

# Structural Assessment of a Tissue Engineered Scaffold for Bone Repair

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## ABSTRACT

The limitations of current grafting materials have driven the search for synthetic alternatives to cancellous bone. A variety of biodegradable polymer foams composed of poly(lactide-co-glycolide) [PLAGA] have been evaluated for such uses. However, structural limitations may restrict the clinical use of these scaffolds. We have developed a sintered microsphere scaffold composed of 85:15 poly(lactide-co-glycolide) with a biomimetic pore system equivalent to the structure of cancellous bone. Analysis of the structural data, indicated that the microsphere matrix sintered at a temperature of 160°C with a microsphere diameter of 355-425 µm resulted in a optimal, biomimetic structure with an approximate pore diameter of 75 to 275 µm, 35% porosity, and a compressive modulus of 272 MPa. The *in vitro* evaluation of human osteoblasts on the sintered matrix indicated that the structure was capable of supporting the attachment and proliferation of the cells throughout its pore system. Immunofluorescent staining of actin showed that the cells were proliferating 3-dimensionally through the pore system. The stain for osteocalcin showed that the cells had maintained the phenotypic expression for this bone specific protein. Through this work, it was shown that an osteoconductive PLAGA scaffold with a pore system equivalent to the structure of cancellous bone could be fabricated through the sintered microsphere method.

## INTRODUCTION

Bone grafting is a surgical technique used to repair large skeletal defects. Currently, the most common graft material is cancellous bone taken from the patient's iliac crest. The other types of commercially available grafting materials are allografts such as demineralized bone matrix particles and deproteinized cancellous chips, and synthetic alloplasts such as calcium sulfate pellets and porous calcium phosphate materials. Although autograft is the gold standard, synthetic bone graft materials are increasing in popularity. This is due to the intrinsic problems associated with using autogenous bone grafts. Limitations such as supply, donor site pain and infection, and unpredictable healing have stimulated the development of synthetic matrices engineered specifically for bone replacement applications.

The majority of the tissue engineering scaffolds currently being researched are based on the biodegradable copolymer known as poly(lactide-co-glycolide) [PLAGA]. Our laboratory has conducted several studies evaluating the ability of PLAGA to promote osteoblast attachment, spread, and proliferation over its surface [1,2]. With a 30 year FDA approval, it has been extensively tested *in vitro*, *in vivo*, and in clinical settings. It is a degradable copolymer whose properties can be tailored based on the ratio of lactic acid to

glycolic acid, and the molecular weight. Using a variety of techniques, PLAGA has been fabricated into 3-dimensional, porous scaffolds that are often combined with growth factors and/or osteoblasts for bone repair applications.

The structure of many of these PLAGA matrices is based on foam techniques. A foam structure is characterized by a high % porosity (80-95% porous) and low mechanical properties (compressive modulus < 50 MPa). Although these structures have been shown to possess high porosity, the clinical use of these implants is restricted due to certain characteristic properties. The primary factor is that the mechanical properties of the implant may preclude its use in surgery.

In our lab, we have taken a different approach to matrix fabrication. Using PLAGA microspheres, we have developed a sintered microsphere matrix with pore diameters ranging from 100-300 µm and mechanical properties in the mid range of cancellous bone [3]. In this technique, PLAGA microspheres of a narrow size range were thermally fused together in a 3-dimensional porous structure. Due to the nature of sphere packing, the resulting pores system had an interconnectivity of 100% and a % porosity of 30-40%. The goal of this study was to optimize the structure of the sintered microsphere matrix and examine the osteoconductivity of the optimal structure with respect to primary culture human osteoblasts.

## MATERIALS AND METHODS

### *Microsphere Preparation (Solvent Evaporation Technique) [85/15 PLAGA]*

In a glass vial, 85/15 PLAGA (Purac,  $M_w=420,000$ ) was dissolved in methylene chloride. A 1% solution of poly(vinyl alcohol) [PVA] was used as a surfactant. The PLAGA solution was added drop-wise to 1% PVA solution with stirring (300 rpm for 10 hours) allowing for the complete evaporation of the solvent. The microspheres were isolated by vacuum filtration, washed with de-ionized water, and vacuum dried for 24 hours. Using micron sieves, the following microsphere diameter size ranges were isolated: <212 µm, 212-250 µm, 250-300 µm, 300-355 µm, 355-425 µm, 425-500 µm, 500-600 µm, 600-710 µm, and >710 µm.

### *Fabrication of Sintered Microsphere Matrices*

Matrices were formed by pouring pre-fabricated 85/15 microspheres of a specific diameter into a stainless steel mold, which was heated at 160° C for the appropriate time. Once heating was complete, the mold was allowed to

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slowly cool to room temperature (approximately 3 hours) before the samples were removed.

#### *Compression Testing*

Samples with a 2:1 aspect ratio (10 mm by 5 mm) were tested using an Instron 4511 mechanical testing system with a crosshead speed of 1 mm/min. Compressive modulus was calculated from the slope of the linear region of the stress vs. strain curve (n=6).

#### *Mercury Porosimetry*

Porosity measurements were obtained using a Micromeritics Mercury Intrusion Porosimeter 1100. Samples were sealed in penetrometers, weighed, and subject to analysis. Porosity was measured for six samples per structure and reported as average % porosity, pore size distribution, and log differential intrusion.

#### *Primary Culture of Human Osteoblasts*

Primary human osteoblasts were obtained from the femoral head of hip replacement patients (Department of Orthopaedics, Thomas Jefferson University, Philadelphia, PA). Cancellous bone chips were removed from the interior of the femoral head using aseptic techniques and digested with a collagenase solution for 4 hours. Following digestion, bone chips were rinsed with PBS and placed in a tissue culture flask. Explants were cultured using F-12 media supplemented with  $\text{CaCl}_2$ , fetal bovine serum, ascorbate, and an antibiotic solution of penicillin and streptomycin. After four weeks, cells were lifted using trypsin and isolated from the bone explants.

#### *Cell Seeding*

Matrices were fixed to tissue culture wells and seeded with 100,000 cells/matrix. Matrices were then cultured for 7 and 16 days. Cell morphology and proliferation was assessed by scanning electron microscopy.

#### *Indirect Immunofluorescence of Osteocalcin*

Osteoblasts seeded on the microsphere matrices were incubated with 0.5 ml of the monoclonal antibody of osteocalcin (1:200 dilution) [Zymed Laboratories, San Francisco CA] for 30 minutes. Constructs were rinsed with PBS and then incubated with 0.5 ml of the secondary fluorescing antibody (1:50 dilution) for 30 min [goat anti-human antibody - Zymed Laboratories, San Francisco CA]. Another PBS rinse was conducted and the samples were stored in PBS and kept out of the light until microscopic evaluation.

#### *Immunofluorescence Staining for Actin*

The cellular cytoskeletal component, actin, was stained using rhodamine-phalloidin. Human osteoblasts were plated on the sintered microsphere matrix at a density of 100,000 cells per matrix for 16 days. Cell/matrix constructs were rinsed three times with PBS and 0.5 ml of the rhodamine-phalloidin solution was added and incubated for 30 minutes. Samples were rinsed in PBS and visualized with confocal laser scanning microscopy.

## **RESULTS**

### *Optimization of the Sintered Microsphere Matrix [85/15 PLAGA]*

The sintered microsphere technique resulted in a semi-uniform three-dimensional structure as shown in figure 1. These electron micrographs shows the typical structure of the matrix with examples of the type of sphere packing commonly seen.

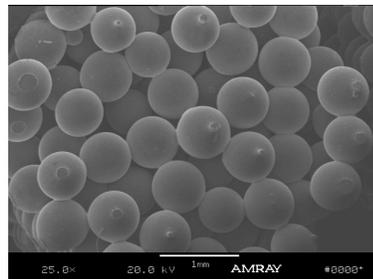


Figure 1. Typical SEM image of three-dimensional sintered microsphere matrix.

Optimization of the sintered matrix was conducted to create a pore system biomimetically equivalent to cancellous bone with mechanical properties in the mid range of cancellous bone. Structural optimization was conducted by modifying microsphere size and heating time. Examining the effect of diameter on modulus, the data showed a decrease in modulus with an increase in microsphere diameter at the 4 hour heating time. However, analysis showed no statistical difference in the moduli of the matrices with varying diameters with 2 hour of sintering. Examining the heating time optimization, at all microsphere diameters, the increase from 2 to 4 hours of heating resulted in statistically significant increase in modulus.

In addition to mechanical testing, mercury porosimetry was also used to examine the effects of sphere size on matrix structure. The porosimetry data revealed a significant correlation between median pore diameter and microsphere size. Significant difference in the average median pore diameter of matrices were found with different diameters and heating times [Student's T-Test]. As expected, the pore diameter increased with an increase in microsphere diameter. The increase in microsphere diameter caused the size range distribution to shift to higher median values.

Unlike the correlation with average pore size, the effect of processing on the total % porosity was not as evident. The 215-250  $\mu\text{m}$  matrix with a 2 hour heating time showed the highest % porosity at 39% while the 600-710  $\mu\text{m}$  matrix at 2 hour heating showed the lowest at 31%. Although the data did not indicate any significant differences between the % porosity of the matrices with different microsphere diameters or heating times, all structures were above the 30% porosity mark which was representative of the amount of bone tissue found in cancellous bone.

#### *Cellular Evaluation of the Sintered Microsphere Matrix*

Figure 2 shows human osteoblasts on the sintered matrix at 16 days of cell culture. This image shows

significant increase in proliferation over the matrix surface and within the pore system. Cellular attachment was evident as cells bridged several gaps in the microsphere structure. Proliferation had progressed from the secondary microsphere layer to the top surface of the matrix.

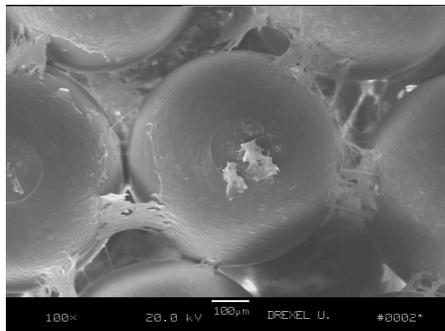


Figure 2. SEM image of human osteoblasts proliferating on the sintered microsphere matrix surface at 16 days of culture. Significant cellular extension is seen.

In addition to electron microscopy, confocal microscopy was used to visualize the cells within the matrix as well as to examine the phenotypic expression of the non-specific protein, osteocalcin. Using immunofluorescence staining techniques, osteocalcin and the structural protein actin were identified on the matrix structure demonstrating that the cells were actively expressing their phenotypes.

The stain for the cytoskeletal protein actin using a primary rhodamine-phalloidin stain was also viewed by CLSM. Since actin is an abundant protein in osteoblasts, the migration of the cells within the microsphere structure could be visualized. The versatility of the confocal microscope allowed for varying depths to be viewed.

## DISCUSSION

This work was focused on modifying the structure and properties of the sintered matrix and evaluating the optimal structure for osteoconductivity. During the optimization process, changes were made to the diameter of the PLAGA microspheres as well as to the duration of the sintering step. Theoretically, in the relationship between sphere size and modulus, it was assumed that an increase in modulus would result from a decrease in sphere size. This was based on surface area and number of fusion points between the spheres. With a significantly greater surface area at smaller diameters, it was thought that microsphere bonding, therefore compressive modulus, would be greater at these diameters. Although the data showed this trend, it was not statically significant. However, the moduli of all structures was significantly higher than other PLAGA scaffolds reported in the literature. For all structures, the range in modulus was from 137 MPa to 297 MPa. This represents a magnitude difference in compressive moduli.

With regards to the heating time modification, the optimization study only examined two times [2 and 4 hours] due to the thermal properties of 85/15 PLAGA. The

mechanical testing data showed a significant increased in the compressive modulus from 2 to 4 hours of sintering. The increase in heating time may have allowed for a stronger fusion between microspheres. Qualitative observations during the testing showed that the matrices were failing at the microsphere bond. Visualization of the fracture surface did not reveal any failures through the actual microsphere structure. Therefore, the strength of the matrix was directly dependent on the combined strength of the microsphere bonds.

In the heating time optimization, only two time were chosen due to the thermal properties of the polymer. Initial characterization of the polymer by differential scanning calorimetry indicated that the copolymer had melting range of 127.5 – 141.6° C but did not exhibit a true glass transition temperature. Therefore, preliminary studies were conducted in order to determine a temperature at which the 85/15 spheres would sinter. Microspheres were heated at temperatures of 135°, 145°, 155°, and 160° C. At 160° C the spheres finally softened and resulted in a bonded matrix. Although the sintering temperature of 160° C was well above the melting range of 85/15 PLAGA, the microspheres did not melt when immediately placed in the 160° C oven. However, at longer heating time of 5, 6, and 24 hours the microspheres melted into a solid polymer mass. This was attributed to an insulating effect from the mold. The thick walls of the mold took a certain amount of time to reach the 160° C temperature. This was particularly evident when using different molds. Heating times were dependent on the dimensions of the mold. This preliminary data indicated that the sintering effects are highly sensitive to temperature and probably should be controlled by a thermocouple within the mold.

The other aspect of structural evaluation was focused on characterization the effects of processing on the porosity of the matrix. The porosity results showed the dependence of pore size on microsphere diameter. All data showed that increases to microsphere size resulted in an increase to pore size. The porosimetry also showed the effects of packing on average pore diameter. Differences in bin sizes from 32 to 70 to 110 had significant impact on the distribution of the pore diameters. As discussed previously, the larger bin sizes resulted in more random packing. The randomness of this packing was represented by the broader pore size distributions.

The porosity data also showed that % porosity was independent of the processing effects. Regardless of processing, all samples had a porosity of 31-39%. This porosity range includes the average percent bone found in cancellous bone. Cancellous bone has been shown to possess a structure with 70% porosity or 30% bone [4]. Therefore, after complete healing, a sintered matrix with a 30% porosity would consist of 70% polymer and 30% bone. Such a biomimetic matrix would allow for the formation of structurally equivalent cancellous bone within the pore system. Once the scaffold completely degraded, the

resulting tissue would have a structural appearance similar to cancellous bone.

Although processing had no effect on % porosity, based on the biomimetic concept of bone regeneration, it was concluded that the absence of relationship between % and processing parameters was not as important as maintaining a structure with a pore system that would result in 30% bone. The modification to heating time was shown to have less of an impact on the structure and properties of the matrix than microsphere diameter. The results demonstrated that heating time only had an effect on the bonding and, hence, the mechanical properties of the matrix, and not the pore structure. This behavior offers an advantage to further optimization. The mechanical properties of the matrix can be altered without resulting in changes to porosity. This allows for the mechanical properties due to heating to be maximized without the consequences of decreasing porosity. This may serve as an effective way of increasing the mechanical properties needed for structural grafting while maintaining the porosity needed to regenerate a significant amount of bone.

In the second part of this study, primary culture human osteoblasts were seeded on the optimal matrix (600-710  $\mu\text{m}$  heated for 4 hours). The culture of human cells on the sintered microsphere matrix also showed some promising results. The SEM and CLSM results indicated that the cells had attached and proliferated through the microsphere pore system. Due to the large size of the pores in this structure, cells were found to be attaching to the secondary layer of the microspheres. This may have been due to the effects of capillary action on the seeding suspension. After addition of the seeding suspension, the matrix may have "wicked" the cells to the secondary layer of microspheres. Only at 16 days of cell culture was it evident that the cells had migrated back towards the surface.

Secondary immunofluorescence staining for osteocalcin showed that the cells had maintained their phenotypic expression of this protein. SEM pictures showed that the microsphere surface and structure provided an excellent scaffold for the attachment and proliferation of the human cells. The results also showed that the cells were capable of bridging the gaps in the microsphere structure. From this data, it was evident that the sintered matrix was capable of functioning as a scaffold for human cells.

Future work will examine the incorporation of growth factors and cells into the sintered matrix in order to further mimic the properties of autograft. An *in vivo* study will examine the regenerative abilities of the scaffold alone and in combination with bone morphogenetic proteins and/or autogenous marrow.

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