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Injectable PolyHIPEs as High Porosity Bone Grafts

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Abstract

Polymerization of high internal phase emulsions (polyHIPEs) is a relatively new method for the production of high porosity scaffolds. The tunable architecture of these polyHIPE foams make them attractive candidates for tissue engineered bone grafts. Previously studied polyHIPE systems require either toxic diluents or high cure temperatures which prohibit their use as an injectable bone graft. In contrast, we have developed an injectable polyHIPE that cures at physiological temperatures to a rigid, high-porosity foam. First, a biodegradable macromer, propylene fumarate dimethacrylate (PFDMA), was synthesized that has appropriate viscosity and hydrophobicity for emulsification. The process of surfactant selection is detailed with particular focus on the key structural features of both polymer (log P values, hydrogen bond acceptor sites) and surfactant (HLB values, hydrogen bond donor sites) that enable stable HIPE formation. Incubation of HIPEs at 37°C was used to initiate radical crosslinking of the unsaturated double bond of the methacrylate groups to polymerize the continuous phase and lock in the emulsion geometry. The resulting polyHIPEs exhibited ~75% porosity, pore sizes ranging from 4 to 29 μm , and an average compressive modulus and strength of 33 and 5 MPa, respectively. These findings highlight the great potential of these scaffolds as injectable, tissue engineered bone grafts.

Introduction

Large bone defects resulting from traumatic injury, tumor resection, or congenital deformities fail to heal naturally and often require reconstructive surgery. Bone grafts promote healing at the defect site by providing a template to guide new tissue formation. Autologous bone continues to be the gold standard for reconstruction due to its high potential for growth and remodeling as well as its ability to osseointegrate; however, its retrieval has anatomical limitations and often results in donor site pain and morbidity. A wide variety of alloplastic materials have also been utilized including stainless steel, titanium, methylmethacrylate resins, polyethylene, silicone elastomers, and hydroxyapatite ceramics. Drawbacks and complications inherent in the use of current alloplastic materials include inadequate tissue integration, limited biodegradability, and stress shielding. In contrast, tissue engineering strategies promote bone regeneration by seeding living cells on or attracting endogenous cells to a biomaterial scaffold and delivering appropriate bioactive cues to aid in cell differentiation and tissue growth.¹ As such, tissue engineered bone grafts

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combine the osseointegration and remodeling of autografts with the availability and tunability of synthetic grafts, thus limiting the complications associated with each of these traditional implants.^{2–3}

Tissue engineering scaffolds typically consist of biodegradable materials that slowly erode at a rate complementary to tissue growth and facilitate full integration of the de novo tissue with the host tissue.¹ In addition to the choice of a suitable biomaterial, the success of tissue engineered constructs depends on the three-dimensional architecture of the scaffold. An interconnected porous structure enables cellular ingrowth and proliferation, vascularization, and the transport of nutrients and metabolic waste.^{4–8} Orthopaedic applications also require scaffolds with adequate mechanical properties to withstand physiological loading and restore tissue function without causing deleterious stress-shielding effects.^{2, 6–7, 9–10} Finally, the ability to match the irregular geometries of these types of bone defects is necessary to promote osseointegration and full healing. Injectable grafts that cure *in situ* are preferable in this aspect to more costly and time-consuming computer-aided design molds. In summary, the advancement of bone tissue engineering strategies is strongly dependent on the development of high-porosity scaffolds that meet these key requirements.

Tissue engineers have developed numerous fabrication strategies to create 3D bone grafts.^{2, 5–8, 11–15} The selected fabrication process dictates scaffold architecture which is a critical design feature given the effect of architecture on construct mechanical properties, degradation rate, and cellular response. In particular, generation of a scaffold with high porosity that retains sufficient mechanical strength for orthopaedic applications remains challenging. The most common techniques to produce porous scaffolds include particulate leaching, gas foaming, thermal induced phase separation, and fiber bonding.^{6, 12, 14–15} Many of these strategies provide exceptional architecture control; however, control of the construct geometry (e.g. to match the contours of the defect) is generally limited to the mold used in fabrication or post-fabrication shaping. In situ forming scaffolds can fill irregular shaped defects, improve contact between the scaffold and surrounding tissue, and eliminate the need for costly molding techniques.^{16–17} Poly(methyl methacrylate) (PMMA) bone cements are perhaps the most widely used injectable material in orthopaedics; however, PMMA is non-degradable which may impede bone healing.¹⁶ Recently, investigators have developed highly crosslinked, degradable networks such as poly(propylene fumarates) and polyanhydrides that can be formed in situ using either thermal or photoinitiated crosslinking.^{18–21} Although biodegradable and injectable, these materials lack the porosity necessary to repair critical size defects. In contrast, in situ curing hydrogels have sufficient mass transport properties but lack the mechanical strength necessary for orthopaedic applications. A scaffold fabrication method that is injectable and porous, yet retains high mechanical strength would provide a significant improvement over current methods.

Polymerization of high internal phase emulsions (polyHIPEs) is a relatively new method for the production of high porosity scaffolds.²² High internal phase emulsions (HIPEs) are characterized by an internal droplet phase volume fraction greater than 74%. Polymerization of the HIPE's continuous phase locks in the emulsion geometry at the gel point to generate a high porosity monolith or polyHIPE.^{23–24} A wide range of porosities (75–99%), pore sizes (1–100 μm), compressive moduli (2 kPa–60 MPa) and morphologies (open- vs. closed-pore) can be produced by varying the HIPE composition and processing variables.^{24–37} A unique feature of the polyHIPE system is that the HIPE retains a viscosity that is suitable for injection prior to cure. An injectable polyHIPE system requires 1) biodegradable macromers with suitable viscosities for emulsion formation and 2) reaction thermodynamics that allow for HIPE polymerization at physiological conditions. Previous research on the development of polyHIPE bone grafts has focused on styrene-based or unsaturated polyester-based macromers.^{11, 22, 25–26, 38–39} Although past styrene-based systems had excellent pore

morphology, they were non-biodegradable which limited their use as tissue engineered scaffolds. Biodegradability was achieved by substituting in unsaturated polyesters; however, the macromers studied were often too viscous to form HIPEs without the use of a toxic diluent, such as toluene.^{27, 36, 40–41}

We have developed a biodegradable and injectable polyHIPE system based on propylene fumarate dimethacrylate (PFDMA) macromers. The viscosity of PFDMA is suitable for HIPE formation and reactive methacrylate end groups enable *in situ* crosslinking into rigid monoliths at 37°C. Furthermore, fumarate-based polymers have shown great promise as bone grafts with established osteoconductivity *in vivo*.^{19–20, 42} PFDMA polyHIPEs exhibited ~75% porosity, pore sizes ranging from 4 to 29 µm, and an average compressive modulus and strength of 33 and 5 MPa, respectively. The ability to synthesize a fully biodegradable polyHIPE without a toxic diluent that can also cure at physiological temperatures is an important adaptation of emulsion templating. These new polyHIPEs have potential application as an injectable, tissue engineered bone graft.

Materials and Methods

Materials

Polyglycerol polyricinoleate (PGPR 4125) was donated by Paalsgard and PEG 600 dilaureate was donated from Unitex Chemical. All other chemicals were purchased from Sigma Aldrich. All chemicals were used as received.

PFDMA Synthesis

PFDMA was synthesized in a two-step process adapted from Timmer *et.al.*⁴³ First, propylene oxide was added dropwise to a solution of fumaric acid and pyridine in 2-butanone (2.75:1.0:0.033 mol). The reaction was refluxed at 80°C until the fumaric acid completely reacted, approximately 19 hours. Residual propylene oxide and 2-butanone were then removed in two distillations steps and the product redissolved in dichloromethane. The solution was then washed in 0.2 M NaOH/brine (6:4 v/v) until basic to remove residual acidic byproducts, washed with brine, and stirred over anhydrous sodium sulfate to remove residual water. Dichloromethane was removed using rotary evaporation to yield the diester bis (1,2 hydroxypropyl) fumarate product as a colorless liquid. The diester was then end-capped with methacrylate groups in an addition process with triethylamine and methacryloyl chloride. Hydroquinone was added to inhibit crosslinking during the synthesis. The molar ratios of the diester, methacryloyl chloride, triethylamine, and hydroquinone were 1:2.1:2.1:0.016, respectively. The reaction was maintained below –10°C to reduce undesirable side reactions and stirred vigorously overnight under a nitrogen blanket. The macromer was filtered to remove triethylamine salt and neutralized overnight in 2 M potassium carbonate. The solution was washed in 0.1 M NaOH/brine (6:4 v/v) to remove residual byproducts, washed with brine, and stirred over anhydrous sodium sulfate to remove residual water. The dichloromethane was removed by rotary evaporation and the PFDMA structure confirmed using ¹H NMR (300 MHz, CdCl₃) δ 1.33 (dd, 3H, CH₃), 1.92 (s, 3H, CH₃), 4.20 (m, 2H, -CH₂-), 5.30 (m, 1H, -CH-), 5.58 (s, 1H, -C=CH₂), 6.10 (s, 1H, -C=CH₂), 6.84 (m, 2H, -CH=CH-). The final product was a low viscosity liquid with a pale yellow to amber appearance.

Surfactant Study

Sorbitan monooleate (Span 80), PEG 600 dilaureate, Tween 80, and PGPR 4125 were studied to observe their effect on PFDMA HIPE formation. Each surfactant had different hydrophilic-lipophilic balance (HLB) values and different hydrogen bond donor sites (Table 1). PFDMA, surfactant, and DI water (2, 0.4, 8g, respectively) were vortexed for 5 minutes.

HIPE formation was indicated by full incorporation of water without evidence of phase separation after mixing stopped. Successful HIPEs were typically characterized by an opaque, white appearance with a notable increase in viscosity, similar to mayonnaise. The scouting compositions which resulted in HIPEs were fabricated full-scale to investigate the effect of surfactant structure on pore architecture.

HIPE Fabrication

HIPEs were prepared using the FlackTek Speedmixer DAC 150 FVZ-K. Briefly, PFDMA was mixed with the surfactant PGPR in the Speedmixer cup prior to emulsification. The PGPR concentrations used in this study are listed in Table 2. Once thoroughly mixed, the aqueous solution of calcium chloride (1% v/v), ammonium persulfate (5 wt %) and deionized water was then added to the organic phase in the speedmixer cup. The calcium chloride was used to prevent Ostwald ripening while the ammonium persulfate initiated radical crosslinking of the macromer chains. HIPEs were then transferred to a 37°C aluminum bead bath for 12 hours to facilitate cross-linking. The resulting polyHIPE foams were placed under vacuum for 24 hrs to remove water prior to characterization.

Gravimetric Analysis

PolyHIPE porosity was measured gravimetrically. Briefly, dried HIPE samples were cut into cubic sections ($9 \times 9 \times 3$ mm) and weighed. Following equation 1, the HIPE porosity can be calculated by comparing HIPE density (ρ_H) with the bulk polymer's density (ρ_P). Reported values are an average of nine sections per polyHIPE composition.

$$\text{Porosity} = 1 - \frac{\rho_H}{\rho_P} \quad (1)$$

SEM Analysis

Scanning electron microscopy (SEM) was utilized to characterize the polyHIPEs pore architecture. Circular specimens were sectioned into quarters, fractured at the center of the quarter, sputter-coated with gold, and imaged using FE-SEM (JEOL JSM-7500F). Images at $250\times$ were utilized to determine the average pore size when the pores were $25\text{--}100\text{ }\mu\text{m}$. Higher magnification ($500\times$, $1000\times$) images were utilized to determine the average pore size when the pores were less than $25\text{ }\mu\text{m}$. Each section was imaged in a raster pattern yielding five images. Measurements were made on the first 10 pores along the image median to minimize user bias. Average pore sizes for each polyHIPE composition are reported ($n=150$). A statistical correction was calculated to account for non-perfect spherical pores, $h^2 = R^2 - r^2$ where R is the void diameter's equatorial value, r is the diameter value measured from the micrograph, and h is the distance from the center. The average diameter values were multiplied by this correction factor resulting in a more accurate description of pore diameter. These values are listed in Table 2.

In vitro Cell Proliferation

Investigation of 3T3 fibroblast viability was done to assess polyHIPE cytocompatibility. Cell viability was determined using the Live/Dead Viability/Cytotoxicity Kit (Molecular Probes). NIH/3T3 Swiss mouse fibroblast were purchased (ATCC-CCL92) and cultured *in vitro* with Dulbecco's Modified Eagle Medium (DMEM), Glutamax, high glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin-Streptomycin solution (Gibco). The polyHIPE sample comprised of 5 wt% PGPR, 75/25 volume fraction, and mixed at 500 rpm was chosen for cytocompatibility testing because it possessed the largest pore sizes and contained the least amount of surfactant which could

potentially disrupt the cell membrane. PolyHIPE foams were prepared for cell seeding as follows: UV irradiation (1 hour per side), ethanol wetting ladder and progressive solvent extraction, and overnight media incubation supplemented with 40 v/v% FBS in DMEM. Following overnight incubation in 37°C, 5% CO₂, medium was removed, specimens were dried in the hood for 30min, washed 1× with PBS and pre-conditioned with growth medium for 15 min. Cells were seeded into wells at 10,000 cells/cm². Live/Dead staining was conducted at 24 hours. Images of each of the three specimens were obtained through raster patterning (n=15) using a fluorescence microscope (Nikon Eclipse TE2000-S).

Mechanical Testing

The foams mechanical properties were all tested with an Instron 3300, equipped with a 1000-N load cell. Three specimens were taken from each sample. The test specimens were cut into flat rectangular shapes (9 × 9 × 3 mm) and compressed at 50 μm/s.⁴⁴ The calculations in ASTM method D1621-04a⁴⁵ were used to determine the compressive modulus. Briefly, a straight edge and computer software were used to determine the linear region of the stress-strain curve by extending a line from the steepest slope of the curve to the zero-load axis. The point at which this line crossed the axis was determined to be where strain equaled zero and all data points were shifted accordingly. The elastic modulus was equal to the slope of the line in the linear region, as outlined in the ASTM method. Reported moduli data was an average of the three sections for each sample tested (n=9).

Statistical Analysis

The data are displayed as mean ± standard deviation for each composition. A Student's t-test was performed to determine any statistically significant differences between compositions. All tests were carried out at a 95% confidence interval ($P < 0.05$).

Results and Discussion

PFDMA Synthesis and Analysis

PFDMA was synthesized by the two-step reaction described previously. The first step produced a diester intermediate, bis-(1,2-hydroxypropyl) fumarate, which was then functionalized with methacrylate endgroups, Figure 1. Following purification, the structure of the resulting PFDMA product was confirmed with ¹H NMR. The integration ratio of methacryloyl protons to fumarate protons in the ¹H NMR spectra, Figure 2, confirmed the structure of PFDMA as a single fumarate unit with two terminal methacrylate groups. The average functionalization was calculated to be ~83%. The methacrylate and fumarate groups provided sites for radical crosslinking of the macromer to permit cure of the HIPE at physiological temperatures. Furthermore, fumarate-based bone grafts have shown promising osteoconductivity results in vivo.^{19–20, 42} The resulting macromer had a sufficiently low viscosity (125 cP) and hydrophobicity to permit HIPE formation.

Model predictions of the octanol-water partition coefficients (logP) were used in these studies as a means of comparing molecular hydrophobicity.⁴⁶ LogP values are a measure of the differential solubility of a compound between two immiscible solvents, typically water and a hydrophobic solvent such as octanol. Log P values range from negative to positive where a negative value corresponds to a hydrophilic molecule and a positive value a hydrophobic one. The method used in these studies for logP prediction was developed at Molinspiration (miLogP2.2 – November 2005). The logP value of each compound was calculated from the sum of its non-overlapping molecular fragments. The group contributions were obtained by fitting calculated logP with experimental logP for a training set of more than twelve thousand molecules. The logP value of PFDMA (3.4) was comparable to macromers that have previously formed stable HIPEs, Table 1.

Selection of HIPE Surfactant

Surfactant choice and concentration play a large role in emulsion stability and successful HIPE formation.^{24, 27, 34, 47} Selection of HIPE surfactants has largely been based on trial and error and historical precedence. One method of characterizing surfactants is their hydrophilic-lipophilic balance (HLB) classification. Typically, empirical testing is used to ascertain what HLB values are suitable for each application with an HLB range of 2–6 designated for water-in-oil emulsions.⁴⁸ Although several investigators have indicated the limitations of the HLB approach, it remains the most method for surfactant selection. In this study, we attempt to identify structural features and predictors that may be used to rationally select surfactants for new HIPE macromers. It was hypothesized that a relationship between surfactant HLB and organic phase hydrophobicity may exist which could then be used to select appropriate surfactants for the PFDMA HIPE. Log P values were utilized as a comparison between established HIPE macromers/monomers and PFDMA, Table 1. The most widely studied polyHIPE system is styrene and divinylbenzene with logP values ranging from 2.8 – 3.6. The surfactant Span 80 (HLB = 4.3) is typically used to stabilize styrene-based HIPEs. Therefore, it was hypothesized that PFDMA HIPEs with a log P of 3.4 should also form stable emulsions with Span 80. Surprisingly, Span 80 did not stabilize PFDMA emulsions despite the similarity between logP and HLB values. Additional surfactants and combinations of surfactants with similar structures as Span 80 but a range of HLB values from 1.8–15 were investigated, Table 2, but these too failed to form stable HIPEs with PFDMA. These studies indicate that HLB alone is insufficient as a selection criteria for stable HIPE formation.

Surfactant structures were compared to determine differences that might affect PFDMA emulsification. It was determined that all of the surfactants tested had hydrogen bond donor sites in the polar head, Figure 3. PFDMA has multiple hydrogen bond acceptor sites in its backbone that could interact with the polar head of the surfactant. It was hypothesized that this hydrogen bonding could prevent the polar head of the surfactant from interacting with the aqueous phase of the emulsion, thereby attenuating its ability to stabilize the organic/water interface. Previous fumarate and ester-based systems that were effectively stabilized with Span 80 utilized a non-polar solvent (e.g. toluene) which may have interrupted the hydrogen bonding.^{11, 22, 27, 49} To avoid using a toxic diluent, a surfactant without hydrogen bond donor sites in the polar head was needed with the appropriate HLB value. The organic soluble emulsifier, polyglycerol polyricinoleate (PGPR), was selected to test this theory based on its comparable HLB (~4.7) and lack of hydrogen bond donor sites in its polar region. This surfactant has also been used to generate water-in-oil HIPEs with ester-based macromers.^{40–41, 50} Successful formation of PFDMA HIPEs resulted with the addition of PGPR suggests that hydrogen bonding does indeed play a critical role in surfactant stabilization of high internal phase emulsions. Further investigation of surfactants that lack donor sites in their polar head could expand the number of biodegradable polymers utilized in emulsion templating given that many biodegradable polymers (e.g. polyesters) have hydrogen bond acceptor sites in their backbone.

Injectable Porous Scaffolds

Stable PFDMA HIPEs were incubated at 37°C to initiate radical crosslinking of the unsaturated double bond of the methacrylate groups. The resulting polyHIPE monoliths exhibited ~75% porosity and an average compressive modulus and strength of 33 and 5 MPa, respectively. SEM analysis was utilized to determine pore size and morphology. Polymerization of the continuous phase of the HIPE locked in the emulsion geometry resulting in a high-porosity foam with a closed-pore morphology and average pore size ranging from 4 – 29 µm, Figure 4. These studies represent an important milestone in the development of an injectable bone graft. Specifically, these polyHIPEs utilize a

biodegradable and osteoconductive polymer, have a suitable pre-cure viscosity for injection; and cure at physiological temperatures to a rigid, high-porosity monolith. The potential utility of this new polyHIPE system as an injectable bone graft is illustrated in Figure 4. Damage to surrounding tissues after deployment of bone cements has been attributed to the high polymerization exotherm. Therefore, the reaction exotherm of the PFDMA was monitored for 4 hours using a thermocouple inserted into the HIPE as it cured at 37°C. The temperature of the HIPE did not exceed 37.3°C which indicates that the mild reaction would not negatively impact surrounding tissues. The cure time of the polyHIPE system (5 wt% PGPR, 75:25) was quantified at 37 °C by monitoring storage and loss modulus in dynamic mechanical analysis. This study indicated that the HIPE has a working time of ~1 h (onset of storage modulus increase) and is fully set within 2 h (slope of storage modulus → 0). It is hypothesized that reactivity of the unsaturated double bond and the concentration of initiator can be used to modulate these times. Finally, cytocompatibility analysis of 5 wt% PGPR specimens yielded 95% viability of 3T3 fibroblasts after 24 hours, Figure 5. Overall, these results indicate that the PFDMA has great potential as an injectable bone graft.

An open-pore morphology is a common design goal for tissue engineering applications to facilitate tissue ingrowth and nutrient/waste transport. Previous studies have investigated the mechanisms that govern pore opening in the polyHIPE system.^{22, 24, 34} Interconnect formation in HIPE systems has been attributed to shrinkage of the thin polymer film separating droplets that occurs as monomer is converted to higher density polymer. If this film between droplets is sufficiently thin, shrinkage results in a window opening and expanding. Therefore, whether a HIPE forms an open- or closed-pore morphology upon polymerization is related to both initial film thickness between droplets and densification during polymerization which initiates pore opening.^{24, 27} Film thickness is dictated by the aqueous phase volume and droplet size; whereas, densification is related to structural features of the polymer. Current studies are underway to investigate these two mechanisms with the goal of generating open-pore, fumarate-based polyHIPEs.

Effect of PGPR Concentration on Pore Architecture

Given that the polyHIPE architecture is dictated by the emulsion geometry prior to cure, modulation of emulsion stability may be used to tune the resulting polyHIPE architecture. This requires a brief review of the thermodynamics involved in both emulsion formation and phase separation. The increase in surface energy of an emulsion compared to the non-emulsified components (ΔW) is a product of both the interfacial energy (σ) and the change in surface area (ΔA) upon emulsification.

$$\Delta W = \sigma \cdot \Delta A$$

ΔW is the free energy of the interface and corresponds to the reversible work brought into the system during emulsification. The magnitude of ΔW can be considered a measure of the thermodynamic instability of the emulsion and drives phase separation as a means to decrease ΔA . From this relationship, it is evident that ultimate stability against coalescence processes is only achieved if σ approaches zero. The surfactant's role during emulsification is to reduce this interfacial tension and form a barrier between the two phases.

Two relationships relevant to polyHIPE architecture can be inferred from this discussion: 1) an increase in interfacial tension ($\uparrow \sigma$) will increase the rate of droplet coalescence due to an increase in W ; 2) an increase in interfacial tension ($\uparrow \sigma$) will correspond to larger initial droplet sizes ($\downarrow \Delta A$) for a given ΔW . It follows that the surfactant which directly impacts interfacial tension can be used to tune pore sizes by changing the initial droplet size and/or

the rate of droplet coalescence prior to cure. To this end, Williams et al. studied the effect of surfactant concentration on both pore size and wall thickness between droplets.⁴⁷ It was reported that a reduction in surfactant concentration could be used to increase pore size by destabilizing the HIPE. In addition, an increase in surfactant concentration was found to decrease wall thickness and induce pore opening upon polymerization. We hypothesized that HIPE stability could be modulated by changing the surfactant concentration to achieve a range of polyHIPE pore sizes and an open-pore morphology. PGPR concentrations from 5 to 40 wt% were utilized to investigate the effect of surfactant concentration on polyHIPE pore architecture. SEM analysis of polyHIPE monoliths was conducted to quantify pore and interconnect size using the 10.7 pixels/ μm ratio at 1000 $\times$, Table 2. Decreasing the concentration of PGPR from 20 to 5 wt% was found to increase average pore diameter in PFDMA polyHIPEs (6 to 29 μm), Figure 6. A narrowing of pore size distributions with increasing surfactant concentration was also observed, Figure 7.

The decreased pore size observed at higher surfactant concentration was attributed to a decrease in interfacial tension with a corollary decrease in droplet size, as discussed above. Assuming conservation of organic phase volume, this increase in surface area also decreases the film thickness between droplets;⁴⁸ however, this wall thinning was insufficient to lead to pore opening in this system. Based on these preliminary results, it was hypothesized that increased densification in combination with decreasing film thickness would be needed to generate open-pore polyHIPEs. This is the subject of current investigation using alternative fumarate-based macromers. A narrowing of the pore size histograms also indicated that a higher surfactant concentration resulted in a more uniform pore size, Figure 7. Williams et al. reported that increased pore size homogeneity was observed with increased surfactant due to a reduction in droplet coalescence.⁴⁷ However, the droplet coalescence observed in these studies was characterized by a few large pores surrounded by many smaller pores. As illustrated in Figure 7, there was a continuum of pore sizes observed rather than the more bimodal distribution reported by Williams et al.⁴⁷ Ostwald ripening has also been reported to increase the pore size distribution of polyHIPEs. In this process, diffusion of water from smaller droplets to larger droplets causes a more gradual broadening of the pore size distribution.⁵¹ Both of these processes are affected by the nature and concentration of surfactant; however, it is unclear whether droplet coalescence or Ostwald ripening is responsible for the observed difference in pore size distribution. Based on the histograms alone, it appears that Ostwald ripening may be more significant; however, additional studies are needed to make a stronger claim on this front.

Effect of Mixing Speed on Pore Architecture

Processing parameters such as mixing speed can also be utilized to tune the pore architecture through manipulation of the emulsion geometry prior to cure. Specifically, we hypothesized that pore size would decrease and homogeneity increase with an increase in mixing speeds. Mixing speeds of 500, 1000, and 2000 rpm on the FlackTek SpeedMixerTM were tested to investigate the effect on pore architecture. Initially, HIPEs with 20 wt% PGPR were utilized resulting in minimal change in pore size with an increase in mixing speed as seen in Table 3. We hypothesized that destabilizing the emulsion with a lower concentration of PGPR would result in a larger pore size distribution, thereby clearly illustrating the effect of mixing speed. Scanning electron micrographs of both 10 and 20 wt% PGPR compositions at varying mixing speeds are found in Figure 8. A decrease in pore size was observed with both 10 and 20 wt% PGPR specimens as mixing speed was increased (500 to 2000 rpm). As discussed, the trend was more evident with the 10 wt% PGPR specimens, Table 3, and further illustrates the effect of surfactant on emulsion stability and pore architecture. The narrowing of the histograms in Figure 9 from 500 to 2000 rpm indicates a more homogeneous pore size distribution which is illustrated in the SEM images. As stated

previously, increased surfactant produced an increase in pore homogeneity due to decreased surface energy. Combining both mixing speed and surfactant had a large effect on pore size homogeneity as indicated by a more narrow distribution in Figure 9.

Conclusions

The application of emulsion templating in the development of tissue engineering scaffolds is a relatively new area of orthopedic research. In this study, polyHIPEs were fabricated without toxic solvents or monomers at cure temperatures appropriate for in situ deployment. These new polyHIPEs have potential application as an injectable, tissue engineered bone graft. Injectable grafts that are also biodegradable and porous offer unique advantages over current alternatives in terms of deployment and tissue integration. In addition, investigation of the effect of surfactant structure on HIPE stability elucidated additional structural features that can be used to rationally select surfactants for new polyHIPE compositions. This transition from a time-intensive trial and error method of selecting surfactants will facilitate additional biodegradable polyHIPE formulations with utility in a wide range of tissue engineering applications.

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References

1. Langer R, Vacanti JP. *Science*. 1993; 260:920–926. [PubMed: 8493529]
2. Rose FRAJ, Oreffo ROC. *Biochemical and Biophysical Research Communications*. 2002; 292:1–7. [PubMed: 11890663]
3. Kenley R, Yim K, Abrams J, Ron E, Turek T, Marden L, Hollinger J. *Pharmaceutical Research*. 1993; 10:1393–1401. [PubMed: 8272399]
4. Mikos AG, Temenoff JS. *Electronic Journal of Biotechnology*. 2000; 3:114–119.
5. Bhatia SN, Chen CS. *Biomedical Microdevices*. 1999; 2:131–144.
6. Hacker M, Ringhofer M, Appel B, Neubauer M, Vogel T, Young S, Mikos AG, Blunk T, Gopferich A, Schulz MB. *Biomaterials*. 2007; 28:3497–3507. [PubMed: 17482257]
7. Karageorgiou V, Kaplan D. *Biomaterials*. 2005; 26:5474–5491. [PubMed: 15860204]
8. Freyman TM, Yannas IV, Gibson LJ. *Progress in Materials Science*. 2001; 46:273–282.
9. Liu X, Ma PX. *Annals of Biomedical Engineering*. 2004; 32:477–486. [PubMed: 15095822]
10. Sikavitsas VI, Temenoff JS, Mikos AG. *Biomaterials*. 2001; 22:2581–2593. [PubMed: 11519777]
11. Busby W, Cameron NR, Jahoda CAB. *Polymer International*. 2002; 51:871–881.
12. Harris LD, Kim BS, Mooney DJ. *Journal of Biomedical Materials Research*. 1998; 42:396–402. [PubMed: 9788501]
13. Kim TK, Yoon JJ, Lee DS, Park TG. *Biomaterials*. 2006; 27:152–159. [PubMed: 16023197]
14. Mistry AS, Cheng SH, Yeh T, Christenson E, Jansen JA, Mikos AG. *Journal of Biomedical Materials Research*. 2008
15. Pham QP, Sharma U, Mikos AG. *Tissue Engineering*. 2006; 12:1197–1211. [PubMed: 16771634]
16. Burdick JA, Anseth KS. *Biomaterials*. 2002; 23:4315–4323. [PubMed: 12219821]
17. Jabbari E, Wang S, Lu L, Gruetzmacher JA, Ameenuddin S, Hefferan TE, Currier BL, Windebank AJ, Yaszemski MJ. *Biomacromolecules*. 2005; 6:2503–2511. [PubMed: 16153086]
18. Svaldi-Muggli D, Burkoth AK, Anseth KS. *Journal of Biomedical Materials Research*. 1999; 46:271–278. [PubMed: 10380006]

19. Peter SJ, Kim P, Yasko AW, Yaszemski MJ, Mikos AG. *Journal of Biomedical Materials Research*. 1999; 44:314–321. [PubMed: 10397934]
20. Peter SJ, Lu L, Kim DJ, Mikos AG. *Biomaterials*. 2000; 21:1207–1213. [PubMed: 10811302]
21. Peter SJ, Nolley JA, Widmer MS, Merwin JE, Yaszemski MJ, Yasko AW, Engel PS, Mikos AG. *Tissue Engineering*. 1995; 1:41–52. [PubMed: 19877914]
22. Christenson EM, Soofi W, Holm JL, Cameron NR, Mikos AG. *Biomacromolecules*. 2007; 8:3806–3814. [PubMed: 17979240]
23. Barby D, Haq Z. 1982
24. Cameron NR, Sherrington DC, Albiston L, Gregory DP. *Colloid and Polymer Science*. 1996; 274:592–595.
25. Akay G, Birch MA, Bokhari MA. *Biomaterials*. 2004; 25:3991–4000. [PubMed: 15046889]
26. Hayman MW, Smith KH, Cameron NR, Przyborska SA. *Journal of Biochemical and Biophysical Methods*. 2005; 62:231–240. [PubMed: 15733583]
27. Busby W, Cameron NR, Jahoda CAB. *Biomacromolecules*. 2001; 2:154–164. [PubMed: 11749167]
28. Barbetta A, Dentini M, De Vecchis MS, Filippini P, Formisano G, Caiazza S. *Advanced Functional Materials*. 2005; 15:118–124.
29. Barbetta A, Dentini M, Zannoni EM, De Stefano ME. *Langmuir*. 2005; 21:12333–12341. [PubMed: 16343011]
30. Cameron NR, Barbetta A, Cooper SJ. *Chemical Communications*. 2000:221–222.
31. Bokhari MA, Birch MA, Akay G. *Experimental Medical Biology: Tissue Engineering, Stem Cells, and Gene Therapies*. 2003; 534:247–254.
32. Hayman MW, Smith KH, Cameron NR, Przyborski SA. *Biochemical and Biophysical Research Communications*. 2004; 314:483–488. [PubMed: 14733931]
33. Cameron NR, Sherrington DC, Ando I, Kuroso H. *Journal of Material Chemistry*. 1996; 6:719–726.
34. Williams JM, Wroblewski DA. *Langmuir*. 1988; 4:656–662.
35. Cameron NR, Sherrington DC. *Macromolecules*. 1997; 30:5860–5869.
36. Lumelsky Y, Silverstein M. *Macromolecules*. 2009; 42:1627–1633.
37. Youssef C, Backov R, Treguer M, Birot M, Deleuze H. *Materials Research Society Symposium*. 2010; 1269:1–6.
38. Umez-Eronini NO, Collins A, Neal DE. *European Cells and Materials*. 2002; 4:77–78.
39. Tai H, Sergienko A, Michael S. *Polymer*. 2001; 42:4473–4482.
40. Lepine O. *Polymer*. 2005; 46:9653–9663.
41. David D, Silverstein M. *Journal of Polymer Science: Part A: Polymer Chemistry*. 2009; 47:9.
42. Mistry A, Pham Q, Schouten C, Yeh T, Christensen E, Mikos A, Jansen J. *Journal of Biomedical Materials Research Part A*. 2010; 92A:451–462. [PubMed: 19191316]
43. Timmer MD, Horch AR, Ambrose CG, Mikos AG. *Journal Biomaterial Science Polymer Edition*. 2003; 14:369–382.
44. Lin-Gibson S, Cooper JA, Landis FA, Cicerone MT. *Biomacromolecules*. 2007:1–8.
45. ASTM, CDoP. 2004
46. <http://www.molinspiration.com/>.
47. Williams JM, Gray AJ, Wilkerson MH. *Langmuir*. 1990; 6:437–444.
48. Williams JM. *Langmuir*. 1991; 7:1370–1377.
49. Cameron NR, Barbetta A. *Journal of Materials Chemistry*. 2000; 10:2466–2471.
50. Cohen N, Silverstein M. *Polymer*. 2011; 52:282–287.
51. Carnachan RJ, Bokhari M, Przyborski SA, Cameron NR. *Soft Matter*. 2006; 2:608–616.

Figure 1.
Molecular structure of (A) bis (1,2 hydroxypropyl) fumarate and (B) PFDMA.

Figure 2.
Nuclear magnetic resonance spectrum of PFDMA.

Figure 3. Comparison of hydrogen bond donor sites (circled): (A) Span 80 has 3 in the polar head, whereas (B) PGPR 4125 has 3 in the hydrophobic tail.

Figure 4.

Injectable PFDMA polyHIPEs can be used *in situ* to space fill complex defects.

Figure 5. 24 hour 3T3 Live/Dead analysis of 5 wt% PGPR polyHIPEs. A) Fluorescent image (green = live, red = dead) B) Comparison of viable cells on tissue culture polystyrene and HIPE (n=15). No significant difference was observed between the polystyrene ($96 \pm 3\%$) and HIPE ($95 \pm 6\%$).

Figure 6. Scanning electron micrographs (A,B,C,D) of PFDMA polyHIPEs with increasing surfactant concentrations. It can be seen that pore sizes decrease as PGPR concentration increases from 5–20 wt%.

Figure 7. Distribution of pore sizes for 75/25 polyHIPEs with varied PGPR concentrations, mixed at 500 rpm. (A) 10 wt%, (B) 15 wt%, and (C) 20 wt%. Pore sizes become more uniform as PGPR content increases.

Figure 8.

Scanning electron micrographs of PFDMA polyHIPEs fabricated at increasing mixing speeds: (A–C) 10 wt% PGPR (A) 500 rpm (B) 1000 rpm (C) 2000 rpm, (D–F) 20 wt% PGPR (D) 500 rpm (E) 1000 rpm (F) 2000 rpm. A decrease in pore size is seen with an increase in mixing speed.

Figure 9. Distribution of pore sizes for 75/25 polyHIPEs fabricated with 10 (black) and 20 wt% PGPR (blue) at 500, 1000, and 2000 rpm.

Table 1

Estimated Octanol-Water Partition Coefficients

Molecule	LogP ^a
Styrene	2.8
Divinyl benzene	3.6
PFDA	2.3
PFDMA	3.4
PPF (n = 5)	2.4
PPF (n = 6)	3.1

^a Octanol-water diffusion coefficient calculated with the Molinspiration miLogP model based on molecular structures.

Table 2

The effect of hydrogen bond donor site location and HLB value on HIPE formation.

Surfactant	Hydrogen Bond Donor Site Location			
	Polar Head	Hydrophobic Tail	HLB value	HIPE Formed
Span 85	1	0	1.8	no
Span 80	3	0	4.3	no
PGPR 4125	0	3	4.7	yes
PEG 600 Dilaurate	0	0	11.7	no
Tween 80	3	0	15	no

Table 2

The effect of surfactant concentration on polyHIPE architecture.

Surfactant (wt%)	Porosity (%)	Average Pore Diameter (μm)
5	75.1 ± 0.4	29 ± 19
10	75.1 ± 0.3	21 ± 11
15	75.1 ± 0.1	14 ± 8
20	74.1 ± 0.1	4 ± 2

Gravimetric porosity, N = 3, average $\pm$ standard deviation. Average pore measurements from image analysis of scanning electron micrographs, N = 150, average $\pm$ standard deviation.

Table 3

The effect of mixing speed on polyHIPE pore structure with a constant volume fraction (75/25) and varied surfactant concentration (10 and 20 wt% PGPR)

[PGPR]	Mixing Speed (rpm)	Average Pore Diameter (μm)
10 wt%	500	21 ± 11
	1000	8 ± 4
	2000	4 ± 3
20 wt%	500	4 ± 2
	1000	3 ± 2
	2000	2 ± 1

Average pore measurements from image analysis of scanning electron micrographs, N = 150, average $\pm$ standard deviation.