Creation of Ovalbumin Based
Scaffolds for Bone Tissue Regeneration

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ABSTRACT

Bio-based materials are a viable alternative to synthetic materials for tissue engineering. Although many bio-based materials have been used, Ovalbumin (OA) has not yet been researched to create 3D structures that promote cellular responses.

Micro-porous scaffolds are a promising construct for bone tissue regeneration; therefore OA crosslinked with three different concentrations (10%, 15% and 20%) of glutaraldehyde (GA) was used in this research. After fabrication, a porous morphology was observed using SEM. Average pore sizes were found to be comparable to scaffolds previously shown to promote cellular response. A TNBS assay determined percent crosslinking in the scaffolds, however there was no significant difference in percent crosslinking despite differing GA concentrations used. Possible explanations include an excess of GA was used.

Using DSC, a glass transition temperature ($T_g$) was found for control indicating the scaffolds are amorphous. Average dry and wet compressive strengths were also found. As expected, differing GA concentrations had no significant effect on $T_g$ and average compressive strengths due to an excess used. Scaffolds were mechanically tested at 37°C with no significant difference found; therefore these scaffolds can be used in the body.

It was shown through cell studies that MC3T3-E1 pre-osteoblast cells significantly increased in number on the 10% and 15% scaffolds, therefore cell proliferation occurred. Because of a positive cellular response, 10% GA scaffolds were used for differentiation studies that showed an increase in osteocalcin at 21 days and alkaline phosphatase levels for scaffolds cultured for 14 days. Overall OA scaffolds have shown to be a promising 3D construct for bone tissue regeneration.
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Table 1: List of Amino Acids in OA

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List of Abbreviations

OA  Ovalbumin
GA  Glutaraldehyde
DTT  Dithiothreitol
DI  Deionized
CSA  Cross sectional area
min  Minute
h  Hour
g  Gram
mg  Milligram
mL  Milliliter
µL  Microliter
M  Molar
mM  Millimolar
mm  Millimeter
cm  centimeter
µm  Micrometer
nm  Nanometer
mol  Mole
mmole  Millimole
psi  Pounds per square inch
rpm  Revolution per minute
W_L  Percent weight loss
W_U  Percent water uptake
EtOH  Ethylene oxide
ECM  Extra cellular matrix
T_g  Glass Transition Temperature
T_m  Melting Temperature
MPa  Megapascal
GPa  Gigapascal
SEM  Scanning Electron Microscope
DSC  Differential Scanning Calorimetry
FTIR  Fourier Transform Infrared Spectroscopy
TNBS  Trinitrobenzene Sulfonic Acid
PBS  Phosphate Buffered Saline
MEM  Minimum essential medium
FBS  Fetal Bovine Serum
ELISA  Enzyme-linked immunosorbant assay
OCN  Osteocalcin
ALP  Alkaline Phosphatase
1. Introduction

Procedures using bone harvested from donor sites, or autografts, has led to the need for an ideal bone graft substitute. Autogenous bone is the most preferred bone grafting material; however, limitations and complications from using autografts include a limited quantity and chronic donor site pain [1, 2]. Bone graft substitutes must have enhanced capabilities to reduce or eliminate the need for an autograft altogether [3] and are necessary to provide support, fill voids, and enhance biologic repair of defects.

The need for tissue engineered constructs is increasing and advances in the field have led to the use of scaffolds, cells, and factors to regenerate organs and tissues [3]. The integration of the biological, physical, and engineering sciences will create the new constructs that regenerate and restore functional state of damaged tissues [4]. Using tissue engineered constructs such as scaffolds as bone graft substitutes has emerged as an approach to regenerate bone.

Both a materials and biological perspective are needed to fully understand the interactions that go on in the body to make biomaterial scaffolds successful. From a materials perspective, the extent of how surface properties and material characteristics affect cell behavior must be determined. It is also important to know the parameters that govern positive cell response to a biomaterial and what in vitro models reproduce these parameters in order to conduct successful research [5]. Using these results as a guideline, a bio-based scaffold for osteoblast adherence and proliferation can be envisioned. Bio-based polymers need to be researched for further tissue engineering advances, specifically their use in bone regeneration [6]. This study aims to create a bio-based Ovalbumin (OA) scaffold and investigate the effects of adhesion, growth and differentiation of osteoblasts on these structures.
1.1 Motivation

Synthetic polymers such as poly (ethylene glycol), glycolide, ε-caprolactone, etc. are commonly used in scaffolds applications [7]. Most synthetic materials alone do not successfully support osteoblast activities and can result in poor cell differentiation and limited bone formation [8]. Acidic degradation products from some synthetic polymers can lead to problems with biocompatibility and toxicity [9]. Hindered osteoblast adhesion, proliferation and differentiation due to synthetic materials can arise because of poor ligand presence. Insufficient ligand presence can result in a decrease in biological activity. This decrease is due to limited integrin-ligand cell interaction without which there is poor cell adherence and growth resulting in little tissue development [10]. Bio-based polymeric scaffolds demonstrate the distinct advantage of biological recognition and preferential cell attachment and differentiation due to receptor-binding ligand presence [11]. Many bio-based materials have also been shown to have better biocompatibility and overall interaction with cells [12]. Several natural biopolymers such as alginate and collagen have been used to create scaffolds; however, to date research has not been done to evaluate OA scaffolds for bone regeneration.

It has, however, been suggested that albumin is released by osteoblasts present in fracture healing and an excess of albumin significantly increased the proliferative state of the surrounding cells. It was possible the albumin influenced and stimulated the adhesion and proliferation of cells [13]. Other studies have shown that supplementing a standard culture medium with varying concentrations of human bovine serum albumin led to a significant increase in the number of MC3T3-E1 pre-osteoblast cells present after culturing [14].

One reason why biopolymers can promote successful bone healing is their origin as natural proteins found in the body. OA, a water-soluble glycoprotein and biopolymer found in
chicken egg whites, has a molecular mass of 45 kDa and is comprised of 386 amino acids (table 1) with 10% of the amino acid sequence conserved when compared to human serum albumin. The protein is comprised mainly of α-helix and β-sheet, but when introduced to an alkaline environment, is transformed to a predominantly β-sheet structure [15]. It can be used to create biocompatible scaffolds for osteoblast adhesion and mineralization into 3D structures [9]. OA is also more readily available and cheaper ($40/kg, Sigma Aldrich) than using synthetic or other natural biopolymers [16].

Table 1. List of Amino Acids in OA.[17]

<table>
<thead>
<tr>
<th>Asp-32</th>
<th>Thr-15</th>
<th>Ser-37</th>
<th>Glu-51</th>
<th>Pro-16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly-18.5</td>
<td>Ala-34</td>
<td>Val-30</td>
<td>Cys-0</td>
<td>Met-13</td>
</tr>
<tr>
<td>Ile-25</td>
<td>Leu-32</td>
<td>Tyr-10</td>
<td>Phe-20</td>
<td>Lys-19</td>
</tr>
<tr>
<td>His-8</td>
<td>Arg-19</td>
<td>(CM) Cys-6.1</td>
<td>(AE) Cys-0</td>
<td>Trp-3</td>
</tr>
</tbody>
</table>

Three dimensional (3D) porous scaffolds are typically used in tissue engineering to promote cell adhesion, proliferation and differentiation to support regeneration of tissue in the body [18]. The need for bio-based, or natural, polymeric materials (biomaterials) is increasing. Because of this need, studies using collagen, alginate and keratin as well as many other natural biopolymers, have been conducted to determine the success of these materials for use in scaffolds in tissue engineering [18-20]. However, the use of OA in biomaterials research is limited for tissue engineering applications.

OA crosslinked with glutaraldehyde (GA) is the main focus in this research and is used to create micro-porous scaffolds. Salt leaching and freeze drying techniques commonly used will create the porous structures needed within the scaffold. Mechanical properties of the structures will be determined using compression tests in order to evaluate the effect of GA crosslinking on
scaffold compressive strength. Thermal properties are found using differential scanning calorimetry (DSC) in order to determine scaffold phases as well as glass transition temperatures and analyze the effect of crosslinking on scaffold properties. Crosslinking will be investigated using Fourier Transform Infrared spectroscopy (FTIR) for chemical structure analysis, and a Trinitrobenzene Sulfonic Acid (TNBS) assay will be used for examining the effect of GA concentration on percent crosslinking in the scaffolds. Biodegradability of the scaffolds will be tested over several weeks to determine mass loss in PBS as well as other enzymes. A well known technique using ethylene oxide gas will be used to sterilize biomaterial scaffolds before cells are seeded onto them. Once MC3T3-E1 osteoblastic cells have been seeded, confocal images will be used to determine osteoblast morphology and proliferation on the scaffolds. An increase in cell number suggests a positive response to the scaffolds allowing for a proliferative state to be reached. OCN and ALP levels will also be looked at to determine cell differentiation. High OCN and ALP levels imply cellular differentiation that can lead to mineralization and tissue formation and if found, suggest a successful OA biomaterial scaffold. Because albumin has been shown to have an effect on osteoblast proliferation, creating OA based scaffolds might be a viable way to regenerate bone tissue.

2. Materials and Methods

2.1 OA Solution

To create an albumin solution, 5.0 g albumin from chicken egg white (OA), grade II (Sigma) and 0.01 g dithiothreitol (DTT), minimum 99% titration was dissolved in 30 mL borate buffer (pH 9.5) and 50 mL deionized (DI) water. The solution was stirred overnight at room temperature to ensure it was completely dissolved then dialyzed using snake skin dialysis tubing.
in tap water at room temperature for three days. The water was changed twice a day for the
duration of the dialysis and the final dialyzed solution was stored in the refrigerator until use.

2.2 Thin Film Fabrication

To create thin films, 1 mL of dialyzed albumin solution was pipetted onto small Teflon
squares. To induce crosslinking several different ratios of albumin solution to GA were used. 100
µL (10%), 142.9 µL (15%) and 200 µL (20%) GA was pipetted and stirred into separate albumin
solutions and left overnight under the chemical fume hood. After approximately 18 hours the
films were removed from the Teflon and placed in excess 100 mM glycine solution at 35 °C for
one hour for GA crosslinking termination.

2.3 Scaffold Fabrication

Scaffold surface characteristics are important for bone tissue regeneration, therefore the
material design is an interconnected microporous scaffold created through a salt leaching and
freeze drying technique [21]. A porous scaffold design was chosen to generate a 3D structure
that will allow for cellular adhesion, proliferation and differentiation [22]. The complex porous
scaffolds are ideal for cell penetration due to high surface area to volume ratio.

To create 3D scaffolds, OA can be crosslinked with GA, a common chemical
crosslinking agent [23]. GA crosslinking is governed by reactions with ε-amino groups of
lysines. The proposed mechanism (figure 1) between monomeric GA and ε-amine groups
involves nucleophilic attack of the aldehyde groups which form a non-conjugated Schiff base
[24]. If the crosslinking reaction occurs under a basic pH, GA polymerizes and another ε -amino
group can be added to the double bond allowing for conjugation of the aldehyde groups of
polymeric GA. Crosslinked scaffolds are washed in glycine to terminate crosslinking and remove excess GA.

Figure 1. GA crosslinking mechanism with lysine.

The crosslinking process has been shown to alter cellular response due to its cytotoxicity [25] and may alter osteoblastic responses through modification of the scaffolds [26]. However, it has been previously reported that using GA as a crosslinker for other biopolymer scaffolds such as collagen, alginate and keratin has not affected biocompatibility [19, 27, 28]. Therefore, small concentrations of GA will be used to prevent cytotoxicity, and OA scaffolds may also be created using this method.

In the past, successful scaffolds that promoted cell ingrowth, cell survival and maintained cell attachment had pore sizes greater than 100 µm [26, 29]. Porosity is imperative in using scaffolds for tissue regeneration as it allows for high permeability for fluid flow, nutrient diffusion and waste removal [30]. Researchers have shown that materials such as hydroxyapatite, keratin and starch based scaffolds with a porosity of higher than 50% led to cell growth, vascularization and tissue regeneration [31]. Scaffold properties can affect the behavior of cells during tissue regeneration as well [26]. The salt leaching technique has been used to create
complex porous scaffolds from biodegradable polymer/hydroxyapatite composite scaffolds for bone formation [32]. Microporous scaffolds can be created through salt leaching techniques, phase separation and freeze drying [22] and [33]. Pore size within the scaffold can affect the cell’s ability to grow within the pores and can also affect cellular adhesion and matrix deposition necessary for tissue formation [34]. Additionally, cell morphology can also be affected by pore shape and size [35].

Separate experiments were run varying particle salt sizes to vary pore sizes, amounts of dialyzed albumin solution and GA to alter crosslinking ratios. A schematic of the fabrication set up is in figure 2.

Figure 2. Set up for scaffold fabrication with increasing crosslinking ratio moving from left to right and decreasing salt size moving from top to bottom in the 12-well plate. Two separate 12-well plates were used for differing OA solution amounts.
For the first experiment, 1 g of sieved salt (particle sizes > 150µm, 90-150 µm, 45-90 µm and < 45µm) was measured into wells of a 12 well plate allowing for three wells for each salt size. Two milliliters dialyzed OA solution was pipetted over the salt of each well plate. To vary crosslinking ratio, 400 µL (20% v/v), 285.7 µL (15%v/v) and 200 µL (10%v/v) of GA was pipetted into the wells. Each well was stirred to ensure equal distribution and placed on a shaker overnight to allow for crosslinking.

For the second experiment the same amounts of salt and crosslinking ratios (10%, 15% and 20%) were used from the first experiment, however 2.5 mL dialyzed albumin solution was pipetted onto the salt. For the crosslinking ratios 250 µL (10% v/v), 357.1 µL (15% v/v) and 500 µL (20% v/v) of GA were used. The solutions were stirred for equal distribution. The well plates were also placed on a shaker overnight to allow for crosslinking.

Once crosslinked the scaffolds were removed from the well plates and placed in an excess of 100 mM glycine solution at 35 °C for GA crosslinking termination. After one hour the scaffolds were removed from the glycine solution and placed in a beaker of DI water to commence the salt leaching process. The beakers were placed on a shaker and the scaffolds remained in DI water for three days. The water was changed twice a day during the process.

After the salt leaching process the scaffolds were placed in the -80 °C freezer overnight. The next day they were placed in a freeze dryer at -80 °C and 10 torr overnight to remove excess water. After 24 hours the samples were removed and placed in a desiccator until use.
2.4 Scaffold Characterization

2.4.1 Scanning Electron Microscope (SEM)

The surface and cross sectional area scaffold morphologies were viewed using the FESEM. Cross sectional areas were able to be viewed by cutting the scaffolds in half with a razor blade. Samples were sprayed with a 15 µm thick conductive Gold-Palladium coating under vacuum in an argon atmosphere. The samples, three for each crosslinking ratio (10%, 15% and 20%) were observed in the SEM under vacuum at 5 kV.

Average pore size was determined by viewing SEM images in Q analysis program and measuring the diameter of 25 different pores. Two samples for each crosslinking ratio were analyzed and pores were identified as areas of void space.

2.4.2 Percent Crosslinking

A Trinitrobenzene Sulfonic Acid (TNBS) assay determines the number of free amino groups in a protein. When primary amines are reacted with TNBS a chromogenic derivative is formed that can be measured at 335 nm. The measurement gives an estimation of how many amines, specifically ε-lysine are present. In the past, many studies have used the TNBS assay to determine degree of crosslinking between biopolymers such as gelatin and collagen with GA [36, 37]. Increasing the amount of GA used should result in an increase in percent crosslinking of the OA scaffolds and a decrease in the amount of free lysine residues.

The assay was performed to measure the number of ε-amino groups in the scaffolds to determine percent crosslinking in the scaffolds. Three scaffolds for each crosslinking density as well as an OA powder as a control were used in the study. For the assay, 11 mg of dry scaffold was placed in a 50 mL screw cap tube. One milliliter of 4% NaHCO₃ and 1 mL 0.5% TNBSA
was added to the vial. The vial was then placed in a water bath with a stir bar at 320 rpm at 40°C for four hours. Three milliliters of 6 M HCl was added to the vial to hydrolyze the reaction then the vial was placed in the autoclave for one hour at 120°C and 15-17 psi to hydrolyze and dissolve the protein. After autoclaving, the vial was taken out and solution diluted with 20 mL of DI water and read on a UV-vis spectrophotometer at 350 nm.

2.4.3 Mechanical Testing

Mechanical strength is another important property of the scaffolds. Previous research demonstrated scaffolds with higher mechanical properties can resist cell contraction during healing as well as external pressures and be able to maintain space during bone regeneration[26, 38, 39]. Crosslinking the scaffolds will increase their mechanical strength while decreasing their fragility [26], and therefore will be the means to increase mechanical properties of OA based scaffolds. Although the ideal mechanical strength of biomaterial scaffolds has yet to be determined, previously researched scaffold compressive strengths have fallen within a 2-9 MPa range [40]. Young’s elastic modulus, or stiffness of the scaffold, is also an important mechanical property to determine how much the material will deform under certain stresses. In previous research, pore size played an important role in the stiffness of the scaffolds created with a salt leaching technique, as modulus decreased with increasing pore size [41]. Testing the mechanical properties of the scaffolds is important because they must mimic the mechanical properties of the tissue being regenerated. In the case of bone regeneration, the scaffolds must be able to withstand compressive strengths. Compact bone compressive strength has been measured to be anywhere from 150-250 MPa due to variability in bone density [42]. The compressive modulus
for bone has been measured to be 5-20 GPa while biomaterial scaffolds vary from 60 MPa-15GPa [40].

The materials were mechanically tested by compression in an Instron 5869 machine using a 100 kN maximum load cell. Compression tests (Figure 3B) were carried out on five samples of each crosslinking ratio (10%, 15% and 20%) with approximately 20 mm diameters and 7 mm (Figure 3A) heights at a crosshead speed of 2 mm/min until maximum extension was reached. The materials were tested in dry and wet conditions. Wet scaffolds were submerged in phosphate buffered saline solution for two days, completely wetting the samples. The slope of the stress versus strain curve, which represents the compressive Young’s modulus, was estimated for all samples.

Compression tests were also performed to varying percent strains to determine the effect of stress on pore structure. Samples were compressed until 20% and 40% then viewed under SEM to view any structural changes in the scaffolds.

2.4.4 Thermal Properties

Differential scanning calorimetry (DSC) can be used to determine physical transformations or phase transitions of the biopolymer scaffolds. A melting or glass transition
temperature of the material can be determined, yielding insight into the physical nature of the scaffolds. If a material has order (crystalline) or some order (semi-crystalline) a melting temperature ($T_m$) would be reached. When a glassy region and glass transition stage is followed by a significant drop in the graph with no other peaks present, an average glass transition temperature ($T_g$) can be determined.

$T_g$’s are commonly associated with amorphous polymers as their molecules are randomly oriented creating no ordered structure. As more crosslinker is used (increase of crosslinking ratio), polymer density increases. As the density of the material increases, molecules are more restricted causing an increase in the $T_g$. Polymers that have been crosslinked tend to be thermosets which reach a $T_g$ but do not reach a $T_m$, verifying their amorphous structure. Perfect amorphous materials do not melt, but degrade with increasing temperature. Because of this, the crosslinking density should also have an effect on scaffold mass loss.

Measurements were performed with a Netzsch 889 analyzer from 30 °C to 250 °C at a heating rate of 10 °C/minute and under a helium atmosphere. Then the temperature was lowered to room temperature at a rate of 10 °C/min. Each sample weight was 2–3 mg with a total of three samples for each crosslinking ratio. Thermal analysis was carried out using the Proteus Thermal Analysis software.

2.4.5 Thermal Mechanical Tests

Thermal mechanical tests were performed on three porous three-dimensional scaffolds with varying crosslinking densities (10%, 15% and 20%) under compression in an Instron 5800R with an environmental chamber. Each scaffold was cylindrical shape with a diameter of 20 mm
and a height of 7 mm. The scaffolds were tested in dry conditions at a physiological temperature of 37 °C and the compressive rate was 2 mm/min. Samples were tested until failure.

2.4.6 FTIR Spectroscopy

Fourier transform infrared (FTIR) spectroscopy is a non-destructive chemical analytical technique that measures the infrared intensity versus wavelength of light and can be used to examine protein structure [43]. The technique detects the vibrations of chemical functional groups in a sample. When an infrared light interacts with the substrate, chemical bonds will stretch, contract and bend as chemical functional groups tend to adsorb infrared radiation in a specific wave number range [44].

Protein analysis is commonly investigated using FTIR spectroscopy [43]. Peaks normally found in protein spectroscopy are Amide I and Amide II. Amide I is usually associated with stretching of the C=O bonds and can be found at a wavenumber range of 1600 and 1700 cm\(^{-1}\) while Amide II is associated with the N-H bond range [43]. Stretching of the C=O bond accounts for a majority of the detected vibrations, while the NH, CN and CNN bond bending accounts for the rest [45]. Previous studies have shown that the shape of the Amide I peak can be directly correlated to the protein secondary structure [43].

Aldehyde groups can also be detected using FTIR spectroscopy. These groups (C=O) are found between 1670-1740 cm\(^{-1}\). Crosslinking OA’s lysines with GA (whose peak contributes to the overall Amide I) peak can be found and analyzed using FTIR. It can be used to determine the presence of crosslinking and structural changes (presence of Schiff base) in the scaffolds by looking at the shape and height of the peaks between 1600 and 1700 cm\(^{-1}\).
Five thin films for each crosslinking ratio (10%, 15% and 20%) and five non-crosslinked OA solution films were used to look at crosslinking chemistry using the FTIR. Each film was crushed into a powder using a mortar and pestle. Untreated OA powder was also viewed as a control. Each film and the control were scanned 128 times.

2.4.7 Contact Angle

Contact angle for the three differing crosslinked scaffolds were evaluated to determine the hydrophobicity of the scaffolds. Ten drops of water were placed on each scaffold and each contact angle measured with a goniometer.

If the water drop on the scaffold is strongly attracted to the solid surface the droplet will completely spread out on the solid surface, complete wetting and the contact angle will be close to 0°. Hydrophilic scaffolds will result in the water having a contact angle between 0° and 30°, but can have a contact angle up to 90°. The smaller the contact angle, the more hydrophilic the scaffold is. If the scaffold is hydrophobic, the contact angle will be larger than 90°.

2.4.8 Degradation

Biodegradability, which determines the durability of the scaffold, is another important property of the scaffolds [26]. Crosslinking has been shown to lengthen degradation time. Degradation can be studied in vitro by monitoring scaffold mass loss as a function of exposure time to an enzyme solution [46]. The use of phosphate buffer solution (PBS) has commonly been used in degradation studies in vitro [47]; however the addition of hydrolytic enzymes to PBS gives a more realistic timeline for in vivo degradation, as shown by other groups [48].

The enzymatic degradation of OA based scaffolds was assessed by immersing three samples of each crosslinking ratio (10%, 15% and 20%) in PBS (1mL for each sample)
containing lipase or collagenase. Each sample was cut into 5.5 mm diameter and 5 mm height cylindrical shapes using a cork borer. The samples were weighed dry to determine initial weight and put into respective tubes containing PBS (control) and PBS+ enzyme solutions (1 mg enzyme/1mL of PBS). The tubes were then placed in a water bath and heated to 40°C at 600 rpm. The samples were collected and dried with kimwipes every two days to obtain a wet weight to determine surface degradation and the solution and enzymes were refreshed.

At weekly intervals up to nine weeks, the samples were taken out, rinsed with distilled water twice then surface dried with kimwipes then weighed. The dry weight was measured by placing the samples in an oven at 30°C for two days. After drying they were weighed to determine bulk weight loss then placed back in the PBS solutions. To determine percent weight loss ($W_L$) and water uptake ($W_U$) by the scaffolds the following equations (1 and 2) were used:

$$W_L = \frac{W_o - W_t}{W_o} \times 100\%$$  \hspace{1cm} (1)

$$W_U = \frac{W_{t,w} - W_t}{W_t} \times 100\%$$  \hspace{1cm} (2)

Where $W_o$ is the initial sample weight, $W_{t,w}$ is the wet weight and $W_t$ is the constant weight after being dried.

2.5 In Vitro Cell Studies

2.5.1 Cell Proliferation

Ethylene oxide (EtOH) plasma treatment can be used to sterilize many biomedical devices, including biodegradable scaffolds. Ethylene oxide is used because other sterilization procedures like irradiation, heat, steam or acid can cause damage and lead to premature polymer
morphological changes and degradation [49]. The scaffolds can be packaged into gas permeable pouches and sterilized with EtOH gas [50]. The sterilization process has shown no morphological or chemical damages in synthetic polymers such as polyesters and poly(lactide-co-glycolide) (PLGA) scaffolds as well as bio-based scaffolds made of alginate and collagen [20, 49].

Osteoblasts are around ten micrometers in diameter and are responsible for the synthesis and mineralization of bone through a protein mixture known as osteoid during initial bone formation and bone remodeling. In most bio-based scaffold bone regeneration studies, MC3T3-E1 pre-osteoblasts are used [6, 18, 51, 52]. Adhesion of cells onto and within the microporous scaffold is critical for cell functions such as synthesis and deposition of proteins in the extracellular matrix (ECM) [53]. The ECM forms the structural support for cells. Cell adhesion to ECM ligands is mediated by integrins, adhesion receptors on the cell surface [54]. Integrin-regulated adhesion involves receptor-ligand interactions, adhesion strengthening and cell spreading [18]. When ligand-binding occurs, integrins associate with the actin cytoskeleton form focal adhesions which contain structural and signaling molecules that links between the cytoskeleton and ECM to regulate cell adhesion and migration [55].

Ethylene oxide sterilized scaffolds were cut into 5.5 mm diameter and 7 mm high cylinders using a sterilized cork borer inside a laminar flow hood for cell culture. Three scaffolds for each crosslinking ratio were cut. Scaffolds were placed in a 48-well plate containing 0.5 mL media containing MC3T3-E1 osteoblast (20,000/mL) cells for 4 and 96 hours. MC3T3-E1 cells were cultured in expansion medium containing α-minimum essential medium (MEM) plus 10% (by volume) fetal bovine serum (FBS) and 1% (by volume) antibiotic/antiomycotic.
Cell staining consisted of fixing the cells for 5 min using 0.5% by volume Triton X-100, 4% by volume formaldehyde and 1 mmol/L CaCl$_2$, 2 mmol/L MgCl$_2$ in phosphate buffered saline, pH 7.4. The scaffolds were then rinsed and post-fixed for 20 min in the same fixative as before without Triton X-100. Cells were stained in the dark for 1 h with 2 µM Texas Red C$_2$ Maleimide and 6 µM DAPI in PBS containing 1 mM Ca$^{2+}$ and 2 mM Mg$^{2+}$. After 5 minutes of air drying, the scaffolds were mounted on glass slides with Vectashield containing DAPI. Confocal imaging or SEM was used to examine cell morphology on the scaffolds and allow for cell number counts between time intervals to give information about proliferation.

2.5.2 Cell Differentiation

Cellular differentiation is the process by which a less specialized cell becomes a more specialized cell type. Osteoblasts express various phenotypic markers such as alkaline phosphatase (ALP) activity and synthesize collagenous and noncollagenous bone matrix proteins such as osteocalcin (OCN) [52]. OCN is a protein secreted by osteoblasts and is known as a late marker of osteoblastic differentiation and mineralization in the bone formation process [26]. ALP is an important marker for bone forming cells as it is produced by osteoblast cells and required for osteoid formation. Therefore bone-forming potential of osteoblast cells can be further analyzed by measuring ALP activity [56, 57].

Pre-osteoblast MC3T3-E1 cells can be seeded onto the scaffolds, incubated, and after several days, the OCN levels determined. Studies have shown that mineralized nodule formation can be increased by the production of OCN [58]. An enzyme-linked immunosorbant assay (ELISA) kit (Biotechnologies, Inc.) can be used to analyze OCN levels. If high levels of OCN are found, osteoblast differentiation and mineralization is occurring within the scaffolds.
To induce osteoblastic differentiation, cells were seeded and cultured for 3, 7, 14, and 21 days. Three scaffolds were tested per time period and an empty well as a control. MC3T3-E1 pre-osteoblast cells were cultured on 5.5 diameter 10% scaffolds as well as 10% films in a 48-well plate in 0.5 mL expansion medium supplemented with ascorbic acid (50 mg/mL) and β-glycerol phosphate (10 mmol/L) to enhance osteoblast differentiation. The medium was changed every 3 days during the differentiation studies. To determine if differentiation occurred, OCN levels were measured for each time interval using an enzyme-linked immunosorbant assay (ELISA) kit (Biotechnologies, Inc.) according to the manufacturer’s directions. Scaffolds were kept for ALP level analysis using a reagent assay.

3. Results

3.1 Scaffold Fabrication

Scaffold integrity was an important factor in determining salt size, amount OA solution and GA crosslinking necessary for scaffold fabrication. Before the salt leaching and freeze drying steps, each scaffold was examined to determine the effect of each variable.

Scaffolds created with 2 mL of solution were studied first. It was found that scaffolds made with the < 45µm salt size and 15% or 10% crosslinking ratio were structurally weak and fell apart when taken out of the well plates. With the > 150µm salt size, there was a large amount of precipitation out of solution as a layer of salt was found on the bottom of the well when the scaffolds were removed. This occurred for all crosslinking ratios.

Scaffolds created with the 2.5 mL scaffolds were studied next. Similar results were discovered for the < 45µm salt particles as the scaffolds fell apart. However, for this experiment there was less precipitation of the > 150µm salt out of solution.
Overall the 2 mL scaffolds were not as thick as those created with 2.5mL and more difficult to handle. Therefore 2.5mL OA solution and > 150 µm salt size were chosen as the optimal scaffold conditions due to scaffold thickness and increased pore size. Crosslinking ratio effects were further studied.

3.2 Scaffold Characterization

3.2.1 Scanning Electron Microscopy & Pore Size

SEM can be used to look at morphology and elemental analysis of the specimens [59]. High energy electrons with 5-10 keV bombard a sample and the secondary electrons are detected to obtain an image. X-rays are also emitted, which allows for elemental analysis of the specimen to show the distribution of elements on the surface [59]. Contaminates such as dust, particles and residual salt on the scaffolds can also be detected and analyzed. Field emission scanning electron microscopy will be used to look at the morphology of the OA scaffolds because it provides a narrower probing beam at low electron energy, resulting in both improved spatial resolution and minimized sample charging and damage.

SEM analysis of the scaffolds allowed for morphology, size of pores and pore interconnectivity to be evaluated. Both the surface and cross sectional areas were viewed for each scaffold with varying crosslinking ratio to determine morphology.

For the 10% ratio scaffolds, a porous structure was viewed on the surface with varying pore sizes (Figure 4A). The structure was intact showing no collapsed pores. The surface average pore size was 147.84 ± 40.36 µm. Cross sectional areas (CSA) were also viewed for the 10% ratio scaffolds (Figure 4B). The images also showed a porous structure. Pore shape for both the
top and CSA were somewhat rounded. An average pore size for the CSA was $111.79 \pm 30.71 \mu m$.

Figure 4. A. Top section of 10% crosslinking ratio scaffolds showing an intact porous structure. B. Cross-sectional area (CSA) of 10% crosslinking ratio scaffolds showing porous structure. Images taken at 100x. C. Top section of 15% crosslinking ratio scaffolds showing an intact porous structure. D. CSA of 15% crosslinking ratio scaffolds showing a similar porous structure. Images taken at 100x. E. Top section of 20% crosslinking ratio scaffolds showing an intact porous structure. F. CSA of 20% crosslinking ratio scaffolds showing porous structure. The structure is somewhat jagged most likely due to cutting the sample for SEM preparation. Images taken at 200x.
Similar images were taken for the 15% ratio scaffolds. The top sections showed an intact, multi-sized porous structure (Figure 4C). The CSA showed a similar porous structure (Figure 4D). Both the top and CSA had rounded pores, but had varying pore shape. Average pore sizes for the top and CSA were $137.66 \pm 48.54 \mu m$ and $111.21 \pm 36.95 \mu m$ respectively.

Finally top and CSA images were taken for the 20% ratio scaffolds (Figure 4E and F). Top sections showed a porous structure with varying pore sizes. Pores were rounded on the edges however varied in shape. Average pore sizes for the top images were $165.05 \pm 52.28 \mu m$ and average pore sizes for the CSA were $191.56 \pm 62.19 \mu m$. Figure 5 shows a comparison of average pore sizes between the top and CSA of the three different scaffolds.

![Average Pore Size](image)

**Figure 5.** Comparison of average pore size for top and cross sectional area of varying crosslinking densities 10%. 15% and 20%. Values were determined not statistically significant.

Interconnectivity was also determined by SEM analysis. All scaffolds, 10%, 15% and 20% showed signs of interconnected pores. Interconnectivity was determined by the presence of pores within pores. Figure 6 shows SEM images show scaffolds with interconnectivity for all three crosslinking ratios.
Figure 6: A. 10% scaffold showing interconnectivity. Membranes of inner pores could also be seen. B. 15% scaffold showing interconnectivity. C. 20% scaffold showing interconnectivity. All images at 200x.
3.2.2 Percent Crosslinking

Using the TNBS assay, percent crosslinking averages for the scaffolds at varying crosslinking ratios were determined. To determine percent crosslinking, the moles of lysine (L) present is first calculated using equation 3 where \( A_{\text{avg}} \) is the average absorbance for the scaffold:

\[
L = \frac{(2\times A_{\text{avg}} \times 0.03)}{([1.46\times10^4] \times 0.011)}
\]  

(3)

Average absorbance was determined by looking at the scaffold wavelengths from 349.07nm-350.11nm and averaging their corresponding absorbencies. This was done for the control, 10%, 15% and 20% samples. The percent crosslinking was calculated by equation 4 where \( L_{\text{control}} \) is the average moles of lysine calculated for the control and \( L_{\text{scaffold}} \) is the calculated average moles of lysine for the scaffold:

\[
\% \text{ Crosslinking} = \frac{L_{\text{control}} - L_{\text{scaffold}}}{L_{\text{control}}}
\]  

(4)

It was determined that the 10% crosslinked scaffolds had a percentage crosslinking of 35 ± 9 %. The 20% scaffolds had a crosslinking percentage at 37 ± 6 %. Finally, the 15% scaffolds had a crosslinking percentage of 23 ± 5%. Figure 7 compares the average crosslinking percentages.

![Figure 7: Comparison of average crosslinking percentages for the three varying crosslinking densities.](image)
3.2.3 Mechanical Testing

Compression tests were done on both dry and wet samples and compression properties determined. Each scaffold was tested until failure, determined by looking at the stress vs. strain curves (Figure 8). Also, each sample exhibited plastic strain.

![Stress vs. Strain Curve](image)

Figure 8. Example of stress vs. strain curve for dry and wet scaffolds. Solid line is an example of dry scaffolds behavior. The dashed line is an example of wet scaffold behavior.

The 10% ratio scaffolds had an average yield strength of 0.025 ± 0.014 MPa. Strain at failure was between 20% and 40% for each scaffold and elastic modulus determined to be 0.060 ± 0.022 MPa. For the 15% ratio scaffolds, the average yield strength was 0.021 ± 0.01 MPa with failure occurring between 20% and 40% strain and had an elastic modulus of 0.046 MPa ± 0.012. The 20% ratio scaffolds had an average yield strength of 0.03 +/- 0.018 MPa and failed between 20% and 40% strain with elastic modulus of 0.062 ± 0.026 MPa. Figure 9 shows the average dry compressive strengths and elastic moduli for the samples.
Scaffolds were also tested wet to determine the effect on yield strength of the scaffolds (Figure 10A). The samples exhibited an average swelling of $1 \pm 0.32$ mm for both the height and diameter of the scaffolds. The average yield strength for the 10% ratio scaffolds was $0.0068 \pm 0.0036$ MPa. For the 15% ratio samples the average yield strength was $0.0039 \pm 0.0013$ MPa. The 20% ratio samples had an average yield strength of $0.0020 \pm 0.0018$ MPa. The elastic moduli for the 10%, 15% and 20% scaffolds were $0.0068 \pm 0.0036$ MPa, $0.0048 \pm 0.0013$ MPa and $0.0026 \pm 0.0026$ MPa respectively (Figure 10B).

Figure 9: A. Comparison of average compressive strength for 20%, 15% and 10% scaffolds. Differences were not statistically significant. B. Comparison of average elastic moduli for 20%, 15% and 10% scaffolds. Differences were not statistically significant.

Figure 10: A. Comparison of average compressive stresses for 20%, 15% and 10% wet scaffolds. Differences were not statistically significant. B. Comparison of average elastic moduli for 20%, 15% and 10% wet scaffolds. Differences were not statistically significant.
After initial mechanical tests and looking at the stress vs. strain curves for each crosslinking ratio scaffolds, it was determined that all dry scaffolds failed between 20-40%. The cross sectional area of samples were viewed under SEM after 20% and 40% strain was induced on the samples to see the effect of stress on the pores and overall integrity of the scaffolds.

At 20% strain, the 10% scaffold porous structure began to collapse and cracks could be seen between the pores. The average pore size was $72.66 \pm 25.81 \mu \text{m}$. At 40% strain the scaffolds also collapsed and lost their rounded shape. Some of the edges became sharp and fractures were seen around the pores. Average pore size was $139.03 \pm 61.72 \mu \text{m}$. Figure 11 compares the SEM images of the porous structure for the control, 20% and 40% strained samples while Figure 12 compares the average pore sizes.
Figure 11: A. CSA 10% ratio control sample with no mechanical stress applied. B. CSA 10% ratio sample under 20% strain. C. CSA 10% ratio sample under 40% strain. D. Crack along a pore seen in CSA of 20% strained samples that could result in failure. E. Crack and tear in the porous structure in a CSA of 40% strained sample that could cause pores to collapse. A-C images taken at 500x. D-E images taken at 2000x.

Fractures were seen between pores at 20% strain in the 15% scaffolds. At 40% strain, fragments of the scaffold could be seen where the material had fractured off and little porous structure could be seen. The average pore sizes for 20% and 40% were 130.35 ± 39.05µm and 119.38 ± 40.11µm respectively. Figure 13 compares the SEM images of the porous structure for the control, 20% and 40% strained samples. Figure 14 compares the average pore sizes for the control, 20% and 40% strain.
Figure 13: A. CSA 15% ratio control sample with no mechanical stress applied. B. CSA 15% ratio sample under 20% strain. Some fracture is seen. C. CSA 15% ratio sample under 40% strain. The porous structure began to collapse. A-C images taken at 500x.

Figure 14: Comparison of average pore sizes between the control, 20% and 40% strain. The averages were not statistically significant.

Twenty percent strain on the 20% scaffolds had similar effects, showing a porous structure with some fracture of the material. The average pore size at 20% strain was 84.55 ± 22.81 µm. At 40% strain pores began to collapse and cracks were noticeably visible along the
structure. The average pore size was $32.21 \pm 8.73 \mu m$. Figure 15 compares the SEM images of the porous structure for the control, 20% and 40% strained samples. Figure 16 compares the average pore sizes for the control and 20% scaffolds at 20% and 40% strain. Figure 17 compares the average pore sizes for the various scaffolds at 20% and 40% strain.

Figure 15: A. CSA 20% ratio control sample with no mechanical stress applied taken at 500x. B. Crack along a pore seen in CSA of 20% strained samples that could result in failure. C. Crack and tear in the porous structure in a CSA of 40% strained sample that could cause pores to collapse. D. Tear in porous structure in 40% strained scaffold that could cause failure. B and C images taken at 2000x. Image D taken at 5000x.
3.2.4 Thermal Properties

Using the Proteus Thermal Analysis software, an onset and end temperature was determined for the $T_g$ phase of each of the scaffolds with varying crosslinking ratios as well as a control. The control was an OA film that was not crosslinked with GA. The control containing no crosslinker had a $T_g$ of 220 °C. This $T_g$ is slightly higher than the reported $T_g$ for OA at 208 °C [60]. Glass transition temperatures for 10%, 15% and 20% were not found between 0 and 250 °C.
3.2.5 Thermal Mechanical Properties

Scaffolds of all three varying crosslinked ratios were tested in compression at 37 °C to determine if an increase in temperature had an effect on mechanical properties. The 10% scaffolds had average compression strength of 0.02 ± 0.006 MPa. The average strength for the 15% and 20% scaffolds were 0.027 ± 0.013 MPa and 0.027 ± 0.029 MPa respectively. Figure 18 shows the comparison between scaffolds previously tested in room temperature verses scaffolds tested at body temperature, 37 °C.

![Comparison of Mechanical Properties at Room vs. Body Temperature](image)

Figure 18. Comparison between scaffolds at room and body temperature. Results were found to be statistically insignificant.

3.2.6 FTIR Spectroscopy

Amide I peaks for all of the scaffolds appeared between the 1600 cm⁻¹ and 1700 cm⁻¹ absorbance discussed in the literature (Figure 19). Non-crosslinked control films had the lowest absorbance at approximately 0.05 and its highest peak could be found at 1640 cm⁻¹. The 10% and 15% scaffolds had similar absorbances at approximately 0.12 and 0.11 respectively. Their wavenumbers are similar to the non-crosslinked film at 1640 cm⁻¹ for both peaks. The 20% scaffolds had the highest absorbance at approximately 0.23 and a peak wavenumber of 1640 cm⁻¹.
3.2.7 Contact Angle

Contact angle for the three differing crosslinked scaffolds were evaluate to determine the hydrophobicity of the scaffolds. The 10% scaffolds had an average contact angle of 44.0 ± 5.3°. The 15% had an average contact angle of 60.6 ± 19.2° and 20% had an average contact angle of 43.3 ± 6.3°.

3.2.8 Degradation

A control in PBS and two enzymes, lipase and collagenase, were used to determine the degradation rate for the three varying crosslinked scaffolds. Percent mass loss was calculated for all scaffolds under the three conditions.

Degradation rate for all scaffolds in PBS were similar (Figure 20). Initially the 20% scaffolds had a faster degradation rate, but leveled off until week 3. Complete degradation of all three scaffolds types took 9 weeks.
Figure 20: Comparison of average percent mass loss for the three varying crosslinking densities in PBS. The differences were determined to be statistically insignificant.

The degradation rate for scaffolds in PBS+lipase enzyme was similar. They also had similar degradation rates compared to the scaffolds in the PBS control (Figure 21). Complete degradation took 9 weeks for the samples.

Figure 21: Comparison of average percent mass loss for the three varying crosslinking densities in PBS+lipase.

Degradation rate for all scaffolds in PBS+collagenase was higher than the PBS control and PBS+lipase. All scaffolds showed rapid mass loss within the first week; however the 15% and 20% leveled off into a slow linear degradation for the remaining weeks. The 10% scaffolds
exhibited a slow degradation rate between weeks 1 and 3 with rapid mass loss resuming after week 3. Scaffolds only took 6 weeks for full degradation (Figure 22).

![Effect of Degradation on Varying Crosslinked Densities in PBS+Collagenase](image)

Figure 22: Comparison of average percent mass loss for the three varying crosslinking densities in PBS+collagenase.

### 3.3 In Vitro Cell Studies

#### 3.3.1 Cell Proliferation

Cells were stained with DAPI and Texas Red to look at cell morphology on the scaffolds. Nuclei were stained purple due to the DAPI binding to the DNA while Texas Red binds to the F-actin of cells staining it red. Because the scaffolds autofloresce, it was impossible to see the stained cell body to determine morphology along the scaffold pores. However, cell numbers for both the 4 hour and 96 hour time intervals could be determined by counting the stained nuclei (Figure 23). At four hours the average number of cells on the scaffolds was 60.8 ± 18.9, 87.6 ± 10.8 and 90.2 ± 8.3 cells per image for the 10%, 15% and 20% scaffolds respectively. For the 96 hour time interval, the average number of cells was 153 ± 4.8, 181.8 ± 49.5 and 137.2 ± 50.7 cells per image for the 10%, 15% and 20% scaffolds respectively (Figure 24).
Figure 23. Comparison of cell viability between three different scaffolds. A. 10% scaffolds at 4 hours. B. 10% scaffolds at 96 hours. C. 15% at 4 hours. D. 15% at 96 hours. E. 20% at 4 hours. F. 20% at 96 hours.
3.3.2 Cell Differentiation

Initial differential studies compared scaffold OCN levels at 3, 7, 14 and 21 day time intervals to standard solutions. A control and crosslinked films were also compared to a standard curve found from the average standard absorbances calculated. Absorbance for all samples (Figure 25) were determined and compared.

Figure 24: Comparison of average cell count for the three crosslinking densities. The results for the 10% and 15% are statistically significant in when comparing the 4 and 96 h cell counts respectively. There is no statistical significance between the three crosslinking densities.

Figure 25. Absorbance of OCN levels for the control, 10% films and 10% scaffolds for the second trial. OCN levels are significantly higher for the scaffolds at 21 days in comparison to the control and films at 21 days.
ALP levels for films and scaffolds (Figure 26) were also calculated to determine if cells were differentiating. Equation 5 was used to determine ALP levels:

\[
ALP (U/L) = \frac{(\Delta Abs) \times (V_{rxn}) \times (1000)}{(18.8) \times (V_s) \times (1)}
\]

(5)

where \(\Delta Abs\) is the average change in absorbance per minute for each scaffold, \(V_{rxn}\) is the total reaction volume in mL and \(V_s\) is the sample volume in mL. The known variables, 18.8, 1000 and 1 are the absorbance coefficient of p-nitrophenyl at 405 nm, conversion from U/mL to U/L and light path in cm respectively.

Figure 26. A) Average ALP levels for films. ALP levels at 14 and 21 days were significantly higher than films at 3 and 7 days. B) Average ALP levels for scaffolds. ALP levels at 14 days were significantly higher than for scaffolds at 3, 7 and 21 days.
4. Discussion

4.1 Scaffold Characterization

4.1.1 Scanning Electron Microscopy

Overall each scaffold, top and CSA, showed a multi-porous structure. The 10% and 15% ratio scaffolds had similar top and CSA pore sizes while the 20% ratio scaffolds had an average higher pore size for both the top and CSA. Average pore size corresponds with research previously mentioned determining optimal pore size for cell adhesion, proliferation and differentiation [15,20,22]. The multi-porous scaffolds are ideal for cell penetration due to high surface area to volume ratio unlike other methods that are limited to flat surfaces. Scaffolds also mimic the 3D environment found in vivo.

4.1.2 Percent Crosslinking

Although the crosslinking results showed the 10% scaffolds had the highest average percent crosslinking followed by 20% then 15%, the values do not correspond with expected results. One would assume the 20% would have the highest percent crosslinking because a higher amount of GA was used. Similarly, the 10% scaffolds should have the lowest percent due to lower amount GA used.

Even though GA is a common crosslinking agent, the mechanism and chemistry involved in crosslinking reaction is not yet fully understood [24]. The inconsistency in the data may be attributed to unknown GA reactions. It has been shown in previous research that varying GA concentration has an effect on crosslinking [61]. Low concentrations of GA allowed for intramolecular crosslinks between lysine groups. Intramolecular crosslinks were the predominant type of crosslinks in this case. When GA concentrations were increased, protein solubility
decreased causing intermolecular crosslinking between the GA chains. In this case the molecular length of the GA polymer is increased in comparison to the initial GA length rather than the number of crosslinking sites that cause intramolecular crosslinking. Therefore it is possible that increasing the concentration of GA for the scaffolds might not significantly increase the amount of crosslinking because after all lysine sites have been crosslinked GA chains are intermolecularly crosslinked and washed out in the glycine rinse. Scaffold properties would therefore not be significantly different.

To determine if an excess of GA was used in the lowest ratio scaffolds (10%) and therefore excess in the other two scaffolds types, the molar ratio between the number of lysines in each scaffolds and the amount of GA used was calculated. For the 10% scaffolds the following equations were used to determine the amount of lysine in one scaffold available for GA crosslinking.

Equation 6 determines the moles of OA in one scaffold:

\[
\text{moles of OA} = \frac{\text{grams OA}}{\text{MW OA}} \quad (6)
\]

\[
\text{moles of OA} = \left( \frac{0.16 \text{ g OA}}{49,670 \text{ g/mol}} \right) = 3.22 \times 10^{-6} \text{ moles of OA per scaffold}
\]

Equation 7 determines molecules OA in one scaffold:

\[
\text{Molecules OA in one scaffold} = \text{(Avagadro's #)} \times \text{(moles of OA in one scaffold)} \quad (7)
\]

\[
\text{Molecules of OA in one scaffolds} = (6.02 \times 10^{23}) \times (3.22 \times 10^{-6}) = 1.94 \times 10^{18} \text{ molecules OA in one scaffold}
\]
Equation 8 is used to determine the molecules of GA needed for crosslinking:

\[ \text{Molecules GA} = \left( \frac{\text{molecules GA}}{1 \text{ molecule OA}} \right) \times \left( \frac{\text{molecules of OA}}{1 \text{ scaffold}} \right) \]  

\[ \text{Molecules GA} = \frac{(19 \text{ molecules GA})}{(1 \text{ molecule OA})} \times \left( \frac{1.94 \times 10^{18}}{(1 \text{ scaffold})} \right) = 3.68 \times 10^{19} \text{ molecules GA} \]

The moles of GA was determined using equation 9:

\[ \text{Moles GA} = \frac{(\text{molecules GA})}{\text{Avagadro's#}} \]  

\[ \text{Moles GA} = \frac{(3.68 \times 10^{19} \text{ molecules GA})}{(6.02 \times 10^{23})} = 6.12 \times 10^{-5} \text{ grams GA} \]

Using 6.12 x 10^{-5} g GA found in the previous equation and using equation 10:

\[ \text{volume GA} = \frac{(\text{grams GA}) \times (\text{MW GA})}{\text{density GA}} \]  

\[ \text{volume GA} = \frac{(6.12 \times 10^{-5} \text{ g GA}) \times (100.19 \text{ g/mol})}{1.06 \text{ g/mol GA}} = 0.0058 \mu\text{L GA} \]

In order to have a 100% crosslinking rate between GA and OA lysines, 0.0058 µL of GA had to be used. Initial experiments used between 250 µL and 500 µL of GA. From these calculations, it was determined that GA was, in fact, in excess for all three scaffolds causing them to have insignificant characteristic differences.
4.1.3 Mechanical Testing

With an increase in crosslinking density, the general trend expected would be that the dry materials tested in compression exhibit an increase in mechanical properties. However, increasing the crosslinking density in the OA scaffolds did not show this trend because of an excess of GA present in the crosslinking reaction.

Although the 20% ratio dry scaffolds did exhibit the highest average compressive strength, the 15% ratio scaffolds had a slightly lower average compressive strength than the 10% ratio scaffolds (Figure 7A). When evaluating the wet scaffolds, it was determined the 10% had the highest compressive strength followed by the 15% and 20% scaffolds respectively. In both the dry and wet samples respectively, the variability in porosity and porous structure within the scaffolds and not the actual material strength might be affecting the compressive strengths of the scaffolds. Variability in porosity is a result of GA and water leaving at different rates from the scaffolds during the freeze drying step. Collapse of the porous structure during testing could result in lower yield strength for the other scaffolds (Figure 8). The difference for both dry and wet scaffolds, however, was ultimately not significant. In conclusion, the average compressive strengths for all the scaffolds were found to be statistically not significant because of the same crosslinking density.

Examining the average elastic modulus for the dry scaffolds showed the 20% scaffolds having a higher elastic modulus than the 10% and 15% scaffolds. However, 10% scaffolds had a higher average elastic modulus than the 15% scaffolds (Figure 7B). Elastic modulus of the 10% wet samples was on the average higher than 15% and 20% scaffolds respectively. Similar to the average compressive strengths, the average elastic modulus differences for each of the scaffolds between all dry, as well as wet, tests were determined to be insignificant.
The difference between all of the dry scaffolds, as well as wet, was found to not be statistically significant. However, comparing the compressive strengths of the dry and wet samples showed there was a significant decrease in average compressive strength between the dry and wet samples. The PBS used to wet the samples plasticized the scaffolds allowing for stretching of bonds without breaking and making them more elastic in comparison to the dry samples. The increase in elasticity decreased brittleness of the scaffolds and the overall mechanical properties.

Although crosslinking ratios varied between scaffolds, it was determined crosslinking ratio did not play a significant role in effecting dry and wet mechanical properties. As previously stated, it is possible that increasing the concentration of GA for the scaffolds might not have significantly increased the amount of crosslinking in the scaffolds due to lysine sites already being saturated at the 10% ratio.

Compact bone is known to have a compressive strength measured anywhere from 150-250 MPa due to variability in bone density as discussed previously. Although the ideal mechanical strength of biomaterial scaffolds has not yet been determined, previously researched scaffold compressive strengths have fallen within a 2-9 MPa range. OA scaffolds, although lower in compressive strength than other bio-based scaffolds as well as natural compact bone, still presents a promising substrate for cell growth and bone regeneration. It is not fully understood to what extent scaffolds must mimic natural bone mechanical properties because as the scaffold degrades its mechanical properties are changed as new bone infiltrates.

Because porosity is imperative for cellular activity within the scaffold, the effect of strain on average pore size was evaluated for all the scaffolds. Average pore sizes for the dry scaffolds before and after a mechanical stress was applied to the scaffolds were analyzed. Environmental
scanning electron microscopy (ESEM) could be done to evaluate average pore size for strained wet scaffolds.

Statistically, the 10% control (which experienced no mechanical stress and had a pore size of 111.79 ± 30.71µm) showed no statistical difference between scaffolds subjected to 40% strain. However, there was a statistical significant difference in average pore size between control and 20% strained scaffolds as well as between the 20% and 40% strained scaffolds. This might be due to the fact that the values are an average between the control scaffolds and the tested scaffolds had a higher average pore size to begin with due to batch to batch variation, causing the pore size to be greater for the 10% scaffolds that experienced 40% strain.

The average pore sizes for both the strained 15% scaffolds were slightly higher than the control scaffold (111.21 ± 36.95µm). Although the pore sizes were larger, there is no statistical significant difference between the control and 40% as well as no significant difference between the 20% and 40% strained scaffolds. However there was a significant difference between the control and 20% strained scaffolds. Because different scaffolds were used in the control measurements and the mechanical testing, there may have been a significant variation in pore size in the scaffolds initially.

Both the strained 20% scaffolds had lower average pore sizes than the control (191.55 ± 62.19µm). It was determined that the average pore size difference between the control, 20% and 40% strained scaffolds was statistically significant. Similar to the 10% and 15% scaffolds, different scaffolds were used in the control measurements and the mechanical testing, and therefore there may have been a significant variation in pore size in the scaffolds.

Comparing the three different scaffolds at 20% strain, the average pore size difference between the 10%, 15% and 20% was statistically significant. At 40% strain the average pore size
for the 20% scaffolds was significantly different than the 15% and 10% scaffolds. These significant differences may be possible because of significant pore sizes between scaffolds before testing. The 15% and 10% scaffolds were insignificantly different.

Because scaffolds were brittle, there were numerous fractures and cracks seen in all scaffolds after a strain was applied. It can be assumed cracks were the mode of failure in the samples causing a low compressive strength. Propagating cracks along the pores due to stress caused them to fracture and collapse and may be the main reason for compressive failure. Although there were significant differences in pore sizes between control and strained scaffolds for all crosslinking ratios, these differences cannot be solely attributed to mechanical testing. As long as pore sizes before and after testing fall within a suitable range (> 100µm) [29] found for tissue engineering applications, the scaffold structure should be successful in promoting cellular activity.

4.1.4 Thermal Properties

DSC curves showed a glass transition stage for the non-crosslinked films at 208 °C. Because no T_g was found for any of the scaffolds (10%, 15% and 20%) between 0 °C and 250 °C, crosslinked scaffolds have a higher T_g than the control films. It can be assumed the scaffolds have an amorphous bulk structure due to crosslinking that occurred as proven by the percent crosslinking assay.

4.1.5 Thermal Mechanical Properties

Although differing crosslinking ratios were used (increase in GA for increasing ratio), there was no significant effect on the dry compressive strength of the scaffolds at body
temperature in comparison to dry scaffolds tested at room temperature. No rearrangement of atoms or thermal degradation of the scaffold was likely to occur.

4.1.6 FTIR Spectroscopy

Using the FTIR spectroscopy peaks, it was determined there was a crosslinking reaction between GA and OA. The initial peak, non-crosslinked OA films, had the lowest Amide I peak of all four peaks. Films of 10% and 15% had similar Amide I peaks, larger than the non-crosslinked films. Finally, 20% films had the largest Amide I peak. Broadening of the peaks is a good indication of Schiff bases presence. The presence of Schiff bases confirm that crosslinking occurred between the GA and lysines in the OA. With crosslinking, peaks increase due to both the aldehyde and amino group frequencies. Aldehyde groups and amino groups can both be found between 1600-1700 cm\(^{-1}\).

Although there was no large difference between the 10% and 15% film peaks, the 20% films exhibited a large peak increase. Because an excess of GA was used to create the scaffolds, it is possible the excess GA was polymerically bound to other GA groups within the scaffolds causing an increase in the peak.

4.1.7 Contact Angle

The 10% scaffolds had an average contact angle of 44.0 ± 5.3°. 15% had an average contact angle of 60.6 ± 19.2° and 20% had an average contact angle of 43.3 ± 6.3°. All scaffolds showed a hydrophilic nature, meaning water was attracted to the scaffolds. The 10% and 20% scaffolds had the lowest average contact angle therefore the highest hydrophilicity. These scaffolds have an affinity for water and can absorb and dissolve in water after 9 weeks. The 15%
scaffolds had the highest contact angle and slightly more hydrophobic. Although the scaffolds had varying average contact angles, the difference between the three scaffolds were not statistically significant and therefore crosslinking density had no significant effect on contact angle and hydrophilicity of the scaffolds. Contact angle measurements showed the scaffolds were in fact hydrophilic in nature, ideal for tissue engineering scaffolds [26].

4.1.8 Degradation

Scaffolds within each group, PBS control, PBS+lipase and PBS+collagenase had similar degradation characteristics. It took 9 wks for full degradation of the scaffolds in PBS and PBS+lipase, but only 5 weeks for scaffolds in PBS+ collagenase. The samples were prone to faster degradation rates when subjected to collagenase because it attacks peptide bonds found in OA. Amide hydrolysis occurs naturally in the body; however degradation rate is very slow and collagenase is needed to increase degradation. Lipase, on the other hand, is a catalyst for hydrolytic degradation of ester bonds found in backbones of fats in the body. Therefore the enzyme had no effect on degradation rate as there were no ester bonds present and the scaffold degraded slowly over time.

4.2 In vitro Cell Studies

4.2.1 Cell Proliferation

For 4 and 96 hour time intervals there was a significant increase in cell number in the 10% and 15% scaffolds. This increase in cell number can be attributed to a positive cellular response to the scaffolds causing MC3T3-E1 cells to proliferate. Because there was a significant
increase in cell number for both the 10% and 15% concentration scaffolds, the scaffold with the lower concentration of GA (10%) was used for differentiation studies.

4.2.2 Cell Differentiation

The initial differentiation study showed no increase in OCN levels for controls, OA films or OA scaffolds at 3, 7, 14 and 21 days. A possible explanation could be a low differential potential of the MC3T3-E1 pre-osteoblast cells used in the study. Scaffolds were seeded with passage 12 or greater cells which possible could have affected differentiation potential. It has been reported for this cell line that a decrease in differentiation can occur at high passage numbers [62, 63]. Detection limits of the assay might also be a cause for seeing no increase in OCN levels.

The study was repeated with lower passage (<P4) MC3T3-E1 pre-osteoblast cells. An increase in OCN levels was observed for the scaffolds cultured for 3, 7 and 14 days in comparison to the control and films; however no significant difference was found. There was a significant increase in OCN levels when comparing the scaffolds, film and control at the 21 day time interval. The films and control saw no significant increase in OCN levels over the three week period of the experiment.

ALP studies showed a significant increase in films at 14 and 21 days in comparison to films at 3 and 7 days. For the scaffolds, a significant increase in ALP was seen at 14 days; however a decrease was seen at 21 days. It has been shown that at two weeks in vitro, ALP is increased and then down regulated when mineralization begins. This corresponds with the scaffold OCN data showing an increase of OCN at 21 days indicating the beginning of mineralization and thus a down regulation of ALP.
From OCN and ALP data it can be concluded that cells positively respond to OA scaffolds resulting in MC3T3-E1 pre-osteoblast differentiation into osteoblasts and the beginning of bone matrix formation.

V. Conclusions

Through past research, it has been discovered that bio-based materials are a more viable and biocompatible alternative to synthetic materials created for tissue engineering applications. Although many bio-based materials have been used, OA has yet to be researched as a possible material to create 3D structures to promote osteoblast adhesion, proliferation and differentiation.

Micro-porous constructs are a promising scaffold for bone tissue regeneration therefore OA crosslinked with three different concentrations GA (10%, 15% and 20%) was the choice material in this research. After successful scaffold fabrication, morphology was determined using SEM allowing for scaffold CSA and top porous structure to be viewed. Average pore sizes were also analyzed and found to be comparative to porous scaffolds used previously in research that promoted cellular adhesion, proliferation and differentiation for tissue engineering applications. A TNBS assay was conducted on the scaffolds to determine percent crosslinking of GA with lysines present in OA. It was determined glutaraldehyde crosslinking did occur, however there was no significant difference between percent crosslinking in the scaffolds created with different GA concentrations. This is because there is an excess of GA used in comparison to amount of lysines available for crosslinking. For all three scaffolds there was no difference in the percent crosslinking despite different amounts used. Further calculations confirmed that an excess of GA was used to create the scaffolds and a 1:1 molar ratio between number of lysines and GA for crosslinking should be studied in the future.
Using DSC, an average glass transition temperature was found for the control non-crosslinked film. It was determined that the control had a $T_g$ similar to the known $T_g$ of OA. Because $T_g$ temperatures were not found between 0 °C and 250°C, the scaffolds were assumed to have a higher $T_g$ than the control and an amorphous structure. Average compressive strength was also found for the scaffolds and similar to other results, there was no significant difference between average compressive strengths and elastic modulus for the three scaffolds showing GA concentration had no effect on mechanical properties. The scaffolds were mechanically tested at 37°C and no significant difference was found between the results for scaffolds at room and body temperature; therefore the scaffolds can be used in the body.

Although the average compressive strength is lower than bone, the needed compressive strength for scaffolds is not known and therefore OA scaffolds cannot be ruled out as a possible substrate for cell growth and differentiation. In fact it was shown through cell studies that MC3T3-E1 pre-osteoblast cells did significantly increase in number on the 10% and 15% scaffolds, and therefore a proliferative state of the cells was reached. Because of a positive cellular response on 10% GA scaffolds, of the lower amount of GA was used to create scaffolds for differentiation studies. Differentiation studies showed a significant increase in OCN levels at 21 days for scaffolds. A significant increase in ALP at 14 days was seen for the scaffolds as well as a decrease at 21 days corresponding with the increase in OCN at 21 days. These results signify the beginning of mineralization. Overall ovalbumin scaffolds have shown to be a promising 3D material construct to induce the proliferation and differentiation of MC3T3-E1 pre-osteoblast cells for bone tissue regeneration.
VI. References


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