>2</sub> sensor, solenoid valves, a breadboard and circuit components, and plastic tubing to allow gas flow. A complete list of materials and expenses can be found in Appendix B.

## Methods

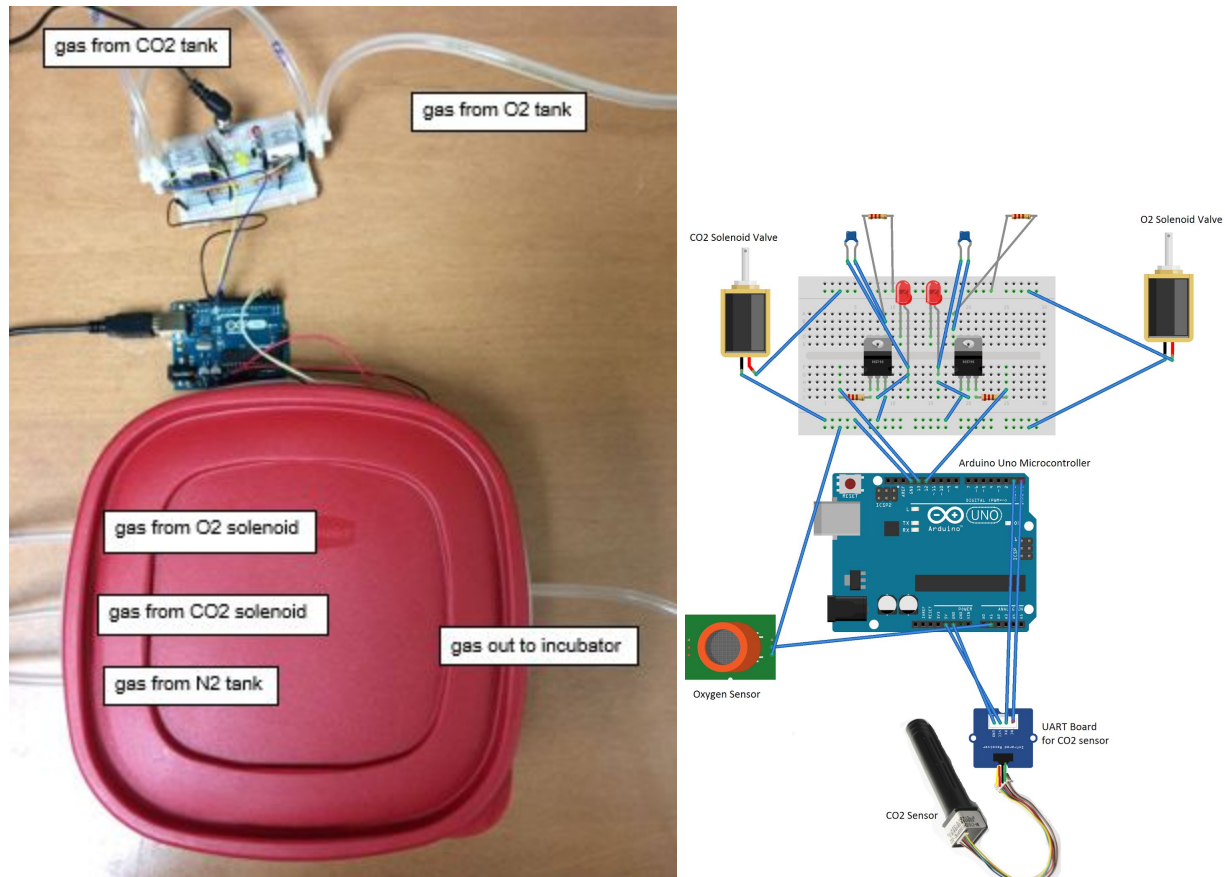
The first chamber, which was used for cell culture, required four holes ( $\frac{1}{8}$  in diameter) for the gas tubing: three on one side for the incoming gas ( $O_2$  and  $CO_2$  regulated from solenoid;  $N_2$  directly from gas tank) and one on the opposite side for the outgoing gas to the gas sensing chamber (Figure 7). The second chamber, used for gas sensing, required one hole for the incoming hypoxic gas mix. Holes were drilled with a CNC Mill (800 rpm, drill bit size D), supported by wood and vices to prevent cracking.

An Arduino Uno controlled the gas sensing system (complete code can be found in Appendix C). Gas sensors were used to read the gas concentration of  $O_2$  and  $CO_2$ , and then two 2-way barbed solenoid valves turned on/off if the gas concentrations were lower/higher than specified, respectively.

## Final Prototype

Our low-cost bioreactor system allows researchers to culture cells in hypoxic conditions (Figure 7). The gas sensor chamber contains  $O_2$  and  $CO_2$  sensors that monitor the gas concentration. When concentrations become too low or high, solenoid valves allow gas from  $O_2$  and  $CO_2$  tanks to pass through or be blocked from the cell culture chamber, which is housed in the Thermo Scientific 3100 incubator, effectively regulating the gas concentration back to specified values.  $O_2$  and  $CO_2$  concentrations are consistently monitored on the Arduino serial monitor.  $N_2$  passes directly from the tank into the cell culture chamber.

Outgoing gas from the cell culture chamber leads into a gas sensing chamber, which is located outside of the incubator. Although the incubator maintains a consistent temperature of  $37^\circ C$ , humidity is maintained within the cell culture chamber through a petri dish of water.



**Figure 7:** Left: Bioreactor prototype system with O<sub>2</sub>, CO<sub>2</sub>, and N<sub>2</sub> regulation. Right: Circuit set-up with communication to Arduino breadboard.

## Validation

The oxygen and carbon dioxide sensors were validated by sensing the gas concentrations in open air and comparing to ambient concentrations. Atmospheric concentrations are 21% oxygen and 0.04% carbon dioxide.

We then validated the solenoid function and Arduino code by setting the oxygen and carbon dioxide tensions higher than atmospheric, for example, 24% O<sub>2</sub> and 5% CO<sub>2</sub>. LED lights on the solenoid circuit confirmed that the solenoids turned on in response to gas concentrations lower than those set by the user. We could also detect gas flow from the solenoid when it was connected to the gas tank and turned on. Detection was simplified by submerging the hose outputting gas from the solenoid into a container of water. Bubbles were indicative of gas flow.

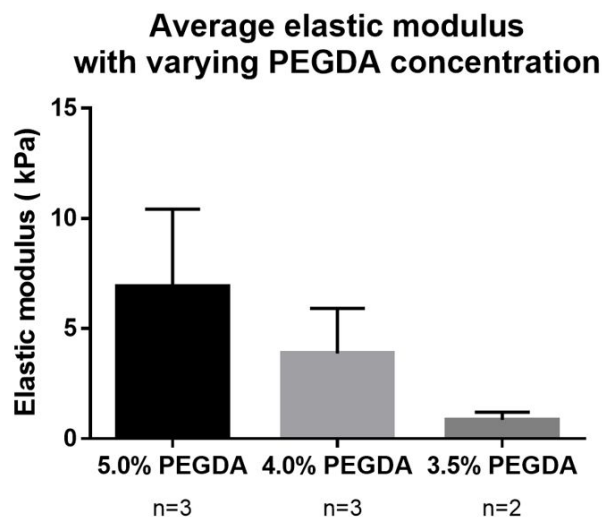
### III. Testing and Results

#### Scaffold

##### PEGDA Mechanical Testing

Mechanical compressive testing was performed on scaffolds using an MTS machine with a 1 kN load cell. The program compressed to a predetermined displacement (1mm in experiments described) and unloaded. The output of this test is a load-displacement curve, which was used to determine the elastic modulus with a MATLAB code that creates a stress-strain curve and allows the user to select the linear portion to determine the slope, and thereby the stiffness of our hydrogel. The MATLAB code can be found in Appendix D.

Testing on gels of various PEGDA polymer concentrations has been performed, and the average elastic moduli of 5%, 4% and 3.5% v/v% PEGDA (Mn 700, 1.12g/mL) gels are all within the ranges found for bone marrow in literature. The data presented in Figure 8 demonstrates a decreasing elastic modulus with decreasing PEGDA concentration, as expected. However, the values obtained at these values are not statistically significant by ANOVA.



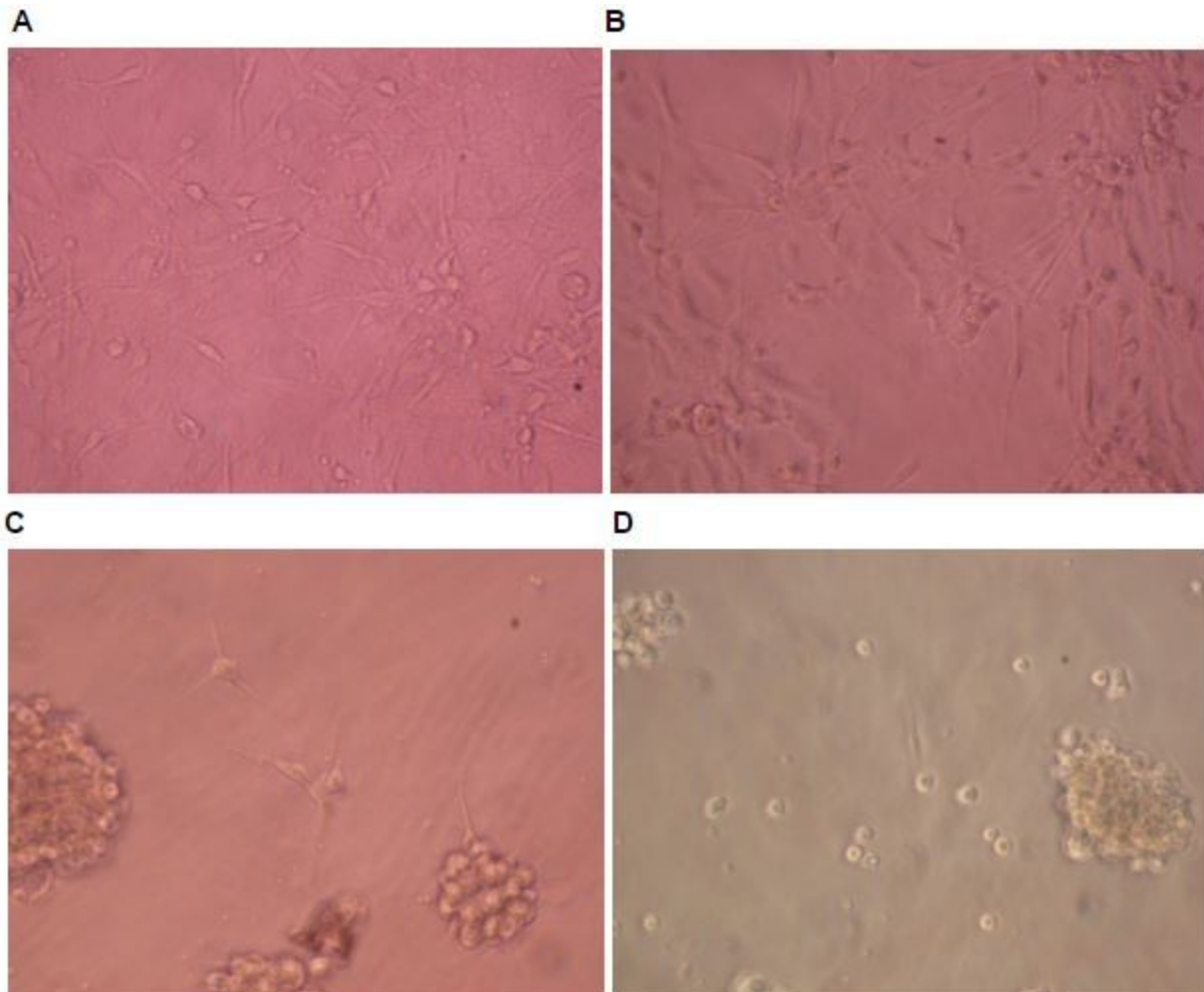
**Figure 8:** Average elastic modulus with varying PEGDA concentration shows higher polymer v/v% concentration increases the elastic modulus (kPa). Differences in elastic moduli not statistically significant.

From the preliminary testing of PEGDA gels without cell encapsulation or tethered RGD sequences, we have shown that we are able to reach stiffness values found in literature for

bone marrow. However, we did not obtain the lower end of this range, 100-200 Pa, which may be more representative of the bone marrow niche.

### Cell Adhesion Testing

All PEG-based gels failed to support adhesion of hMSCs to the surface of the scaffold. This includes PEG-RGD, PEG-GRGDS, and PEG-gelatin gels. Only gelatin gels, shown below in Figure 9, supported adhesion. Still, it appears that 5 w/v% gelatin produces hMSCs with the appropriate morphology (relative to tissue culture plastic control), while the 10 w/v% and 20 w/v% conditions do not permit appropriate adhesion and cell morphology.



**Figure 9:** A: hMSCs plated on tissue culture plastic control. B: hMSCs on 5 w/v% gelatin. C: hMSCs on 10 w/v% gelatin. D: hMSCs on 20 w/v% gelatin.



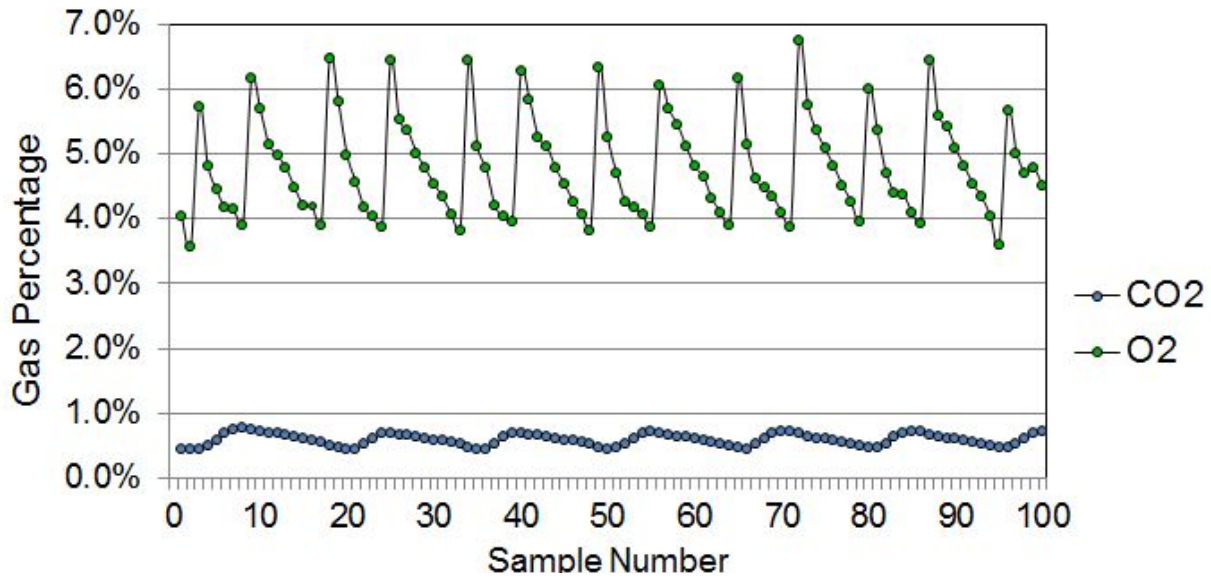
## Bioreactor

### Gas Sensor Validation

After assembling our entire system, we monitored oxygen and carbon dioxide concentrations in the hypoxia chamber over a period of time. The goal of this testing was to evaluate the maintenance of the user-set gas concentrations. Our target feedback range was to have each gas concentration remain within 1% of the user-set value. The Arduino serial output, including time points at which the solenoid valves opened, is shown in Figure 10. Graphs of the resulting concentration fluctuations are shown in Figure 11.

```
Current Sensor Percentages: CO2: 0.518% | O2 percent: 4.098%
Current Sensor Percentages: CO2: 0.495% | O2 percent: 3.895% O2 HIGH      CO2 HIGH
Current Sensor Percentages: CO2: 0.462% | O2 percent: 6.165% CO2 HIGH
Current Sensor Percentages: CO2: 0.454% | O2 percent: 5.148% CO2 HIGH
Current Sensor Percentages: CO2: 0.522% | O2 percent: 4.606%
Current Sensor Percentages: CO2: 0.617% | O2 percent: 4.471%
Current Sensor Percentages: CO2: 0.691% | O2 percent: 4.335%
Current Sensor Percentages: CO2: 0.720% | O2 percent: 4.098%
Current Sensor Percentages: CO2: 0.715% | O2 percent: 3.861% O2 HIGH
Current Sensor Percentages: CO2: 0.682% | O2 percent: 6.740%
Current Sensor Percentages: CO2: 0.638% | O2 percent: 5.758%
Current Sensor Percentages: CO2: 0.622% | O2 percent: 5.352%
Current Sensor Percentages: CO2: 0.600% | O2 percent: 5.081%
Current Sensor Percentages: CO2: 0.577% | O2 percent: 4.810%
Current Sensor Percentages: CO2: 0.552% | O2 percent: 4.505%
Current Sensor Percentages: CO2: 0.523% | O2 percent: 4.268%
Current Sensor Percentages: CO2: 0.493% | O2 percent: 3.963% O2 HIGH      CO2 HIGH
Current Sensor Percentages: CO2: 0.458% | O2 percent: 5.995% CO2 HIGH
Current Sensor Percentages: CO2: 0.458% | O2 percent: 5.352% CO2 HIGH
Current Sensor Percentages: CO2: 0.528% | O2 percent: 4.708%
Current Sensor Percentages: CO2: 0.625% | O2 percent: 4.403%
```

**Figure 10:** Arduino output of oxygen and carbon dioxide concentrations in the bioreactor system over time. The farthest right columns indicate time points at which the oxygen or carbon dioxide solenoids were opened (“HIGH”).



**Figure 11:** Graph of oxygen and carbon dioxide concentrations in the bioreactor system over time. Peaks represent periods when the respective solenoid valve was open, delivering gas to the system.

Peaks and troughs occur in a regular pattern on the graph. Peaks represent periods when the solenoid valve was open, allowing gas flow into the cell culture chamber. The opening of the solenoid valve was prompted by a previous gas sensor reading below that of the set gas tension. After the peak, the following readings represent the gas concentration slowly declining. Once the concentration drops below the user-set value, the solenoid valve opens again and another peak can be seen.

In this experiment, oxygen tensions are maintained within 2% of the user-set value, which was 5%. In an extended experiment, the O<sub>2</sub> concentrations ranged from 3.556% to 7.045%. Carbon dioxide tensions are maintained within 0.3% of the user-set value, which was 0.5%. The CO<sub>2</sub> concentrations in the extended experiment ranged from 0.433% to 0.785%.

## IV. Discussion

### Scaffold Implications

#### PEGDA Mechanical Testing

Based on literature values of PEG elastic moduli, particularly a low molecular weight PEG like the Mn 700 we used, the MTS results we obtained may not be trustworthy. The MTS was likely not sensitive enough to produce accurate measurements. Mn 700 PEGDA should have elastic modulus values in the tens to hundreds of kPa. Mn 3000 PEGDA could theoretically enable us to reach between 1-10kPa, but this was not attempted this semester.

In future studies, all mechanical measurements should be performed on a parallel plate rheometer (Malvern Kinexus). Shear strain should be applied to hydrated PEGDA hydrogels between two 50 mm diameter parallel plates appropriately spaced at a rate of 1% per second and removed at the same rate to create shear stress–strain plots.

## Acrylation of Peptide

During dialysis, the RGD peptide may have escaped through the pores of the membrane. There was DMF in the sample at a high w/v%, so the solvent could compromise the cellulose ester membrane (which is not compatible with DMF). Regenerated cellulose membranes are more stable with solvents like DMF, though it is still not ideal to use such a harsh solvent on any dialysis membrane. Additionally, the dialysis membrane we were using had a MWCO of 100-500 Da, meaning that the pores are effective at keeping proteins greater than 500 Da from diffusing across the membrane. As a result, our peptide, less than 500 Da, would likely be lost (398 Da RGD-MA), even if the DMF did not compromise the membrane.

The GRGDS-MA peptide may still have been lost during dialysis, as it was only slightly larger than the MWCO (541.6 Da when the methacrylate is attached). Verification of whether or not there is protein in our sample using BCA reagents or mass spectrometry would allow assessment of the efficacy of the methacrylation.

## UV Light Source

The UV light source we used to polymerize the gels was likely the cause of many of our complications, which can be shown by the many iterations of gels we fabricated. While the peptide could have been lost and prevented cell adhesion on PEG-RGD and PEG-GRGDS, the PEG-gelatin gels should have permitted adhesion. The gelatin-MA used for this fabrication was provided by the Masters lab, and did not require dialysis or any other modifications. Because the cells did not adhere to these gels, it is much more likely that the incomplete polymerization we observed interfered with crosslinking PEGDA and gel-MA together.

Tony Berger in the Masters lab later confirmed that the UV source we were using had presented similar complications when it was still being used as equipment in his lab.

## Bioreactor Implications

The bioreactor provides a low-cost alternative to culturing cells in a hypoxic environment. Our first prototype is the first step towards developing a stand-alone system that delivers a user-set oxygen concentration.

Validation of the bioreactor over the course of 30 minutes demonstrated that the system was able to maintain the gas concentrations within the cell culture chamber to within +/- 2% of the user-set concentration, providing a relatively stable system in which to culture cells (Figure 11).

Validation of the CO<sub>2</sub> and O<sub>2</sub> sensors would ideally be performed using calibrated O<sub>2</sub> and CO<sub>2</sub> sensors, but since these tools were not available, the sensors were assumed to be accurate based on their readings at atmospheric concentrations.

A component that needs improvement in the bioreactor system is the carbon dioxide sensor. Even though the sensor was advertised to reach 10,000 ppm (10%), we could not reach readings higher than 4%, lower than the 5% CO<sub>2</sub> concentration that is required for standard cell culture. Correspondence with Sandbox Electronics suggests our CO<sub>2</sub> sensor is defective, possibly due to mechanical shock or incorrect calibration. Although the sensor has not been abused and the calibration appeared complete, an alternate option for CO<sub>2</sub> sensors with Arduino Uno compatibility is recommended.

## Future Work

### Scaffold Fabrication

When fabricating the final biomaterial, we suggest that our client attempt to develop fibronectin-conjugated PEG. Conjugation of fibronectin (FN) to PEGDA, producing a proteolytically stable and functionally active form of FN attached to the PEGDA polymer. This is our best recommendation for a biomaterial to be chemically-defined and still mimic the bone marrow niche through a reductionist technique. We selected fibronectin due to the abundance of the protein in bone marrow, making it a physiologically-relevant addition to our bone marrow microenvironment, and due to its ability to mediate cell adhesion, migration and proliferation [23]. We recommend following the chemical methods of Zhang et al. to create the final FN-functionalized PEGDA gels. FN is a dimer with repeating units, where the ninth and tenth type III units incorporate cell-binding domains. Human plasma fibronectin (HPFN) can be conjugated to PEGDA at reduced cysteine residues which make up 2.6% of the amino acid residues, concentrated in the 70kDa amino-terminal of the protein, and should not affect the type III repeat regions which have only two cysteines. A Michael reaction will occur between the HPFN and PEGDA. HPFN will be added to a beaker at a concentration of 90ug/mL and mixed with β-mercaptoethanol (BME) to disrupt disulfide bonds. This will be incubated for 15 min at 37°C before adding an equal volume of PEGDA solution to the BME/HPFN mixture. A second incubation of 3 hours at 37°C will occur, allowing acrylate ends of PEGDA macromers to bind thiols in cysteine residues of HPFN. Once the FN is appropriately conjugated to PEGDA, the gels can be fabricated as described previously by Zhang et al.

To create a three-dimensional scaffold, NaCl crystals could be incorporated into the solution to allow for salt-leaching and pore creation. Once the gels have cured, they would be placed in deionized water for several days in order for the NaCl to be completely removed. These gels would then be dehydrated and frozen until use. Practically, we experienced many difficulties using the UV lamp available in the teaching lab. It is uncertain whether the lamp itself is the issue or if another factor is at play. The concentration (w/v%) of Irgacure 2959 could be too low to facilitate proper polymerization of the entire volume. This was an issue without the salt

crystals added, causing only a fraction of the entire volume to be polymerized at the bottom of the well. Once the salt crystals were added, the volume did not polymerize at all, but instead, remained a PEGDA solution slurry with the salt crystals. As a result, primarily due to lack of time left on the project, we decided to make non-porous gels that we could seed cells on top of. This approach would eliminate some confounding variables in the experimental data. For example, it could be that three dimensional culture confers additional benefit to the hMSCs, or it could be the varied stiffness parameter. By decoupling these two, and only seeding cells on the two dimensional surface, we could directly compare to the negative tissue culture plastic control culture.

## Bioreactor Fabrication

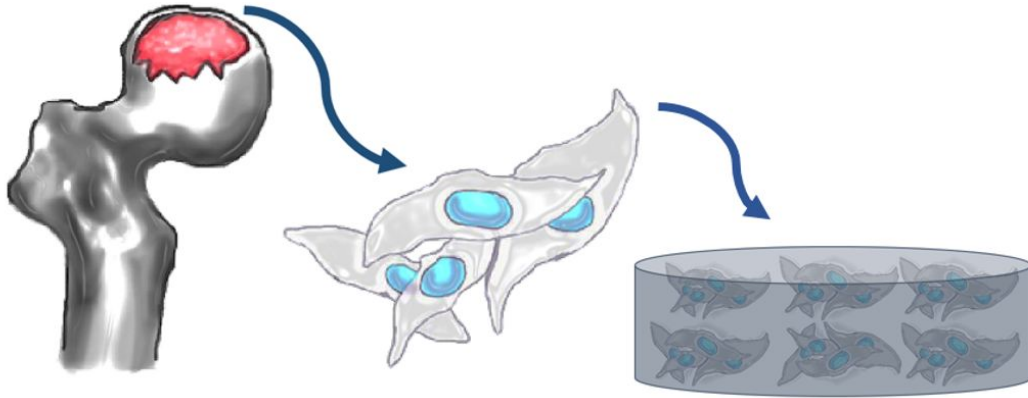
The bioreactor could be improved by fine-tuning the regulation of gas concentration. This could be done by altering the gas flow out of the gas tanks, the time the solenoid valves are open, and the time between solenoid valve openings. By adjusting these variables in different combinations, tighter maintenance of the user-set oxygen and carbon dioxide percentages could be maintained.

Two additional bioreactor chambers like the one created already could be fabricated. This would allow higher throughput evaluation of the effects of oxygen tension on hMSC quiescence, since cells could be cultured in multiple oxygen tensions simultaneously. Ultimately, the device system would also enable researchers to move away from the traditional *in vivo* animal models that are often inaccurate and not the best representatives of human physiology [12].

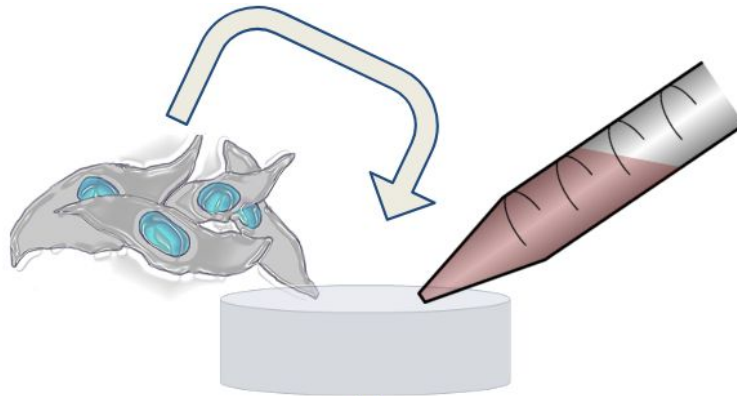
## Evaluation of hMSC Quiescence

The main objective of Dr. Li's inquiry is to understand whether or not manipulation of scaffold stiffness and oxygen tension impacts the percentage of quiescent cells. Initial experimentation with stiffness values which span multiple orders of magnitude (<1 kPa, 1-10 kPa, 10-100 kPa, and possibly >100 kPa), as well as oxygen tensions ranging from 1-10% O<sub>2</sub>, would be informative, ultimately demonstrating whether further investigation into the impacts of stiffness and oxygen tension on hMSC quiescence is warranted.

For these experiments, cells were isolated from human bone marrow removed from femoral heads of patients undergoing hip surgery, as shown in Figure 12. To Wang in the Li lab assisted with cell isolation, passaging, and experimental culture. These cells were expected to be seeded into our PEG-based biomaterial and cultured. To seed the cells, hMSCs suspended in media will be plated onto the PEGDA scaffolds for 30 min to permit time to adhere and swell the biomaterial (Figure 13). Media will be aspirated and new media will be added to the gels. hMSCs will also be seeded on tissue culture plastic for culture in various hypoxic conditions. We will analyze the activity of hMSCs after culturing them on our novel biomaterial for 7 days.



**Figure 12:** Isolation of hMSCs from femoral heads and seeding into PEGDA scaffold.



**Figure 13:** hMSCs seeded on the PEG-based, adhesive scaffolds, to culture as a 2D monolayer.

For this experiment to evaluate the effect of stiffness or oxygen tension on hMSC quiescence, we would extract total RNA using the Nucleo Spin RNA II kit (Clontech). 100 ng of RNA would be converted into complementary DNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Finally, we would perform quantitative PCR using the iQ SYBR Green Supermix (BioRadA) with primers for p16 and p21, which enable us to evaluate hMSC senescence. Relative mRNA levels of these genes associated with stemness would be determined using the  $2^{-\Delta Ct}$  method with ubiquitin C (a housekeeping gene) as an internal reference. Quiescent cells would demonstrate low levels of senescent behavior.

A BrdU assay would also be performed to evaluate the percentages of cells that are proliferating. Ideally, flow cytometry for the marker Ki67 would be used to show the percentages of cells in the G0/G1 phases, meaning they are not proliferating or synthesizing DNA. This would point more clearly to quiescence. However, the BrdU assay is a standard protocol used in quiescence studies, and would serve as a proof-of-concept. A Cell Proliferation ELISA BrdU Colorimetric Assay (Roche Applied Science) would be used to quantify hMSC proliferation. The cells would be seeded in the PEGDA hydrogels, with bromodeoxyuridine (BrdU) label added after 24 hours of incubation. After an 18 hour incubation, the cells would be

fixed and denatured for 30 minutes, followed by a 1 hour incubation with anti-BrdU antibody in dilution buffer. The cells would then be washed with PBS, followed by an incubation with peroxidase conjugate until color sufficient for photometric detection is developed, at which point the reaction would be stopped with 1 M  $H_2SO_4$ . Fluorescence would be measured with a spectrophotometric plate reader.

## Multivariate Testing

After evaluation of quiescence with the methods described above, multivariate testing including scaffolds of various stiffness and various oxygen tensions will be performed on hMSCs. The combination of a biorelevant scaffold stiffness and oxygen tension creates a more biomimetic environment overall for cell culture, which will ideally allow the culture of quiescent, multipotent hMSCs for extended time in the laboratory.

## Ethical Considerations

hMSCs are derived from umbilical cord blood, adipose tissue, amniotic fluid, or urine [7]. Although this presents an opportunity of increased access to researchers, regulation in terms of consent, control, and commercialization is needed in industries of stem cell banking and disease modeling [24].

As with all new therapies, and stem cell-based therapies especially, there is the possibility for rejection by the general public. For example, some individuals reject newly developed therapies because they claim scientists and healthcare professionals are “playing God” [25]. These ethical considerations are implicated in all medical advancements, though. hMSC use in regenerative medicine does not present any unique concerns.

## V. Conclusions

Regenerative medicine using hMSCs has the potential to develop therapies that target the disease mechanisms of degenerative musculoskeletal diseases such as OA. To better study these disease mechanisms, researchers need a culturing system that can recapitulate the bone marrow microenvironment. Current culture methods are variable in their ability to maintain hMSCs in a multipotent and quiescent state, and do not attempt to recreate the physiological conditions that prevent differentiation. For this reason, there is a need for a culture system that sustains cell multipotency and quiescence by mimicking the bone marrow niche through scaffold stiffness and oxygen tension.

Overall, our first step towards creating a culturing system to mimic the bone marrow niche through substrate stiffness and oxygen tension is quite satisfactory. Initial experimentation point towards a fibronectin-conjugated PEG hydrogel to best mimic the bone marrow microenvironment. The bioreactor provides functional regulation of oxygen, carbon dioxide, temperature, and humidity, and can be practically implemented in a budget-conscious research

setting. Cell culture evaluations on both the scaffold and bioreactor will ideally yield substrate stiffnesses and oxygen tensions of interest for maintaining hMSC quiescence. These two variables could then be combined by culturing cells on substrates of various stiffnesses in various oxygen tensions. This multivariate testing captures more components of the human bone marrow microenvironment, and will ideally allow better maintenance of hMSC quiescence than either factor alone.



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## VII. Appendix

### Appendix A. Scaffold Fabrication Protocol

**Acrylated Peptide Preparation Purpose:** Produce acrylated peptide through aminolysis of N-hydroxy succinimide ester of acrylic acid by the alpha-amine terminus of the peptide sequence

(arg-gly-asp). This will produce an amide linkage between the peptide and the methacrylate group:

#### Reagents:

N-acryloxysuccinimide (1g stock reagent) (169.13 da)

DMSO

RGD sequence (1mg) (346.34 da)

GRGDS sequence (1mg) (490.6 da)

Phosphate buffer (PBS)

#### Procedure:

1. Dissolve peptide to final aqueous concentration of 1 mg/mL in 50mM of sodium bicarbonate buffer, pH 8.2.

- Dissolve entire RGD (1mg) in 1 mL sterile sodium bicarbonate buffer, keep sterile
- 4.2 g Sodium Bicarbonate ( $\text{NaH}_2\text{CO}_3$ ) in 1000 ml ddH<sub>2</sub>O, stir à sterile filter!

2. Dissolve the N-acryloxysuccinimide in DMSO at a 10:1 N-acryloxysuccinimide:peptide molar ratio and add dropwise to aqueous peptide solution

RGD:

- Molar mass RGD: 346.34 g/mol
- Moles RGD:  $0.001\text{g}/(346.34\text{ g/mol}) = 0.00000288733\text{ mol}$
- Need  $0.00002887336\text{ mol}$  à  $0.00003887336\text{mol}*(169.13\text{g/mol}) = 4.88335161\text{mg}$  N-acryloxysuccinimide
- Dissolve 50 mg N-acryloxysuccinimide in 1mL DMSO to make stock
- Add 100 uL of N-acryloxysuccinimide in DMSO stock dropwise to the aqueous RGD.

GRGDS:

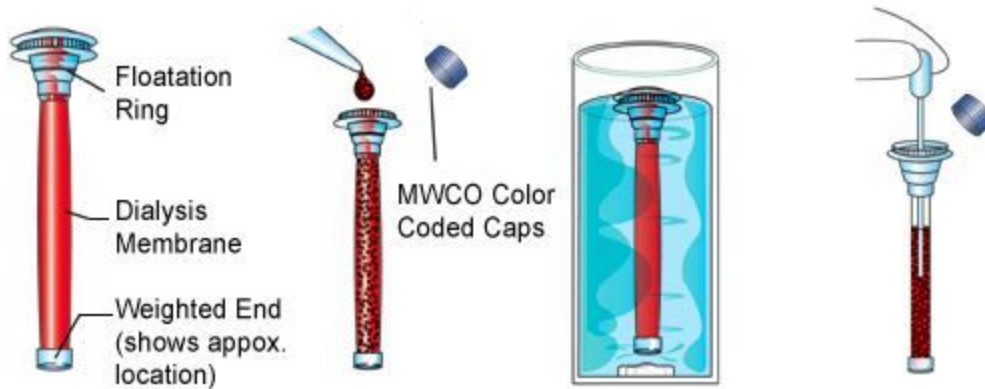
- Molar mass GRGDS: 490.6 g/mol
- Moles GRGDS:  $0.002\text{g}/490.6\text{g/mol} = 0.00000407664\text{ mol}$
- Molar GRGDS:  $0.00000407664\text{mol/mL} = 0.00407664084\text{ M}$
- Want 10X N-acryloxysuccinimide:  $0.04076640847\text{ M}$
- Want:  $0.0000407664\text{ mol/mL}$  N-acryloxy succinimide à  $0.0000407664*(169.13\text{g/mol*mL})$  à  $0.006895\text{ g/mL}$  à  $6.895\text{ mg/mL}$

- Make this 10X so that you can use 100uL in the reaction: 68.95 mg/mL N-acryloxysuccinimide

3. Agitate reaction vial continuously at room temperature for **10 hours**.

4. Separate peptides (from acrylic acid and hydroxyl succinimide byproducts) by dialysis in deionized water for **48 hours** with bath changes.

- This is done to remove acrylic acid which could add carboxylic acid to the hydrogel during polymerization.



- Follow image above (courtesy of Spectrum Labs [26]). First rinse the Float-a-lyzer (for 0.1 – 0.5 kD targets) with sterile water. Then load the sample with pipette. Dialyze.

5. Lyophilize dialysis product **overnight** or, if concentrated enough to mix into the final polymer solution, skip lyophilization step and use the product of dialysis directly if desired.

### Scaffold Preparation

**Purpose:** Fabricate RGD-conjugated PEGDA scaffolds with varied Young's Moduli to test effect of stiffness on MSC quiescence.

#### Reagents:

PEGDA MW 4000 (1 g stock reagent)

PEGDA MW 700 (100mL)

Igracure 2959 (10 g) (25 mL of 0.5 w/v% Igracure 2520, 0.125g in 25 mL PBS, sterile filter)

Acrylated-RGD sequence

NaCl crystals

1X PBS

#### Procedure:

1. Pestle NaCl crystals in a mortar and sieve through cell strainers with a nylon mesh of 100-200 nm pore size. Use vacuum to pull NaCl crystals through mesh.

2. Dissolve PEGDA in a saturated NaCl solution to predetermined w/v%. Make 1mL of solution for each w/v% sample in a 10mL beaker and stir for 1 hr in the dark at 130 rpm.

- Start with ~10 w/v%, then experimentally determine with rheometer, approximating a range of Young's moduli values between 100 Pa and 1000 Pa.
- Suggested values: 30 w/v%, 20 w/v%, 10 w/v%  
Example: add 1g PEGDA to 10mL of saturated NaCl solution to make a 10% w/v% solution. (For the MW 4000, add 1mg to 1000uL saturated NaCl).

3. Add 40 uL of 500uM RGD to the 1mL PEGDA solution and stir until further use (get final RGD concentration of 20uM). Determine how many grams of RGD-MA you get out of the dialysis.

RGD-MA:

- Find total moles of RGD-MA: molar mass of entire RGD-MA:  $(3 \times \text{mmC} + \text{mmO} - \text{mmH} + \text{mmRGD}) = (3 \times 12 + 16 - 1 + 346.34) \sim 398 \text{ g/mol}$
- Total moles: grams recovered after lyophilization / (398g/mol)

GRGDS-MA:

- Total moles (assume 0.002 g recovered)  $0.002 / 541.6 = 0.00000369276 \text{ mol}$  in 1 mL =  $0.00369276218 \text{ mol/L}$  (~3693 uM)
- A 500uM RGD solution means 0.0005M, 0.0005mol/L or 0.5mmol/L

4. Combine PEGDA solution with Irgacure 2959 photoinitiator (Sigma-Aldrich) at 10 - 20 v/v% of the final solution (sterile filter diluted Igracure)

- Ex. 183uL Irgacure + 1040uL PEGDA solution + RGD à Igracure ~15 v/v%

5. Add PEGDA solution + photoinitiator directly to the RGD-MA (at appropriate w/v%) and vortex for 5 seconds.

6. If you wish to fabricated 3D porous, salt-leached scaffolds, mix 800mg of sieved NaCl crystals with 200 uL of the PEGDA precursor solution, and transfer 50 uL to each well of a 48-well plate. Otherwise, perform step 7 and the gels will be prepared for sterilization and 2D culture.

7. Crosslink gels using UV.

8. Salt leach the salt crystals from the gel using deionized and sterile-filtered water for 7 days.

9. Freeze gels in ethanol for 2 days at -20°C and freeze dry them for future use in cell culture.

## Appendix B. Materials and Expenses

Bioreactor
------------

<b>Product name</b>	<b>Specifications</b>	<b>Product number</b>	<b>Manufacturer</b>	<b>Quantity</b>	<b>Price (\$)</b>
Arduino Uno R3	ATmega328P	50	Adafruit	1	34.06
Grove O2 Gas Sensor	0-25%	1010200002	Seeed	1	59.00
Grove CO2 Gas Sensor	2000 ppm	101020067	Seeed	1	99.90
MH-Z16 NDIR CO2 Sensor	100,000 ppm	SEN-000037	Sandbox Electronics	1	92.95
Rubbermaid Tupperware	Polypropylene	253010521	Target	4	12.64
Gas Tubing	1/8 in outer diameter				Free
Solenoid Valve	2-way, 5V, 100 mA	2258401	Jameco	6	53.70
Transistor	TIP102	32977	Jameco	12	4.68
Soderless Breadboard	Self-Adhesive (White)	PRT-12002 ROHS	Sparkfun	1	Free
Diode				2	Free
100 Ω Resistor	1/4 watt	COM-10969 ROHS	Sparkfun	2	Free
5V Power Supply				1	Free
LED	5 mm	COM-12062 ROHS	Sparkun	2	Free
<b>Scaffold</b>					
N-Acryloxy succinimide	1G	19930-1	Polysciences, Inc.	1	65.00
Polyethylene glycol diacrylate (PEGDA 4000)	1G	15246-1	Polysciences, Inc.	1	59.00

N,N-Dimethyl formamide	100mL	AA43997AE	Fisher Scientific	1	39.30
<b>TOTAL COST</b>					<b>520.23</b>

## Appendix C. Arduino Code for Gas Control

```

#include "Arduino.h"
#include "SoftwareSerial.h"
#include "NDIR_SoftwareSerial.h"

//O2 Sensor:
const float VRefer = 3.3;    // voltage of adc reference
const int pinAdc = A5;      //the pin to read O2 concentration from.

//set O2/CO2 tension desired in chamber
float setO2tension = 14;
float setCO2tension = 5;

//solenoid control from Arduino digital pins 12 and 13
int solenoidO2control = 13;
int solenoidCO2control = 12;

float O2Conc, O2percent, CO2Conc, CO2percent = 0;
//CO2 Sensor:
//Select 2 digital pins as SoftwareSerial's Rx and Tx. For example, Rx=2 Tx=3
NDIR_SoftwareSerial mySensor(2, 3);

void setup()
{
    //initialize Solenoids
    pinMode(solenoidO2control,OUTPUT);
    pinMode(solenoidCO2control,OUTPUT);
    // Initialize Gas sensors/ serial comms
    Serial.begin(9600);
    if (mySensor.begin()) {
        Serial.println("Wait 10 seconds for sensor initialization...");
        delay(10000);
    } else {
        Serial.println("ERROR: Failed to connect to the sensor.");
    }
}

```

```

        while(1);
    }
    Serial.print("Set O2: ");
    Serial.print(setO2tension,3);

    Serial.print("%, Set CO2: ");
    Serial.print(setCO2tension, 3);
    Serial.println("%,");
}

//Helper method for reading the O2 percent
float readO2Percent() {
    // Vout samples are with reference to 3.3V
    long sum = 0;
    for(int i=0; i<32; i++) {
        sum += analogRead(pinAdc);
    }
    sum >>= 5;
    float MeasuredVout = sum * (VRefer / 1023.0);
    //float Concentration = FmultiMap(MeasuredVout, VoutArray,O2ConArray, 6);
    //when its output voltage is 2.0V,
    float Concentration = MeasuredVout * 0.21 / 2.0;
    float Concentration_Percentage=Concentration*100;
    return Concentration_Percentage;
}

//helper method for reading CO2 percent.
float readCO2percent() {
    CO2percent = 0;
    if (mySensor.measure()) {
        CO2Conc = mySensor.ppm;
        CO2percent = CO2Conc / 10000;
    } else {
        Serial.println("Sensor communication error.");
    }
    return CO2percent;
}

void loop() {

```



```

//CO2 sensor control block
CO2percent = readCO2percent();
//Serial.print("Current Sensor Percentages: CO2: ");
Serial.print(CO2percent, 3);
//Serial.print("% | ");

//O2 Sensor Control Block
O2percent = readO2Percent();
//Serial.print("O2 percent: ");
Serial.print(",");
Serial.print(O2percent, 3);
Serial.print(",");
//Serial.print("% ");

// Solenoids Control block
// if the measured CO2 and O2 percentages are less than the set percentages,
// then open the valves to increase the percentages.
if(O2percent < setO2tension){
    digitalWrite(solenoidO2control,HIGH);
    Serial.print("O2 on,");
}
if(CO2percent < setCO2tension){
    digitalWrite(solenoidCO2control,HIGH);
    Serial.print("CO2 on,");
}
Serial.println("");
//leave the valves in their current state for 5 seconds.
delay(2000);
//close the solenoid valves if they were opened
if (digitalRead(solenoidO2control) == HIGH) {
    digitalWrite(solenoidO2control,LOW);
}
if (digitalRead(solenoidCO2control) == HIGH) {
    digitalWrite(solenoidCO2control,LOW);
}

delay(5000);
}

```

## Appendix D. MATLAB Code for MTS Testing.

```

close all;
clear all;

%% LOADING
% Load your data file
ask = uigetfile('.txt', 'Select
file');
data1 = load(ask);

% import your data
disp1 = data1(:,1);
% in mm
force1 = data1(:,2); % in N
time1 = data1(:,3); % in s

% Plot your raw data and
% inspect it to make sure it
% looks as you expect

plot(disp1, force1);
xlabel('Displacement');
ylabel('Load');
title('Load vs. Deflection');

% Enter the geometric
% measures of your bone
% cross section
d = input('Enter diameter
of gel in mm: ');
A = pi * (d/2)^2;
%area of gel in mm^2

t = input('Enter thickness
of gel in mm: ');

stress = force1 ./ A;
strain = disp1 ./ t;

%stress vs. strain graph
figure;
plot(strain, stress);
ylabel('Stress (MPa)');
xlabel('Strain (mm)');
title('Stress-Strain');

% ginput(2) -> enables
% you to identify 2 points
% from the current axes
% returns their x- and y-
% coordinates in the x and
% y column vectors.
[x,y] = ginput(2);
slope = (y(2)-y(1)) / (x(2)-
x(1))
E = slope %in MPa

```

## Appendix E. Circuit Schematic for Gas Control

