1 INTRODUCTION

More than 120,000 people in the United States are currently on the waiting list for life-saving organ donations; over 6,000 of these people die annually without receiving needed treatment. Tissue engineering may help address issues of donor organ shortage and transplant rejection. However, applications have been limited by the ability to design complicated scaffolds that reproduce the architecture present in the cellular microenvironment. 3D bioprinting (3DBP) holds promise for addressing these shortcomings by producing biologically-inspired structures based upon computer-generated models. Unfortunately, low cell viability due to lack of nutrient transport through thick scaffolds is a current barrier to clinical use. Without blood vessels to facilitate nutrient and oxygen transport, cells at the center of the constructs die. There is an **urgent need** to improve methods such as 3DBP to develop implants to treat these patients.

One method to address this problem involves artificial blood vessels created by physical channels through the scaffolds, seeding with endothelial cells, and adding growth factors. By this method, scaffold design must be further complicated by including vasculature. However, introducing oxygen-releasing polymers directly into the scaffolds may be a simpler way to address the need for oxygen transport.[1] My **research objective** is to investigate the ability of oxygen-releasing microspheres to decrease cell necrosis for adipose-derived stem cells in printed implants.

Using oxygen-releasing microspheres to reduce cell death rates for tissue engineering applications has only been referenced in one publication at the time of this application. This system involves a two level approach. First, core-shell microspheres are created with the shell consisting of poly(lactide-co-glycolide) (PLGA) and the core containing H_2O_2-modified poly(vinyl pyrrolidone) (PVP). This shell system allows for slow release of H_2O_2-modified-PVP for up to two weeks.[3] Second, the microspheres are then encapsulated in a hydrogel with catalase enzyme and cardiosphere-derived cells (CDCs). Catalase reacts with the H_2O_2 bound to PVP to generate oxygen, which is then free for use by the cells. This system has been shown to eliminate significant CDC death.[4] This promising result may also be replicable in other cell types.

Among the most propitious lineages of adult stem cells are the adipose-derived stem cells (ASCs) due to their ease of acquisition and ability to be chondrogenic, osteogenic, and adipogenic. Despite these advantages, ASC survival rates following *in vivo* implantation are low. Reduced viability may be due to lack of oxygen at the implant or inject site, which could be addressed by oxygen-releasing polymers.[2]

The steps involved in making a clinically relevant printed scaffold include material choice, cell type choice, printer type choice, scaffold material characterization, *in vitro* testing, preclinical animal testing, and clinical testing. Using this approach, I will complete the first six steps to adapt the PVA-H_2O_2 system for use in 3DBP and test its ability to prevent necrosis in adipose-derived stem cells using various hydrogel formulations. **I hypothesize that using oxygen-releasing polymers will increase ASC viability in engineered tissue systems.** To do this, I must address tissue engineering concerns such as refining 3D printing parameters, determining whether microsphere addition alters the mechanical properties of the gel, and selecting the gel type by printability and cell viability.

2 RESEARCH PLAN

2.1 Gel manufacture: For 3DBP applications, inks must solidify quickly, have appropriate mechanical properties, be non-immunogenic, and promote proliferation. Before manufacturing and encapsulating the microspheres, I will test several gel formulations including poly(ethylene glycol) diacrylate to determine an appropriate method for 3DBP. Printability will be measured by

the degree to which the print matched the design specifications by calculating the percent error of the printed scaffold compared to the 1 cm^2 design. I will select the weight percent of each hydrogel by testing printability.

2.2 Manufacture of microspheres and gel encapsulation: To create the H_2O_2-modified polymer, H_2O_2 will be mixed with PVP in multiple molar ratios. The core-shell microspheres will be electrosprayed using coaxial electrohydrodynamic atomization using a protocol described by Nie et al.[3] Both the flow rate and voltages will need to be optimized to create particles of uniform size and morphology. I will use the previously determined weight percentage of hydrogel to encapsulate the ASCs, catalase, and microspheres. The optimum concentration of catalase will be determined by studying oxygen release kinetics in a hypoxic, acellular environment and measuring which concentration of enzyme sustains oxygen release for the longest time and at the highest levels. This will be determined over a 21-day period by using $Ru(Ph_2Phen_3)Cl_2$, a luminescent molecule sensitive to O_2 concentration, while using rhodamine b, a fluorescent molecule insensitive to O_2 to correct for background absorbance.

2.3 Testing printed materials: Ensuring that scaffolds are safe for cells *in vitro* prior to further testing is essential. Cell viability in the scaffolds printed with H_2O_2/PVP will be tested *in vitro* in a hypoxic environment using Live/Dead, MTS, DNA content, and IHC assays; they will then be compared to control scaffolds without microspheres and containing microspheres with no incorporated H_2O_2. Should cell mortality persist, I will attempt to adjust the gel porosity, printing methods, and O_2 release system to increase viability. If viability improves with the microspheres *in vitro*, I will test their efficacy during a subcutaneous study in mice.

2.4 Timeline and Proposed Laboratory: To conduct this study, I would like to work in Dr. Warren Grayson's lab at Johns Hopkins. Due to the close alignment of our research interests, when I met him at the BMES conference this year, he expressed considerable enthusiasm in working with me and funding my project should I be awarded the NSF GRFP. I anticipate this project to take five years: two for gel and microsphere manufacture and testing and three for *in vitro* and *in vivo* material studies.

3 INTELLECTUAL MERIT AND BROADER IMPACTS

Currently, only Li et al. have used microspheres to deliver O_2 to implant sites to prevent cell morbidity. Instead, I will develop and test a rapid, accurate, and programmable method to fabricate these tissues using 3DBP. **There have been no published papers that utilize microspheres, oxygen release, hydrogels, and 3DBP in conjunction to print biomaterials; integration of these techniques could greatly increase versatility of 3DBP in tissue engineering applications.** As cell death is one of the primary concerns of tissue engineering in general and 3DBP specifically, finding a solution to this problem could advance the field from constructing thin tissue sections, to larger tissues, and eventually organs.

Developing a standard method for incorporation of oxygen-releasing microspheres into a hydrogel-based printed scaffold is a novel approach that has potential applications in areas such as cardiac, bone, and cartilage tissue engineering. Osteoarthritis treatment is a particular challenge because of the hypoxic environment, but the H_2O_2-polymer complex can be a source of oxygen for implanted stem cells while they heal the native tissue. Should this method prove effective, it could be used to encapsulate other materials such as growth factors or nutrients. To eventually reach clinical application, I will collaborate with surgeons at Johns Hopkins to develop materials that have clinical utility. Finally, I will present the results of my work at national and international conferences.

[1]Camci-Unal, G., et al. (2013) Polym Int. [2]Tsuji, W., et al. (2014) World J Stem Cells. [3]Nie, H., et al. (2010) J Biomed Mater Res A. [4]Li, Z., et al. (2012) Biomaterials.