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Injectable Macroporous Microparticles for Soft Tissue Augmentation

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Abstract— **Macroporous polymeric microparticles have been fabricated using a combination of particulate leaching and gas foaming techniques. Controlling the concentration of ammonium bicarbonate particles and the spin speed of the microemulsion in poly (ε-caprolactone) (PCL) yields a range of macroporous microparticles with interconnected pores (10-50μm) that may promote cell and tissue ingrowth *in vivo* when implanted subcutaneously. This fabrication technique introduces a novel template which can be modified to meet a diverse set of material and biological specifications.**

I. Introduction

Synthetic polymers are used to make scaffolds for biomedical engineering applications because they are biocompatible and easily modified for specific applications. However, synthetic polymers have a lack of porosity due to the formation of long, inter-twining polymer chains. Without interconnecting pores, the body cannot sufficiently interact with the material and integrate the material into the existing tissue [1]. In addition to the lack of porosity, the size of typical implants make them difficult to modify for soft tissue augmentation and subcutaneous injection. Synthetic polymer scaffolds typically require an invasive surgical procedure to be implanted which causes extended healing times and scar formation. To reduce the invasive-ness of a typical procedure, materials which can be miniaturized for injection would be optimal. Therefore, there is a need for a macroporous, injectable scaffold system which can be tailored to meet the specifications of biological applications.

Macroporous scaffolds have been developed as a way to promote cell infiltration and integration with the existing tissue. Porous scaffolds have been fabricated using various techniques including particulate leaching, gas foaming, and solid freeform fabrication [2]. Particulate leaching can be achieved using a salt or sugar particle of a specific size. This process combined with sufficient packing density can yield a highly porous structure [3]. Gas foaming is aided by the use of an effervescent salt which creates gas bubbles upon contact with an appropriate solvent [4]. Three-dimensional printing utilizes solid freeform fabrication as a means to make a porous structure by polymer infiltration after a three-dimensional model has been created. The goal of these techniques is to create interconnecting pores that allow cell infiltration and subsequent matrix deposition throughout the scaffold. The drawback to these porous, polymeric scaffolds is their large size and that they must be implanted with a surgical procedure.

To overcome these limitations, researchers have developed techniques to fabricate polymer scaffolds which are small enough to inject. This has been achieved using emulsion techniques to create microparticles [5]. Microparticles can be easily injected and have been used for many applications including drug delivery and dermal augmentation [6-7]. By manipulation of the processing parameters during fabrication, microparticles of varying size and structure can be created which provide temporal control over the interaction with the body [8]. Currently strategies to create porous microparticles result in nanopores on the surface of the particles [9-10]. The small pore size could be useful for drug delivery or diffusion through the pores, however, nano-sized pores do not allow cell infiltration. Fabrication of macroporous microparticles has been investigated by various groups, however most are created using complex processes which are not viable in a simple lab setting [11-14]. To maximize the utility of macroporous microparticles, there is a need for a simple, reproducible method to achieve large, highly interconnected surface and interior pores. There are many potential benefits to the fabrication of a macroporous scaffold which has the versatility of being injectable.

Biomaterials have been extensively studied with the ultimate goal during fabrication and testing being to achieve the most inert, biocompatible material possible [15]. The biocompatibility of a material which is to be implanted is tested for cell attachment, proliferation and differentiation. If a material is cytotoxic it is considered to be non-biocompatible [16]. When tested *in vivo*, the desirable effect of a material is a non-effect. A non-effect means that a material should have good tissue integration and not have a significant inflammatory response. Microparticles are often used because their smaller size can lead to less frustrated phagocytosis and more tissue integration in the body.

This study focuses on combining the benefits of both the particulate leaching/gas foaming technique and the microemulsion technique to create macroporous microparticles as an injectable polymer scaffold system. To the best of our knowledge, this is the first study which demonstrates a simple procedure to obtain macroporous polymer microparticles which subsequently exhibit optimal

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tissue integration through the interconnected pores of the microparticles.

II. Materials and Methods

A. Materials

Poly (ε-caprolactone) (PCL), Mn 70,000-90,000 and polyvinyl alcohol (PVA), Mw 31,000-50,000, 98-99% hydrolyzed were purchased from Sigma (St. Louis, MO). Dichloromethane (DCM) was obtained from Acros Organics. Ammonium bicarbonate was purchased from Fisher Scientific (Pittsburgh, PA).

B. Preparation of Macroporous Microparticles

Macroporous microparticles were fabricated with a water/oil/water (W/O/W) double emulsion method. PCL (200mg) was dissoluted in 3mL of dichloromethane overnight. Varying amounts of ammonium bicarbonate in 1mL nanopure water were added to the polymer solution and the resulting emulsion was vortexed for 2 minutes. Immediately following the vortexing, the primary emulsion was poured into a beaker containing 125mL of 0.1% (w/v) PVA solution. This second emulsion was then spun for 2 hours at 400rpm and 30°C to allow the solvent to evaporate. The resulting macroporous microparticles were washed 3 times with nanopure water to remove excess PVA solution. Finally, the particles were lyophilized with a freeze dryer overnight.

C. Characterization of Microparticles

Freeze dried microparticles were visualized using a scanning electron microscope. Particle sizes were analyzed on Image J software (US National Institute of Health). Surface porosity and interconnectivity were performed using threshold intensity of pixels in scanning electron micrographs using ImageJ software. The internal pores were visualized by embedding the PCL microparticles in Optimum Cutting Temperature (O.C.T.) solution, freezing the samples at -80°C, then having them processed by cutting 10μm slices on a microtome. The slides were imaged using optical microscopy. The surface roughness of the microparticles was characterized using MeX software with stereo imaging on the scanning electron microscope.

D. Microparticles in a Murine Model

C57BL/6 mice (age 10-13 weeks) were purchased from Charles River Laboratories, Wilmington, MA. The animals were handled in compliance with the institutional regulations established and approved by the Animal Research Committee at the University of California, Los Angeles. For scaffold implantation, mice were anesthetized with isofluorane and a 1-cm long dorsal midline incision was made to create a subcutaneous pocket. A thin (~200μm), laser-cut PCL sheet was rolled into a tube and filled with macroporous microparticles then inserted in the subcutaneous pocket about a centimeter away from the incision, and the skin was sutured with 3-0 Vicryl (Ethicon, Somerville, NJ). One scaffold was implanted in each mouse.

The animals were sacrificed at 7 and 14 days post-implantation. The scaffolds, along with the adjacent subcutaneous tissue, were harvested and fixed promptly in 10% formalin overnight at 4°C.

E. Immunohistochemistry

The fixed samples were embedded in paraffin and sectioned at 6 μm. The deparaffinized sections were stained with hematoxylin and eosin. Hematoxylin and eosin (H&E) sections were used to verify overall cell infiltration and tissue integration of the PCL microparticles.

III. Results and Discussion

Microparticles fabricated using a double emulsion technique with an effervescent salt were macroporous and had a range or particle sizes with consistent interconnected pores. The W/O/W emulsion had ammonium bicarbonate in the W_1 phase to create the pores, PCL dissolved in dichloromethane as the O phase, and 0.1% PVA as the W_2 phase. The W_1 and O phase was vortexed to achieve the primary emulsion which was then poured into the W_2 phase and re-emulsified resulting in microparticles. Bubbles of carbon dioxide and ammonia gases are formed when the ammonium bicarbonate comes into contact with water. These gas bubbles leave voids in the polymerizing microparticle as the oil-based solvent evaporates in the secondary emulsion. This process results in the creation of macropores in the microparticles. Unlike similar techniques for macroporous microparticles, our technique gives interconnectivity of pores through the combined particulate leaching/ gas foaming technique. The interconnectivity of surface pores and the size of internal pores were examined with scanning electron microscopy and cryo-sectioning. Pore size range was large with an average pore size of ~20 μm.

These particles and pores sizes were modulated by altering the material processing parameters. PCL macroporous microparticles had larger sizes at both slower spin speeds and higher concentrations of ammonium bicarbonate (Table 1). The gross morphology as well as the surface morphology and the cross-sectioned slices of particles show the qualitative difference seen with addition of the effervescent salt to the double emulsion technique (Fig. 1). When there

Table 1 – Size of PCL Microparticles

NH_4HCO_3	*Spin Speed*	*Microparticle Diameter*	*Average Pore Diameter*	*% Area Porous*
0%	400 RPM	128± 57 μm	-	-
5%	400 RPM	338± 90 μm	11.9 μm	19.90%
10%	400 RPM	384± 86 μm	10.7 μm	24.20%
20%	400 RPM	465± 95 μm	10.2 μm	16.90%
40%	200 RPM	1003 ± 330 μm	12.0 μm	20.10%
40%	400 RPM	520 ± 79 μm	10.0 μm	17.70%
40%	600 RPM	*shredded	-	-

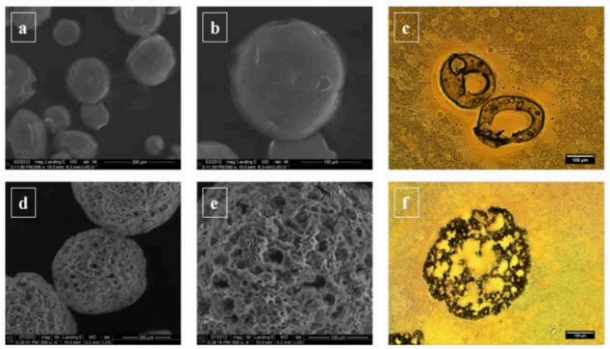

Figure 1. **a-c** PCL microparticles with 0% ammonium bicarbonate. **d-f** PCL macroporous microparticles with 40% ammonium bicarbonate. **a, d** Gross morphology of microparticles 200X on SEM. **b, e** Surface morphology of microparticles 500X on SEM. **c, f** 10um cross-sections from cryo-sectioned microparticles, 100um scale bar.

was an absence of ammonium bicarbonate, the resultant microparticles had a smoother texture and much smaller particle diameters. This data shows that as the percent ammonium bicarbonate was increased, the overall size distribution of particles also increased. A typical histogram of particle size distribution can be seen in fig. 2, with a summary of size distribution in table 1. The effect of the ammonium bicarbonate producing gaseous bubbles in the primary emulsion causes the increase in particle diameter. As the percent of ammonium bicarbonate increases, so does the number of gas bubbles formed. The pressure the bubbles produce pushes against the polymer as the solvent evaporates, therefore enlarging the microparticles as they are formed. As the polymer phase is solidifying (due to dichloromethane evaporation) and viscosity is increasing, the bubbles create less stress on the polymer as it stiffens and the gas phase eventually escapes through the interconnecting pores. Surface and interior pore sizes, as well as percentage of surface pores interconnected remain consistent throughout most processing parameters (Table 1, Fig. 2). The gaseous bubbles surrounding the microparticles as they form, prevents the coalescence of particles. This emulsion is achieved without addition of surfactants due to the stabilization that the gas bubbles have on the microemulsion [14]. Without the gas formation, particles would be less stable in the material fabrication process leading to aggregation of polymer microparticles.

SEM image analysis on micrographs of the PCL microparticles results in an average particle size of 128± 57 µm, 338± 90 µm, 384± 86 µm, 465± 95 µm, and 520 ± 79 µm with a concentration of 0%, 5%, 10%, 20%, and 40% ammonium bicarbonate, respectively. Surface pore sizes for the microparticles averaged 10 ±1 µm for each of the sets with ammonium bicarbonate (Table 1). Interior pore diameters averaged to be 29 ± 18 µm when visualized by 10 µm slices of cryo-sectioned particles (Fig. 1, Fig. 2). When the percent ammonium bicarbonate was altered, there was no significant change in particle morphology, however, the surface roughness decreases with increases in ammonium bicarbonate (unpublished data). This follows with the small decrease in surface pore size seen with the addition of ammonium bicarbonate.

The *in vivo* response to the PCL macroporous microparticles has been investigated. Small packets of microparticles in a PCL pouch were implanted subcutaneously in a mouse model. A representative explant can be seen in fig. 3. The microparticles were implanted and not injected to fully immobilize the particles, however, subcutaneous injection is possible and was performed in a few animals (Fig. 3a). Upon harvest of the polymer microparticles and surrounding tissue, immuno-

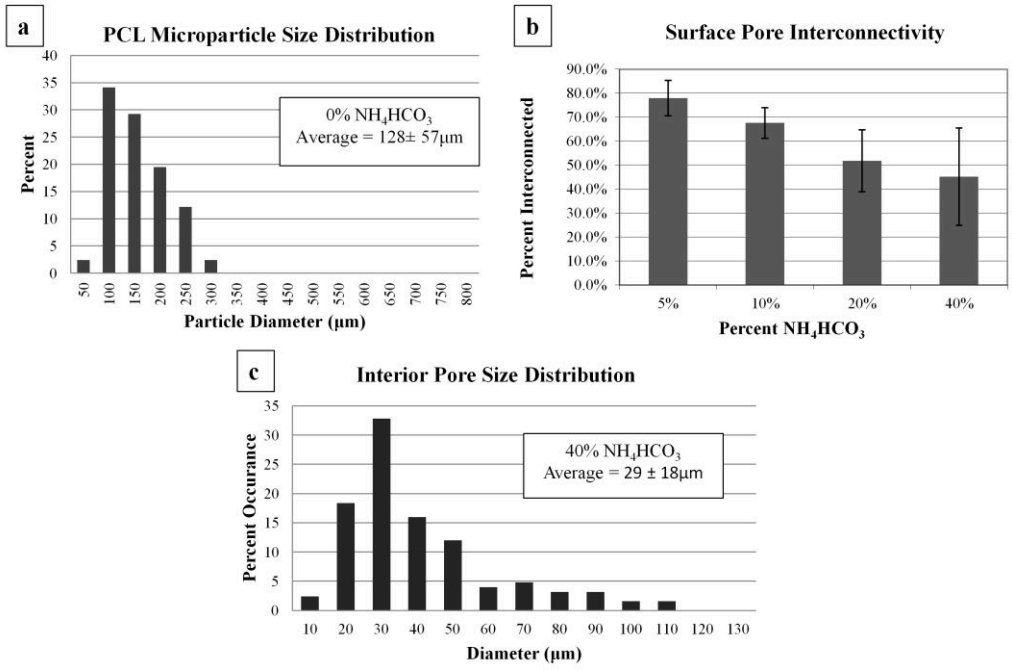

Figure 2. a. Typical histogram of PCL microparticle size distribution **b.** Surface Pore Interconnectivity with varying concentrations of ammonium bicarbonate **c.** Internal Porosity of microparticles made with 40% NH_4HCO_3

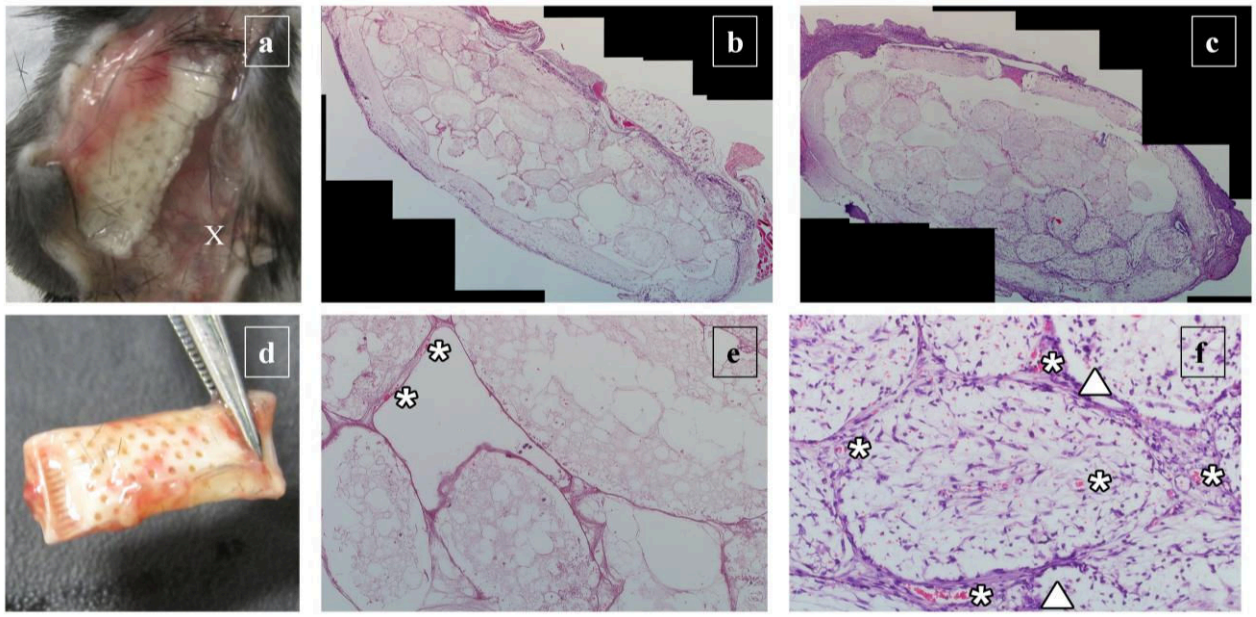

Figure 3. **a, d.** Typical explants of PCL pockets filled with microparticles at one week post implantation. **X** indicates where macroporous PCL microparticles can be seen when injected adjacent to a PCL pocket implant. **b, e.** H&E staining of paraffin sections of one week implants. **c, f.** H&E staining of paraffin sections of two week implants. * denotes blood vessels. **Δ** denotes areas of foreign body giant cell formation

histochemistry was performed to analyze cell/tissue infiltration/integration, as well as blood vessel formation throughout the PCL microparticles. From the H&E stained slides shown in fig. 3, it is apparent that there is some cell infiltration at day 7, however, by day 14 there is blood vessel formation and more tissue integration. The ability of the microparticles to integrate into the existing tissue is due to the size and interconnectivity of the pores. With high levels of tissue integration, there is improved acceptance of the material into the body and the ability for soft tissue augmentation without significant inflammation. This response is desirable and applicable in a variety of medical fields including dentistry and dermatology.

IV. Conclusion

The fabrication of macroporous PCL microparticles establishes a simple material processing technique which can be utilized as an injectable polymer system. The combination of gas foaming and particulate leaching achieved polymer microparticles with interconnected pores which allowed for tissue in-growth upon implantation. The versatility of application for this technology spans many areas of biomedical engineering.

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