

STUDIES ON THE DEVELOPMENT OF A TISSUE ENGINEERED MATRIX FOR BONE REGENERATION

Cato T. Laurencin^{1,2,3,4,*}, Frank K. Ko^{2,4}, Mohamed A. Attawia¹, and Mark D. Borden^{1,2}

¹Department of Orthopaedic Surgery, Allegheny University of the Health Sciences, Philadelphia, PA

²School of Biomedical Engineering, Science and Health Systems; ³Department of Chemical Engineering; and

⁴Department of Materials Engineering, Drexel University, Philadelphia, PA

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Abstract

Alternatives to conventional bone grafts are needed. Using a degradable polymer/ceramic composite system, our laboratory has developed porous matrices for use as trabecular bone graft replacements. Composed of 50:50 poly(lactide-co-glycolide) (PLAGA) and hydroxyapatite (HA), these matrices possess a three-dimensional (3D), porous structure based on microsphere technology. Our current studies examine the relationship between processing conditions and internal matrix structure. This has led to the development of three novel processes capable of fabricating a 3D porous structure: (1) a sintered microsphere method, (2) a solvent cast microsphere method, and (3) a gel microsphere method.

Preliminary characterization of matrix structure was carried out by scanning electron microscopy (SEM). The solvent cast and sintered microsphere methods used pre-fabricated microspheres isolated through a solvent evaporation technique. SEM characterization of the sintered and solvent cast methods indicated a 3D network of fused microspheres with a range in pore diameter from 100-450 μm and 50-250 μm , respectively. The gel microsphere method also used microspheres from the solvent evaporation technique, however, matrices were created from microspheres isolated before complete solvent removal. SEM characterization indicated a 3D internal porous network ranging in size from 100-300 μm . It was determined that the porous structure was due to the aggregation of "gel-like" microspheres.

Key Words: Tissue engineering, biomaterials, polymer, composite, poly(lactide-co-glycolide), pore network.

*Address for correspondence

Cato T. Laurencin
Allegheny University Hospitals - MCP Division,
Department of Orthopaedic Surgery,
3300 Henry Ave., Philadelphia, PA 19129

Telephone number: (215) 842-7360

FAX number: (215) 848-1355

E-mail: Laurencin@allegheny.edu

Introduction

The need for surgical reconstruction or replacement is often the result of trauma, pathological degeneration, or congenital deformity of the tissue [6]. Reconstructive surgery is based upon the principle of replacing these types of defective tissues with viable, functioning alternatives. In bone replacement applications, surgeons have historically used two types of grafts, autografts and allografts. Unfortunately, conventional bone autografts are limited by donor site morbidity and limited amount of bone available for harvesting, while allografts (usually from a cadaver) are limited by possible disease transmission and unpredictable osteoconductivity.

An alternative to bone grafts is the use of porous, biodegradable polymer matrices. Using the biocompatible copolymer poly(lactide-co-glycolide) (PLAGA) and its homopolymers poly(lactic acid) and poly(glycolic acid), several researchers have fabricated matrices for use in bone replacement applications [4, 5, 10, 11]. To date, the major drawback of many of these structures has been the relatively low mechanical properties of the matrices which may lead to problems with implant failure and stress overloading. In order to function as a synthetic bone replacement, matrices should be biomechanically similar to the type of bone they are replacing and have an internal pore structure to allow for bone ingrowth. Keaveny and Hayes [9] found compressive moduli in the range of 0.01 to 2.0 GPa for trabecular bone, and 14 to 18 GPa for cortical bone. A pivotal study by Hulbert *et al.* [7] showed that optimal pore size for bone ingrowth was in the range of 100-250 μm .

In our previous study, we developed a process capable of producing porous PLAGA matrices that used particulate hydroxyapatite (HA) as a reinforcing agent [5]. This resulted in a structure with a compressive modulus in the upper range of trabecular bone (1.5 GPa) and ranges in pore diameters from 18-20 μm and 110-150 μm for micropores and macropores, respectively. Additional *in vitro* studies showed that osteoblasts attached and proliferated throughout the three-dimensional (3D), porous network while maintaining their characteristic phenotype [1, 2]. A degradation study found that the compressive modulus of

the matrix decreased to the low range of trabecular bone during a six week period [5]. In order to improve the mechanical properties and increase control of matrix structure, a solvent evaporation technique was incorporated into the fabrication of matrices. This technique has been used as a method for formation of polymer microspheres [3, 8, 12]. Incorporation of this technique into matrix processing has led to the development of three novel methods for producing a 3D porous matrix. The purpose of this study was to examine the internal structure resulting from the different processing methods.

Experiment

Microsphere preparation (solvent evaporation technique)

In a glass vial, 2.0 g of PLAGA {50:50 lactide: glycolide, molecular weight (MW) = 50,000, American Cyanamid Company, Wayne, NJ} was dissolved in 10 ml of methylene chloride in a 1:5 weight/volume (w/v) ratio. A 1% solution of poly(vinyl alcohol) (PVA; MW = 25,000, 88% mole hydrolyzed; Polysciences, Inc., Warrington, PA) was used as a surfactant. The PLAGA solution was added drop-wise to 300 ml of the 1% PVA solution with stirring. The PLAGA/PVA emulsion was stirred at 250 rpm for 10 hours. This allowed for complete evaporation of the solvent. The microspheres were isolated by vacuum filtration, washed with deionized water and air-dried for 24 hours. Free flowing PLAGA microspheres were then sieved into the following size ranges: < 117 μm ; 117-152 μm ; 152-178 μm ; 178-229 μm ; 229-297 μm ; 297-590 μm ; and > 590 μm .

Sintered microsphere matrix

Pre-fabricated PLAGA (50:50) microspheres (2.0 g, diameter of 117-229 μm) and NaCl (0.5 g, particle size < 75 μm) were thoroughly mixed to create a 5:1 PLAGA:NaCl mixture. This microsphere/salt mixture was cast into a compression mold (Fred S. Carver, Inc., Wabash, IN), and heated at 75°C for 1 hour. During the sintering and cooling periods, the matrix was subjected to 0.92 lb. (~ 4.5 psi or 0.03 MPa) of compression. The mold was cooled to room temperature, and the sample was demolded. The matrix was then placed in 300 ml of deionized water (37°C) for 24 hours in order to leach out NaCl particles. The sample was then placed in a lyophilizer (5-10 μm Hg vacuum; -55°C) for 24 hours. This resulted in compressed microsphere matrices with diameters of 13 mm and thickness of 3 mm.

Solvent cast matrix

Pre-fabricated PLAGA (50:50) microspheres (2.0 g, diameter of 178-229 μm) were mixed with 2.0 g of NaCl (178-229 μm) and 2.0 g of HA (74-104 μm ; Howmedica, Inc., Rutherford, NJ) to give a 1:1:1 compositional ratio of PLAGA:HA:NaCl. Approximately 0.5 ml of methylene

chloride was added drop-wise to the mixture. The addition of each drop caused aggregation of the mixture in the area where it made contact. The mixture was stirred during the addition of the solvent. After the solvent had been added, the mixture had a putty-like consistency, and was transferred to a Carver mold. The mold was compressed at room temperature with a force of 5000 lb. (~ 24,303 psi or 168 MPa) for 1 minute. Due to the low amount of solvent, the sample was immediately removed from the mold and placed in a lyophilizer (5-10 μm Hg vacuum; -55°C) for 24 hours. After removal from the lyophilizer, the sample was placed in 300 ml of deionized water (37°C) for 48 hours with a change in water after 24 hours. After the salt leaching step, the sample was dried in a vacuum for another 48 hours.

Gel microsphere matrix

In a glass vial, 2.0 g of PLAGA (50:50, MW = 50,000) was dissolved in 10.0 ml of methylene chloride to form a 1:5 w/v polymer solution. The polymer solution was poured into a 1% PVA solution with stirring. The resulting emulsion was stirred for 1.25 hours. Once stirring was complete, excess PVA was removed leaving a minimal amount for transfer. The microspheres were then transferred to a conical tube and allowed to settle to the bottom. The remaining PVA was then suctioned-off leaving microspheres isolated at the bottom of the tube. At this point, 2.0 g of NaCl (178-229 μm) and 2.0 g of HA (74-104 μm) were added. The mixture was thoroughly stirred and then cast into a cylindrical mold. The sample was allowed to air dry for 24 hours. The matrix was demolded and placed into a lyophilizer (5-10 μm Hg vacuum; -55°C) for an additional 48 hours. Following lyophilization, the sample was placed in 300 ml of deionized water (37°C) for 48 hours for the salt leaching process. The water was changed after 24 hours. The sample was removed from the water bath and placed in the lyophilizer for an additional 48 hours.

Optimization of this method dealt with modifying the stirring time of the microspheres. With microsphere stability dependent on solvent removal during the stirring period, matrices were fabricated using stirring times of 1.00, 1.25, 1.50, and 1.75 hours.

SEM Analysis of matrix structure

Matrices examined by SEM were cut in cross-section (three cross-sections per sample) and sputter-coated with gold using a Denton Desk-1 sputter coater (Denton Vacuum, Inc., Moorestown, NJ). Coated sections were affixed to SEM stages and visualized with an Amray 1830/D4 scanning electron microscope (Amray, Inc., Bedford, MA) operated at an accelerating voltage of 20 kV. Matrix samples were qualitatively characterized and pore size estimates were obtained by direct measurement from SEM images of matrix cross-section. For each sample, pore

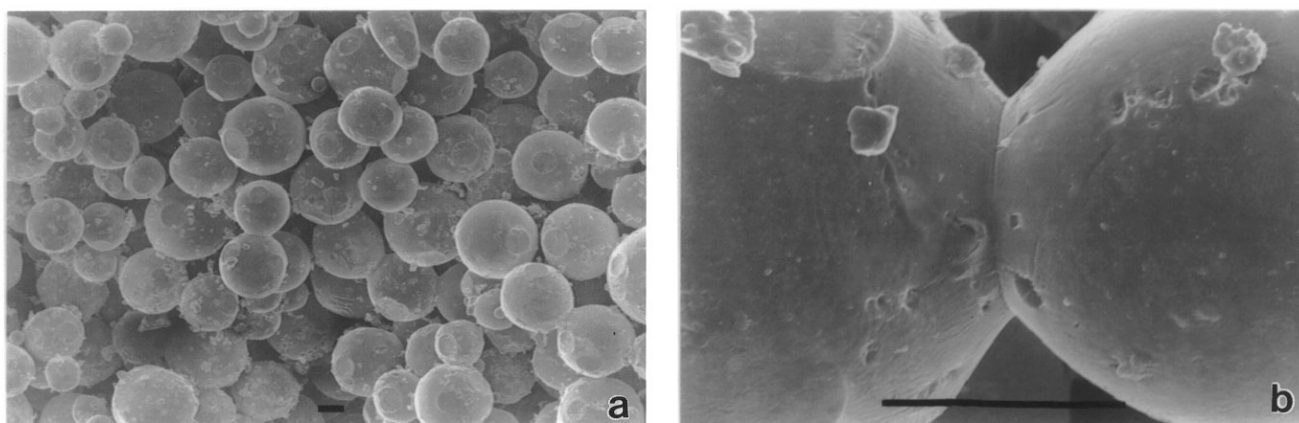


Figure 1. Scanning electron micrographs of the sintered microsphere matrix before compression. **(1a)** At low magnification, matrix is seen as composed of network of PLAGA microspheres thermally fused together. **(1b)** At high magnification, fusion between two adjacent microspheres is shown. Bars = 100 μm .

diameter was assessed by measuring the largest axis of the pore. The range in size indicated the smallest and largest pore diameter and was an average of the three cross-sections.

Results

SEM Results of the sintered microsphere method

Scanning electron micrographs of the fracture surface of a sintered microsphere matrix before compression are shown in Figures 1a and 1b. The low magnification image in Figure 1a shows that the uncompressed matrix is composed of a network of microspheres thermally fused together. Microsphere fusion appears to occur only at the contact points between adjacent microspheres. This fusion zone is seen as a flat plane on the surface of the microspheres. From this SEM image, it appears that the microspheres were randomly packed together resulting in a porous structure. Since SEM images indicated that PLAGA microspheres maintained their spherical structure and all salt particles were successfully leached out, it is apparent that the pore structure of the sintered matrix was completely interconnected.

Figure 1b shows a high magnification image of two microspheres fused together. The microspheres appear to be completely connected by an interface approximately 90 μm long. The indentations on the microsphere surface range in size from 5 to 15 μm and are probably due to the presence of NaCl particles during the processing. The flat planes on the surface of the left microsphere represent the area of a microsphere fusion prior to fracture.

SEM images of the sintered microsphere matrix after compression are shown in Figures 2a and 2b. These images were taken from a transverse cross-section of the disk

showing its thickness. The upper portion of this cross-section was removed to show a fracture surface. Figure 2a shows a low magnification image of the cross-sectional region. As seen from the image, fusion between the microspheres has increased in comparison with the uncompressed microsphere matrix (see Fig. 1b). The compressive force of the piston during sintering gave the spheres an elliptical shape thus increasing the contact points between the microspheres. The short axis of the spheres is aligned in the direction of this compressive force. Although the fusion between microspheres increased, the structure still remained porous. Pore size was estimated to range between 100-450 μm . The pores appear to be connected to one another as seen at the left edge of the image (arrows).

Figure 2b shows a low magnification image of the fracture surface. In this image, microsphere packing is apparent. Microsphere surface appears rough, probably due to the presence of NaCl particles (< 75 μm) during the sintering step. Fracture of the intra-sphere connections can be seen as a flat plane across the microsphere surface. An external pore (arrows) approximately 90 μm in diameter appears to link the internal pore network to the outside.

SEM Results of the solvent cast method

A scanning electron micrograph of a solvent cast disk is shown in Figure 3. The sample examined had a compositional ratio of 1:1:1 PLAGA:HA:NaCl and was in the shape of a thin disk with a diameter of 30 mm and a thickness of 4 mm. This image shows a cross-sectional view of the disk. The matrix appears to have a fairly even distribution of pores and HA particles. Pore morphology varies from circular to oblong shapes with an estimated range in size from 50 to 250 μm . The rectangular shape of

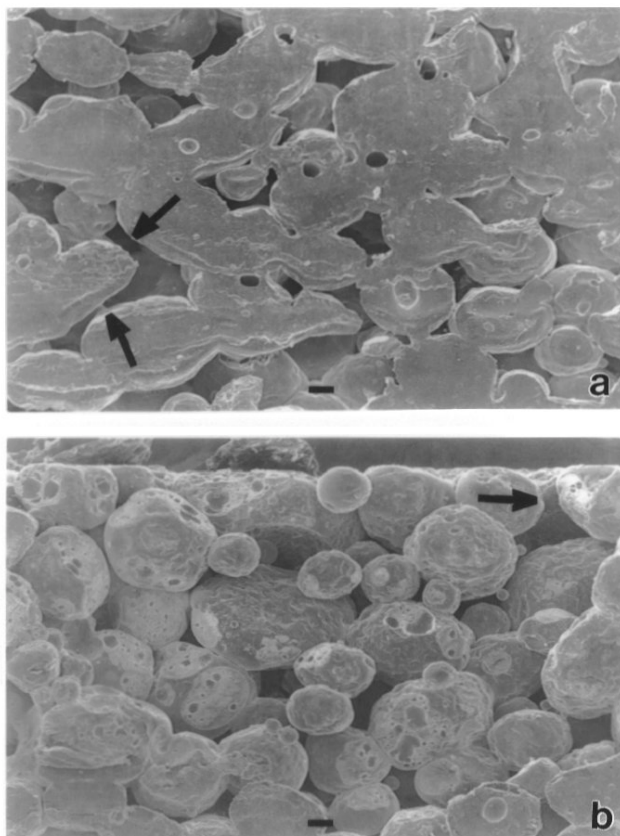


Figure 2. Scanning electron micrographs showing (2a) the cross-section (interconnection between pores is indicated by arrows); and (2b) the fracture surface {a 90 μm external pore can be seen connecting internal pore system to exterior (arrow)} of a compressed microsphere matrix. Bars = 100 μm .

some of the pores (arrows) can be attributed to the voids left from the NaCl particles (size range 178-229 μm). Although this matrix was compressed with 5000 lb. (~ 24,303 psi or 168 MPA), the image indicates that the matrix maintained its porous structure even under these extreme conditions.

SEM Results of the gel microsphere method

With the addition of the solvent evaporation technique [3, 8, 12] to the original salt leaching/microsphere method, the gel microsphere method was created. This method was optimized by making modifications to processing conditions. This modification dealt with determining the degree of microsphere stability needed to allow for microsphere aggregation while maintaining a 3D porous network. With microsphere stability dependent on the amount of solvent removed during the stirring step of matrix processing, matrices were fabricated using stirring times of 1.00, 1.25, 1.50 and 1.75 hours.

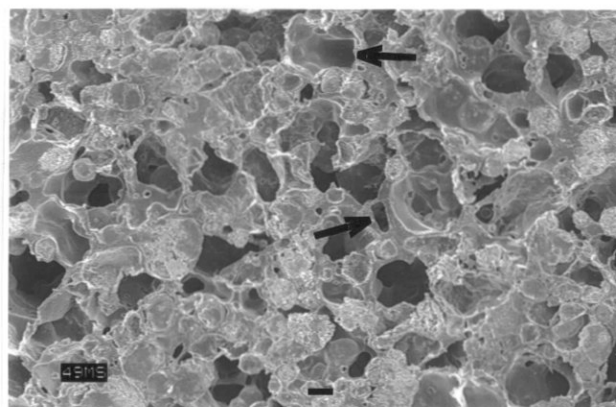


Figure 3. Scanning electron micrograph showing the cross-section of a solvent cast matrix. Rectangular pores are due to NaCl leaching (arrows). Bar = 100 μm .

Stirring time modification (1.00 hour) The low magnification image in Figure 4a shows a combination of micropores (< 100 μm) and macropores (100-450 μm). The pore structure is irregular and appears to deeply penetrate the matrix as seen by HA particles located within the pores (arrow). HA particles appear as white spheres incorporated into the polymer and evenly distributed throughout the matrix. This cross-sectional image shows an irregular internal matrix structure with no evidence of characteristic microsphere forms.

Stirring time modification (1.25 hours) In Figure 4b, the low magnification image shows a PLAGA/HA matrix with fewer micropores (< 100 μm) than the 1.00 hour matrix. Pore morphology appears to be roughly circular with a range in macropores from 100-300 μm . HA particles can be seen completely embedded within the PLAGA matrix and on the surface of some of the pores. The pores run deep into the matrix.

Stirring time modification (1.50 hours) Figure 4c shows a low magnification image of the 1.50 hour matrix. With an increase in stability at 1.50 hours of stirring, the microsphere structure is evident. Microsphere diameter ranged from 200-500 μm with both spherical and oblong shapes. Aggregation of the microspheres in their gel-form and subsequent solvent removal has created large pores ranging in size from 80-400 μm . The pores are connected by channels running between microspheres. This increase in stability reduced the incorporation of HA into the polymer. Most particles are partially embedded in the matrix covering the surface of the pores. A residual void left by a salt particle can be seen lining the surface of a pore (arrow) indicating the interconnection of the pore network.

Stirring time modification (1.75 hours) The low magnification image in Figure 4d shows a microsphere

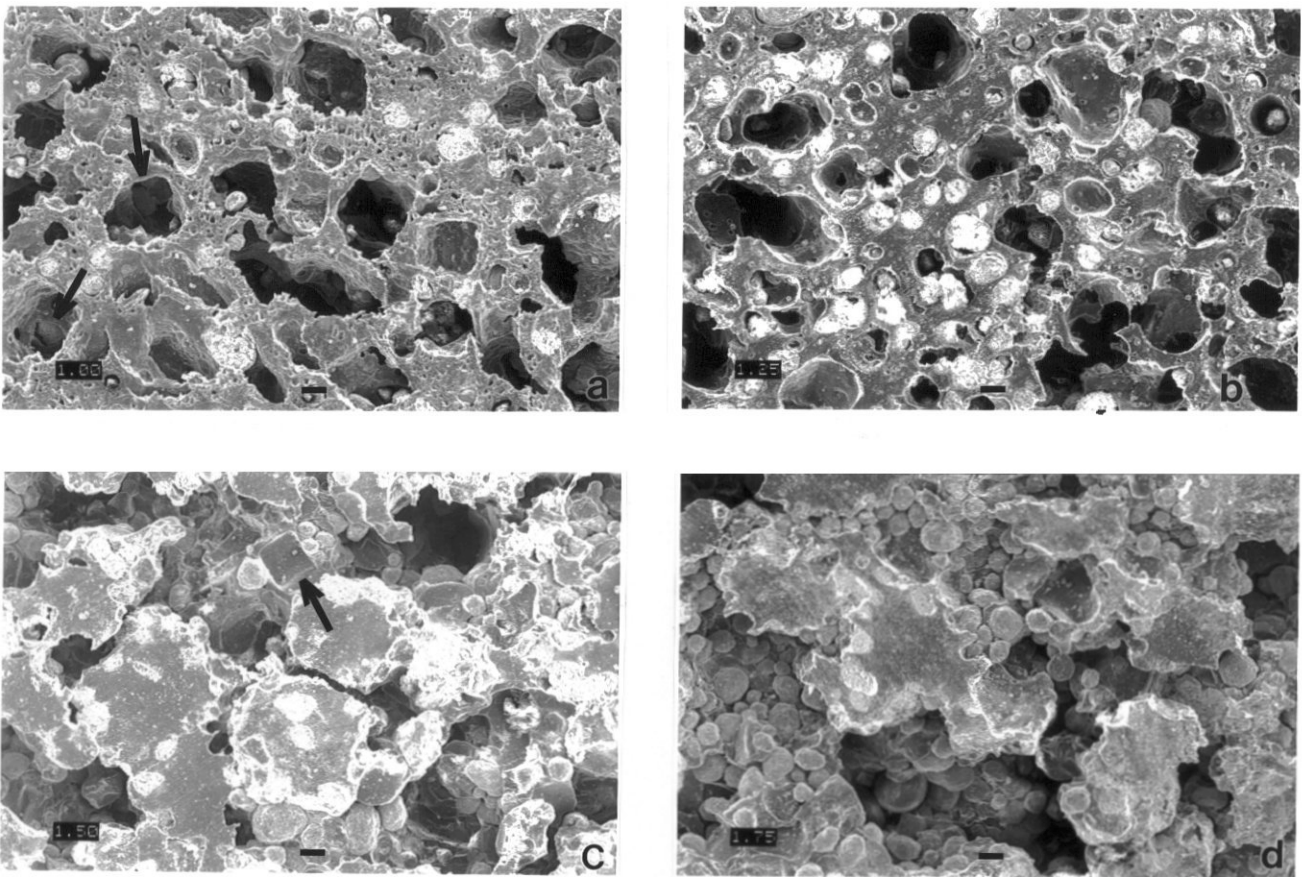


Figure 4. Scanning electron micrographs showing matrix structures after: (**4a**) 1.00 hour stirring {HA particles are seen deep within matrix pores (arrows)}; (**4b**) 1.25 hour stirring (HA particles are seen as white spheres); (**4c**) 1.50 hour (the arrow indicates an NaCl void formed in the surface of a pore); and (**4d**) 1.75 hour stirring (due to the high stability of the microspheres, HA particles are found only on the surface of the pores). Bars = 100 μm .

structure with a moderate degree of aggregation. Microspheres are large (diameter ranges from 200-600 μm) and have both an oblong and spherical shape. The moderate degree of aggregation has led to the formation of large, highly connected pores (40-670 μm). HA particles are dispersed over the surface of the microspheres. HA particles coat the surface of the pores with slight incorporation into the polymer.

Discussion

This work examined several methods for the creation of a synthetic bone replacement. The basis of matrix development was that the fabrication of a 3D, porous structure could be accomplished through the random packing of polymer microspheres. In the sintered method, microspheres were heated past the glass transition temperature of PLAGA to thermally fuse the microspheres

into a porous matrix. NaCl of a relatively small particle size (< 75 μm) was used to fill the voids created by the random packing of the microspheres. This was done to maintain the pore structure and prevent its collapse during heating. Although the sintered disk was only subjected to a minimal amount of compression {0.92 lb. (~ 4.5 psi or 0.03 MPa)}, a highly fused and highly porous microsphere structure was created. Since the compression was applied during the entire sintering and cooling periods, this amount was sufficient to change the shape and degree of fusion of the microspheres. SEM examination indicated a highly structured matrix with apparent interconnectivity between the pores.

The solvent cast method resulted in a porous structure formed from the solvent mediated aggregation of pre-fabricated microspheres. A minimal amount of solvent was used to dissolve the outer shell of the microsphere. This dissolved outer coating allowed the microspheres to aggregate together thereby creating a 3D structure. NaCl

particles were used to control porosity by acting as void forming spacers while HA particles were used to reinforce the structure. This matrix was molded using 5000 lb. (~24,303 psi or 168 MPA) of compression. SEM characterization indicated that porosity was maintained even though the sample was subjected to this high compressive force. Since only the outer shell of the polymer microspheres was dissolved, the hard inner core was able to maintain microsphere spacing during compression preventing the collapse of the pore.

The third method developed was the gel microsphere technique. The gel microsphere method incorporated the solvent evaporation technique into matrix processing to increase the stability of the microspheres. Microspheres were isolated before complete solvent removal occurred. In this gel-form, the microspheres aggregated together to form a 3D structure. Similar to the solvent cast method, NaCl was used as a void forming spacer and HA was used as a reinforcing particle. SEM characterization indicated that matrix structure was dependent on the stability of the spheres. As the stability of the microspheres increased, aggregation decreased which led to a more porous structure. The optimal stirring time was chosen based on the lack of micropores and heavy microsphere aggregation. Based on the SEM images showing a lack of micropores and pore size in the range of 100 to 300 μm , the 1.25 hour matrix was chosen as an optimal structure.

Based on the results of the SEM characterization, it appears that all matrices had pores within the optimal size range of 100-250 μm . This is the first step in developing a synthetic bone replacement. Further quantification of matrix porosity and interconnectivity is vital in order to determine whether the matrices have the ability to serve as tissue scaffold. Additional mechanical and *in vivo* characterization is also needed to examine the biomechanical and biological response to the matrices. The ultimate goal of this work is to produce synthetic alternatives to conventional bone grafts that have the phenotypic and initial biomechanical characteristics of trabecular bone. In an optimal situation, the matrix would provide a scaffold for tissue regeneration while maintaining the necessary biomechanical support. Matrix degradation would be tailored to closely match bone formation such that during degradation the matrix would slowly load the newly forming bone. The regeneration of bone within the scaffold would serve to reinforce the degrading matrix. Upon complete degradation, the matrix would no longer be visible and the implant site would be filled with natural bone. With the incorporation of growth factors and antibiotics in dynamic polymer matrices such as these, synthetic matrices could eventually rival the capabilities of conventional bone grafts.

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Discussion with Reviewers

Reviewer I: Data on composition and mechanical strength should be included in the text.

J.O. Hollinger: (Biomechanical) data should be provided as well as statistical testing to present variability among the three techniques.

F. Takeshita: Please provide the data regarding the compression results.

Authors: This paper represents a preliminary study examining matrix structure. Due to the complexity of correlating mechanical strength to matrix structure, characterization by mechanical testing is beyond the scope of this paper. These data will be presented in a future publication (in preparation) examining the relationships between structure, porosity, degradation, and mechanical properties.

K.A. Uhrich: Why is “gel” used to describe the third method? Does the resulting material have other gel-like properties?

Authors: In the gel method, the microspheres are isolated before complete solvent removal. At this point, the microspheres consist of a polymer/solvent gel. The amount of solvent removal will determine the properties of the gel and resulting stability of the microspheres. In this gel-form, the microspheres aggregate together to form a 3D matrix. Similar to the sintered and solvent cast methods, the term “gel” microsphere is used to describe the process which fuses the microspheres together.