

Analysis of Chitosan-Alginate Bone Scaffolds

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Abstract

A potential alternative to grafting bone is implanting a cellular scaffold, a structure that is placed directly into the body to support growing bone cells at the injury site. The scaffold must withstand compression, degrade harmlessly into the body, and foster cell growth to be considered successful.

In order to find the most advantageous scaffold, we compared scaffolds composed of various proportions of chitosan and sodium alginate. After producing these scaffolds, we conducted compressive tests on samples of each ratio, first in a dry environment at room temperature (21°C) then in an environment simulating normal body conditions (37°C and pH 7.2 buffered solution). Additionally we co-cultured embryonic stem (ES) cells with a monolayer scaffold to observe which scaffold samples allowed for the most cell adhesion.

We determined that a scaffold with a chitosan to alginate ratio of 60:40 was the mechanically strongest scaffold under physiological conditions. Furthermore, it promoted the adherence of ES cells. Therefore, the 60:40 scaffold is the optimal scaffold with potential for medical application.

1 Introduction

Current medical procedures utilize bone grafts to repair damaged bone tissue. Bone injuries can be repaired using autogenic or allogenic grafts. Autogenic grafts, or transplantations of bone tissue from the injured person's own body, are the preferred method for repairing damage. However, the procedure is limited by the amount of donor tissue available for grafting. Allogenic grafts, or transplantations of

bone tissue from another person's body, may result in the rejection of the implanted bone by the patient's body. [1] Additionally, this medical procedure can be very costly to the patient.

Tissue engineering can be used instead to create a scaffold with ideal physical and chemical properties to encourage cellular growth. By seeding a cellular scaffold with embryonic stem (ES) cells, new bone cells can be generated to replace damaged ones [2]. With this method, the patients will "grow" their own bone. The scaffold should be biodegradable and non-toxic; therefore, it should pose no potential harm to the patient. The implantation of a scaffold instead of the transplantation of bone will also eliminate the risk of rejection by the patient's body. However, in order to make this technology feasible, we must find a scaffold that will have optimal performance in the human body.

For our scaffolds, we decided to use organic materials, specifically chitosan and sodium alginate. These materials have opposite ionic charges: chitosan has a positive charge while sodium alginate has a negative charge. Together, the two form a stable chemical bond that increases structural integrity. They also form scaffolds within a relatively short period of time and can be easily manipulated to regulate the level of porosity. Additionally, the materials allow for the cells to easily adhere to the scaffold due to its slightly opposite charge from the cell. Chitosan causes the scaffolds to have a slightly positive charge while the cell surface has a slightly negative charge.

Previous research regarding the use of organic and inorganic materials, including chitosan and sodium alginate, in scaffolds has been conducted. However, little research has

been documented on the mechanical stability and strength of scaffolds composed of differing ratios of chitosan and sodium alginate. In this experiment, we examined the scaffold's structural integrity and adhesion of cells to it under various conditions.

2 Background

Cellular scaffolds are designed to promote the growth of new bone tissue that will eventually be integrated into the rest of the bone, thus regenerating bone. In order to encourage the cells to proliferate throughout the scaffold and form bone tissue, the scaffold must be porous. The pores should be interconnected to allow the cells to grow together thus enabling nutrients to reach the cells via newly formed blood vessels. The properties of the pores, such as size, shape, and surface area, must be modified depending on the type of injury, location of the injury, and health of the patient. Furthermore, the strength of the scaffold is dependent upon the injury. The scaffold should be strong enough to support the body, especially while the new bone tissue is growing. However, if the scaffold is too strong it will bear most of the load from the body. The surrounding bone weakens as it accustoms itself to lesser load. [1]

Additionally, cells need to be able to attach to the scaffold as most cells cannot survive without adhering to a surface. Cell adhesion is affected by the properties of the surface, including charge, texture, and rigidity. [3] To forgo the need for further surgery beyond the implantation of the scaffold, the scaffold will also need to degrade in the body during or after healing so that only the newly generated bone takes the place of the injured bone. If it does not dissolve, the scaffold will hinder the growth of the new bone. Therefore, in order to have the scaffold safe for use, it must be made of a biodegradable and non-toxic material. [1]

Various materials are used to construct bone scaffolds. They are classified as synthetic

or natural materials. Synthetic materials commonly used for tissue engineering include polylactic acid, polyglycolic acid, or a combination of the two. They are all fibrous, non-toxic, biodegradable plastics used to make products such as biodegradable packaging and resorbable sutures. Synthetic materials can be easily manipulated but do not encourage cell adhesion as well as organic materials do. Some organic substances include: chemicals from cornstarch, used for packaging and plastics; coral, which has been approved for some applications by the U.S. Food and Drug Administration; collagen, a fibrous structural protein present in skin and bone; and chitosan, a chemical derived from crab shells. Organic materials are generally non-toxic and promote cell adhesion. [1]

Chitosan is an organic substance derived from arthropod exoskeletons. It is biodegradable, renewable, and non-toxic. Furthermore, it is not rejected by the body as a foreign substance [4] and has a surface with a positive charge that promotes cell adhesion. As such, it has been used to make scaffolds and has been shown to support bone formation. However, chitosan alone is relatively weak and unstable and swells in solution. Alginate is another biodegradable substance extracted from seaweed. [5] Due to its negative charge, it is able to chemically bond with positively charged chitosan, forming a stronger scaffold material.

To induce the formation of bone tissue, stem cells can be seeded onto the scaffold. Stem cells are non-specialized cells that can differentiate into many types of cells. Embryonic stem (ES) cells in particular are pluripotent and differentiate into any type of cell in the body. Therefore, we can induce the differentiation of ES cells into two major bone cells types: osteoblasts and osteoclasts. Osteoblasts function in the formation of the bone, while osteoclasts function in the removal of bone (resorption). Together, osteoblasts and osteoclasts continually reform and reshape

bone. An imbalance in bone formation and bone resorption could increase or decrease bone mass, resulting in osteopetrosis or osteoporosis, respectively. [2] If we can promote the growth of osteoblasts and osteoclasts onto a scaffold, we can potentially induce the formation of bone tissue.

3 Method

3.1 Construction of Scaffolds

To make the chitosan-alginate scaffolds, 0.3gms of chitosan first was mixed into 100 μ L of acetic acid and 30 mL of water. The acetic acid dissolved the chitosan, creating a solution. Similarly, 0.3 gms sodium alginate was dissolved into 30 mL water. Chitosan and sodium alginate solutions were combined to create 10 mL samples in the following proportions (chitosan: alginate): 90:10, 80:20, 60:40, 40:60, 20:80, and 10:90. To thoroughly mix these samples, we placed each solution into a 20 kHz sonicator, which was set to pulse with 40% amplitude for 3 minutes at 30 second intervals with a 10 second pause.

In preparation for the freeze-drying process, each concentration of chitosan and sodium alginate was divided into five to six 200 μ L microtubes. These vials were placed into a centrifuge for five minutes. They were then frozen overnight and freeze-dried for two days. The freeze-dryer sublimated the ice crystals within the scaffold, leaving minute pores in the chitosan alginate mixture. Before conducting compression tests, the dried samples were divided in half, effectively producing ten to twelve samples for each ratio. The height, mass, and porosity of each sample were obtained.

3.2 Compression Testing

To measure the strength of each scaffold, force was applied to each sample with a compressor. The force required to displace the sample up to 10% of its height was recorded at several intervals of displacement. As a secondary test,

the recovery of each sample was measured by applying force to the sample at 50% of its displacement and measuring the force over a 5 minute time period. The mechanical properties of each ratio were first tested in the laboratory (dry) at 21°C and then in an environment emulating typical physiological conditions of 37°C and 7.4-pH phosphate-buffered saline solution.

Stress, strain, and elastic modulus were calculated for each ratio in order to evaluate the mechanical strength of each scaffold. Stress was calculated by dividing the force applied by the surface area, while strain was determined by dividing the displacement by the original height of the samples. Stress versus strain was graphed, and the corresponding elastic modulus was derived from the slope of the graph. The elastic modulus was our measurement of mechanical strength.

3.3 Cell Cultures

In order to observe the effect of the scaffold on the adhesion of cells, mouse embryonic stem cells were plated on chitosan-sodium alginate coated 24-well plates. As a control stem cells were plated on uncoated tissue culture plastic. Approximately 50,000 cells placed into each of the wells along with 0.33 mL of media. The media was aspirated and replaced regularly in order to remove dead cells and ensure the growth of cells that adhered to the scaffold.

After 96 hours we stained the samples with propidium iodide, which binds to dead cells, and dimethyl sulfoxide, which binds to live cells. This process is known as live/dead staining. The chemicals used for live/dead staining contain proteins that either bind to the nucleotides of dead cells or detected calcium in living cells. The dye was mixed with the media in a 1 μ L dye: 1mL media ratio. Approximately 300 μ L of this dye solution was added to each well. The samples were imaged with a microscope, taking care to observe the presence and adhesion of stem cells to scaffold samples of each different chitosan-alginate ratio.

4 Results and Discussion

4.1 Physical and Mechanical Properties

Each of the samples we obtained after the freeze-drying process had an average volume of approximately 0.2 cm^3 and a mass of 0.006 g . To calculate the porosity we used the following formula:

$$\text{Porosity} = \left[1 - \frac{\text{Density of Scaffold}}{\text{Density of Solid Material}} \right] * 100\%$$

*Density of solid material was 1.5 g/cm^3

As the difference between the quantities of chitosan and alginate increased, the porosity of the scaffold samples increased. Similarly, the porosity of the scaffold decreased as the difference decreased. (Figure 1)

We performed compression tests on scaffolds of each ratio at room temperature in dry conditions and at physiological temperature in buffer solution. (Figure 2-3) Average elastic moduli for scaffolds of each chitosan-alginate ratio in both environments were calculated. For both testing conditions, the elastic modulus of the scaffold was highest for chitosan-alginate samples with a ratio closest to 50:50. Elastic moduli for scaffolds tested at physiological temperature in buffer solution were significantly lower than those of scaffolds at room temperature in dry conditions (by ~2 orders of magnitude). We were unable to collect useful data for scaffolds of ratios 90:10 and 10:90 because they dissolved in the phosphate-buffered solution during compression testing.

Chitosan and alginate form one-to-one chemical bonds; in other words, when the amounts of chitosan and alginate are equal, more bonds are formed, resulting in a denser and stronger material. The scaffolds with chitosan-alginate ratios closest to 50:50 (i.e., 60:40 and 40:60) therefore had lower porosity and higher elastic modulus.

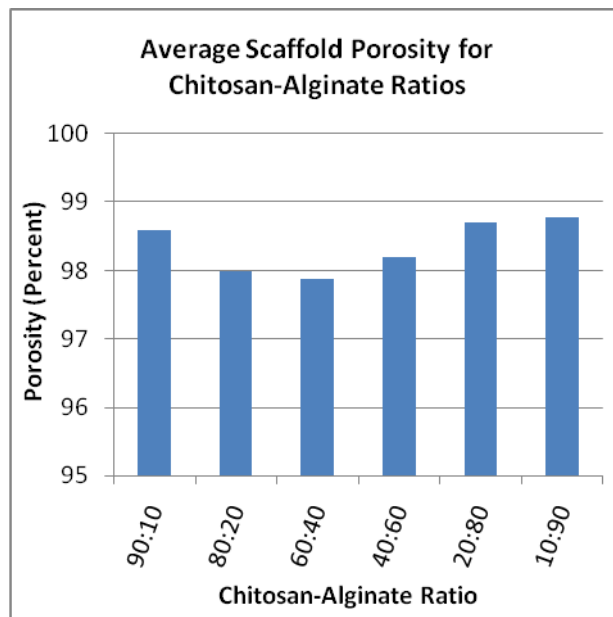


Figure 1: Average porosity of scaffolds of each chitosan-alginate ratio. The 90:10 and 10:90 chitosan-alginate samples had the highest porosities while the 60:40 samples were the least porous.

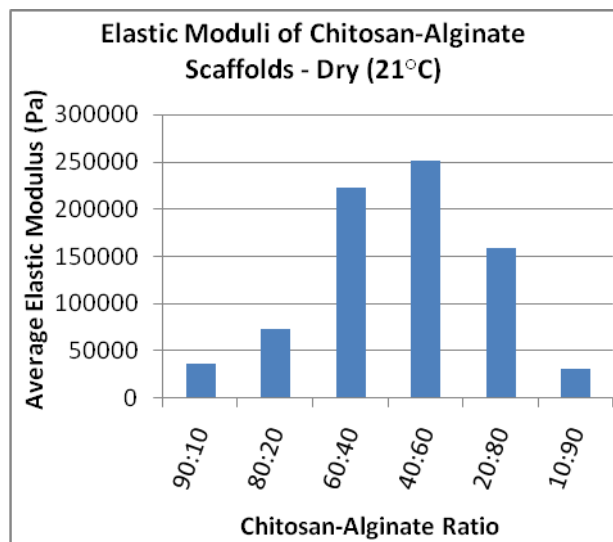


Figure 2. Average elastic moduli of scaffolds calculated by compression tests at room temperature (21°C) in dry conditions. Elastic moduli are highest for chitosan-alginate ratios closest to 50:50.

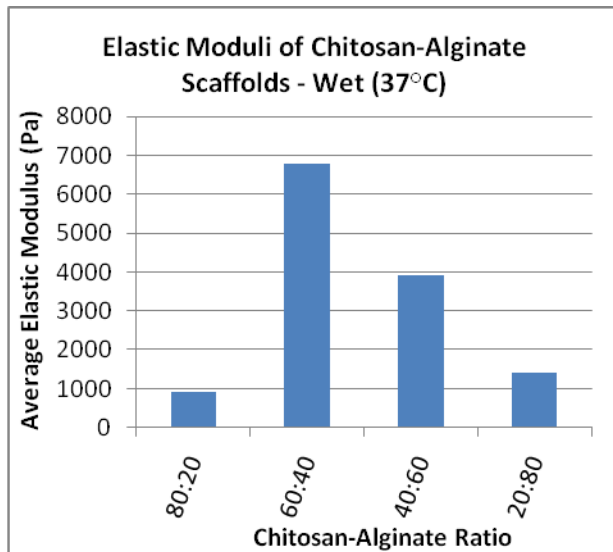


Figure 3: Elastic moduli of scaffolds tested at physiological temperature (37°C) in buffer solution. Elastic moduli are also highest for ratios closest to 50:50, and are significantly lower than those for scaffolds tested in dry conditions.

Additionally, we tested the recovery of each scaffold sample by measuring compressive force at 50% displacement for over 5 minutes. We observed that force decreased exponentially over time. (Figure 4) This was most likely because the scaffolds behave like viscoelastic materials. They demonstrated linear resistance to stress like viscous materials and recovering like elastic materials.

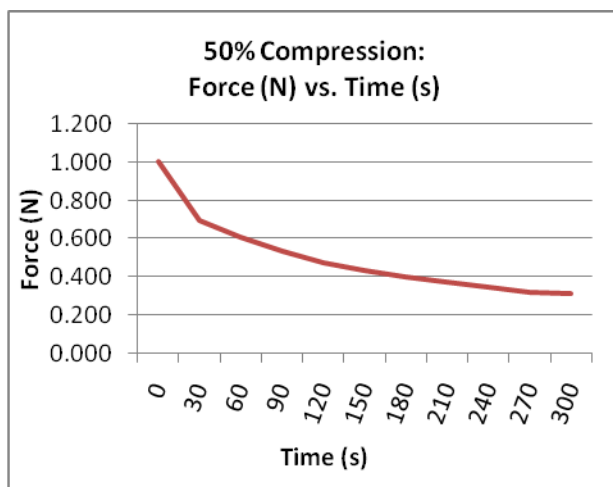


Figure 4: Force on a 60:40 scaffold at room temperature in dry conditions at 50% compression over time. Force decreased with time, showing that the scaffolds were viscoelastic.

4.2 Cell Cultures

We seeded mouse embryonic stem (ES) cells on a monolayer of chitosan-alginate mixtures of the same ratios used in mechanical testing. We also grew embryonic stem cells without chitosan-alginate as a control.

After staining the cells in each well, we used fluorescent imaging to observe how many cells were alive and dead. The stained cells were exposed to certain wavelengths of light. The excitement of the cells' atoms emitted a specific color under a fluorescent microscope. Green indicated live cells, while red indicated dead ones. However, we observed several instances where the cells appeared yellow. This error could be attributed to the laser within the microscope, which emitted a greater variety of wavelengths than the blue and green wavelengths desired. The live cells appear larger because the live stain binds to the calcium in the fluid surrounding the cell's nucleus. On the other hand, the dead cells appear smaller because the dead stain binds to the nucleus. We can conclude that the yellow cells are live cells because their size is consistent with that of live cells.

According to the images of our cell cultures, there appears to be a notable decrease in live cells as the concentration of chitosan decreased. Our control allowed us to observe the affinity of ES cells to adhere to surfaces. Compared to the control, scaffolds of 90:10, 80:20, and 60:40 appeared to have visibly more live cells that adhered to their surfaces than the 40:60, 20:80, or 10:90. This may be due to the charges of chitosan and alginate. Chitosan has a positive charge, while alginate has a negative charge. Scaffolds with more chitosan (90:10, 80:20, and 60:40) have a greater positive charge; therefore, since cells have a slightly negative charge, they will more readily adhere to a positive surface.

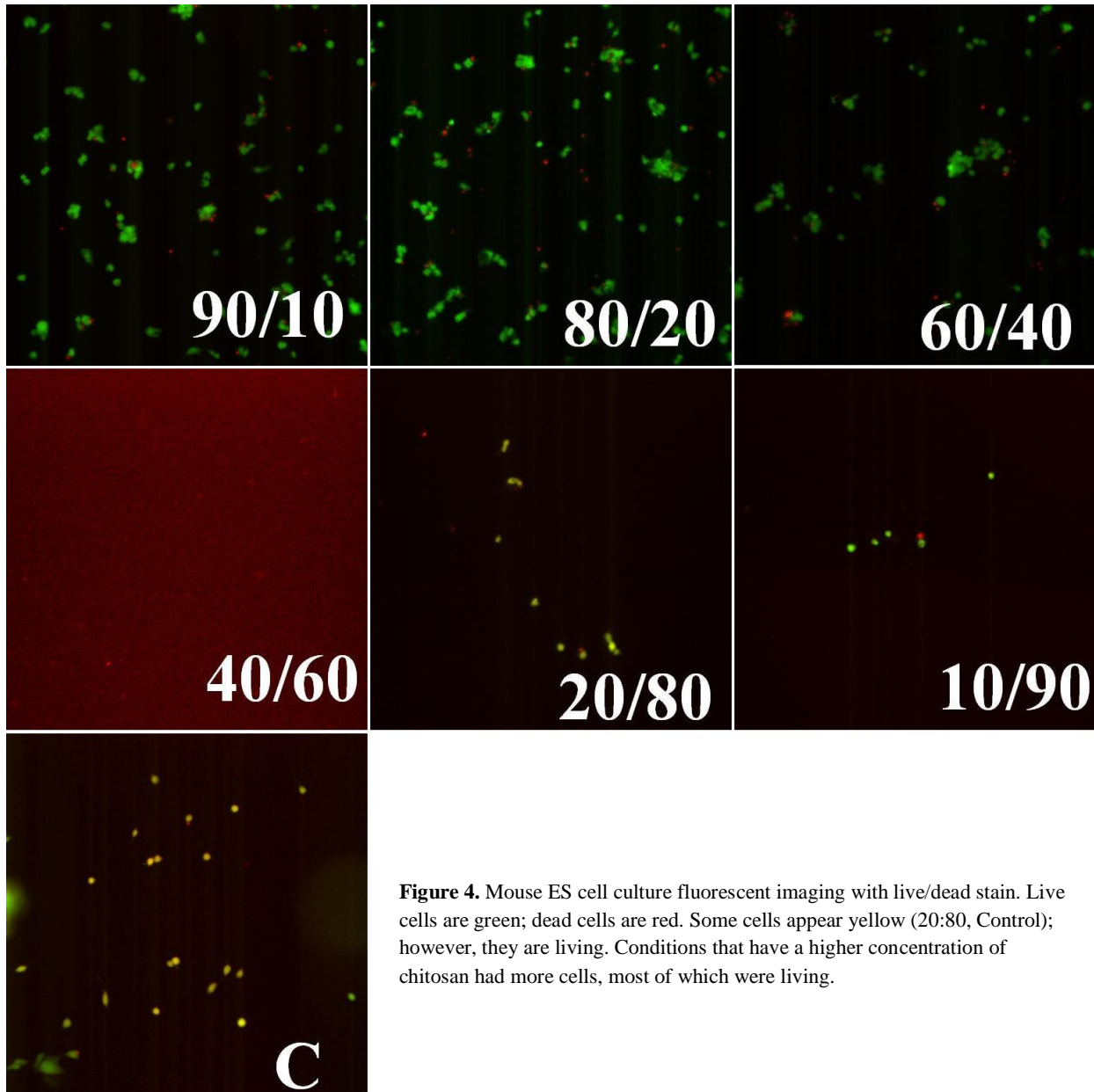


Figure 4. Mouse ES cell culture fluorescent imaging with live/dead stain. Live cells are green; dead cells are red. Some cells appear yellow (20:80, Control); however, they are living. Conditions that have a higher concentration of chitosan had more cells, most of which were living.

5 Related Work

5.1 Mechanical Properties of Chitosan-Alginate Scaffolds

Related research has studied the mechanical properties of chitosan-alginate structures, including scaffolds and sponges. Many compression tests have been performed on

chitosan-alginate scaffolds, reporting their allowable stress. [4] One such experiment included hydroxyapatite, a substance found in bone, in order to increase the strength of chitosan-alginate scaffolds. [4] Other works placed the scaffolds in solutions of varying pH in order to study their swelling behavior. Swelling is an indication of mechanical instability. [5] Additionally, others focused on

the dissolution of chitosan and alginate structures within the human body for the purpose of releasing medication. [6]

5.2 Promotion of Cell Adhesion and Proliferation

Chitosan and sodium alginate structures have been tested for cell adhesion and proliferation. Many observations have been recorded about cell growth in chitosan-alginate scaffolds using cell imaging, transplantation into rats, and staining. Furthermore, osteoblast and osteoclast differentiation has been observed as a further step in determining the viability of chitosan-alginate scaffolds for promoting bone growth. [5] We observed how the ratio of chitosan and sodium alginate affected the adhesion of embryonic stem cells on a chitosan-alginate monolayer.

6 Conclusion

An optimal bone scaffold is very important for a successful bone implantation. A bone scaffold must have the ability to withstand the rigors of the human body, to nurture and house bone cells, and to degrade into the body once new bone has grown into place. To fulfill such requirements, we chose chitosan and alginate to make our bone scaffold because of their strong chemical bond. Furthermore, chitosan and sodium alginate demonstrated an ability to foster the adhesion of bone cells. By using chitosan and alginate, we achieved the optimal chitosan to alginate ratio that could create the strongest and most protective scaffold.

We began by measuring the porosity of our samples and conducting compressive tests on chitosan-alginate scaffolds of varying ratios. We discovered that as the chitosan-alginate ratio approached 50:50, namely for ratios of 60:40 and 40:60, the porosity decreased and strength increased due to bonding between the chitosan and alginate. However, strength of all samples decreased once exposed to physiological conditions (37°C, 7.4 pH).

While strength is a key component of bone scaffolds, the scaffold's ability to house and promote bone cells is equally essential. According to our observations, 90:10, 80:20, and 60:40 are the chitosan-alginate proportions that promoted greater cell adhesion. These samples had more chitosan than alginate and thus an overall positive charge, which attracted cells.

In analyzing results for both mechanical and cell cultures, we have determined that a scaffold with 60% chitosan and 40% sodium alginate is the optimal ratio for mechanical strength and cell growth. The scaffold with this ratio mechanically performed far better than other ratios in physiological conditions. Furthermore, it appeared to promote a moderate adhesion of cells. A scaffold of 60:40 provides the balance of mechanical strength and sensitivity to cell growth needed for medical application.

Although the 60:40 is relatively strong, it may not be strong enough to repair bones that are supposed to bear large compressive loads. There is a great amount of potential for mechanical improvement. Additional materials can be included within the composition of the scaffold to increase its strength. In future experimentation, tests must be conducted on the differentiation of ES cells into bone cells (osteoblasts and osteoclasts). Chemicals could be added to the scaffold in order to induce bone cell differentiation. Further research must be conducted on the lifespan, mechanical strength, and cell growth on 60:40 scaffolds in order to determine its feasibility as an alternative treatment.

7 Acknowledgements

We would like to thank Dr. Devendra Verma, a project mentor, and Diana Arellano, our project advisor.

We would also like to thank the Rutgers University Department of Biomedical Engineering, Lulu Li & Jeff Barminko, our technical advisors, and Dr. Noshir Langrana.

We would like to acknowledge the NJ Governor's School of Engineering and Technology (Donald M. Brown, Director, and Blase Ur, Program Coordinator); Rutgers University School of Engineering (Dr. Yogesh Jaluria, Outgoing Interim Dean, and Dr. Thomas Farris, Dean); and the NJ Governor's School Board of Overseers.

We appreciate the support of our 2009 program sponsors: Rutgers University, the Rutgers University School of Engineering, the Motorola Foundation, Morgan Stanley, PSEG, Silver Line Building Products, and the families of 2001-2008 program alumni.

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