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Injectable PolyMIPE Scaffolds for Soft Tissue Regeneration

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Abstract

Injury caused by trauma, burns, surgery, or disease often results in soft tissue loss leading to impaired function and permanent disfigurement. Tissue engineering aims to overcome the lack of viable donor tissue by fabricating synthetic scaffolds with the requisite properties and bioactive cues to regenerate these tissues. Biomaterial scaffolds designed to match soft tissue modulus and strength should also retain the elastomeric and fatigue-resistant properties of the tissue. Of particular design importance is the interconnected porous structure of the scaffold needed to support tissue growth by facilitating mass transport. Adequate mass transport is especially true for newly implanted scaffolds that lack vasculature to provide nutrient flux. Common scaffold fabrication strategies often utilize toxic solvents and high temperatures or pressures to achieve the desired porosity. In this study, a polymerized medium internal phase emulsion (polyMIPE) is used to generate an injectable graft that cures to a porous foam at body temperature without toxic solvents. These poly(ester urethane urea) scaffolds possess elastomeric properties with tunable compressive moduli (20–200 kPa) and strengths (4–60 kPa) as well as high recovery after the first conditioning cycle (97–99%). The resultant pore architecture was highly interconnected with large voids (0.5–2 mm) from carbon dioxide generation surrounded by water-templated pores (50–300 μm). The ability to modulate both scaffold pore architecture and mechanical properties by altering emulsion chemistry was demonstrated. Permeability and form factor were experimentally measured to determine the effects of polyMIPE composition on pore interconnectivity. Finally, initial human mesenchymal stem cell (hMSC) cytocompatibility testing supported the use of these candidate scaffolds in regenerative applications. Overall, these injectable polyMIPE foams show strong promise as a biomaterial scaffold for soft tissue repair.

1. Introduction

Injury caused by trauma, burns, surgery, or disease often results in soft tissue loss, most commonly skin, muscle, adipose, and cartilage.[1] These tissues have unique properties that drive various functions such as: supporting vasculature, body locomotion, and impact protection.[1, 2] The lack of viable donor tissue to treat these injuries often results in scarring and impaired function. Tissue engineering aims to improve on current options through the use of a porous scaffold combined with stem cells and bioactive factors that are designed to regenerate the damaged tissue. Scaffolds have been fabricated using a variety of

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polymers to restore the function and regenerate these tissues; however, clinical success is often limited by poor graft properties.[2–8]

Soft tissue scaffolds are often designed to have tunable moduli and strengths comparable to the target tissue.[9–12] A common feature of soft tissue that is often poorly replicated in scaffolds is elasticity and an ability to withstand repeated loading without loss of function. [6, 7, 13, 14] Improved biomaterial scaffolds should be designed to match tissue modulus and strength while also retaining desired elastomeric and fatigue-resistant properties. In addition, these materials need to support cells and growing tissue, typically with an interconnected porous structure and biodegradable chemistry.[15–18] Of particular design importance is the interconnected porous structure of the scaffold, which facilitates mass transport. This is especially true for newly implanted scaffolds that lack vasculature to support tissue and cells. The size of a tissue engineered construct is constrained by the diffusion limit of nutrients to cells at the center of the construct.[19, 20] Porosity and pore size need to be easily modulated in a tissue engineered scaffold to allow for increased fluid permeability, and thus nutrient flux, in order to enable larger defects to be regenerated.[20]

Tissue engineers have developed numerous fabrication strategies to create 3D scaffolds that meet these various requirements.[15–17, 21–27] The fabrication process dictates scaffold architecture and the resultant mechanical properties, degradation rate, and cellular response. Some common techniques to produce porous scaffolds include: particulate leaching, gas foaming, thermal induced phase separation, and fiber bonding.[24–29] These methods often utilize toxic solvents and high temperatures or pressures during fabrication.[23, 30–32] The scaffolds must then be purified and shaped to fit the defect, commonly with the use of computer generated molds made from clinical imaging techniques.[28, 29, 33] Molds are reliant upon the accuracy of the images, increase cost, and delay patient treatment. Research has shown that *in situ* forming scaffolds can better fill irregularly shaped defects, thus improving contact with surrounding tissue and eliminating the need for costly molding techniques.[34] An elastomeric, biodegradable, and porous scaffold that can be fabricated at body temperature without the use of toxic components could be injected into the defect without the need for extensive imaging or mold casting and can potentially improve tissue regeneration beyond current methods. Of the fabrication methods mentioned previously, particulate leaching, hydrogels, and gas foaming are the typical options that could be injectable. However, a sintering process is typically required to generate interconnects in particulate leached scaffolds, and thus cannot be done *in vivo*. Additionally, hydrogel scaffolds typically have 10–100 nm mesh sizes that limit cellular infiltration and migration to the rate of scaffold degradation.[35] Hydrogels with larger pores have also been fabricated, but are commonly non-biodegradable or non-injectable.[36] Polyurethane foams have recently been formed *in situ* by injecting isocyanates and polyols which react rapidly to conform to the defect.[37] In animal studies, this material successfully regenerated bone tissue without without notable toxicity or inflammation in the surrounding tissue despite injecting isocyanates directly into the defect site.[37] Other researchers have conducted animal studies of injectable two-component polyurethane foams and found mild short-term side effects.[38]

Emulsion templating is a technique utilizing water-in-oil (w/o) or oil-in-water (o/w) emulsions to generate an injectable graft that cures to a porous foam *in situ*. Briefly, an oil phase composed of a low-viscosity macromer/monomer solution is combined with water and a surfactant to create an emulsion. Emulsions with water volumes between 40–74% are termed medium internal phase emulsions (MIPEs), the polymerized form is termed a polyMIPE. Once cured, the continuous phase “locks in” the emulsion geometry with the suspended water droplets as pore templates. Prior to curing, the emulsion has a mayonnaise-like consistency capable of flow through a syringe and space-filling irregularly shaped

defects.[39] Historically this technique required the use of toxic solvents to control macromer viscosity or high cure temperatures.[29, 40–43] Our lab recently fabricated emulsion templated scaffolds without solvent that cure at body temperature and are biodegradable.[39] Successful deployment through a syringe with microscale integration in a bone defect was also demonstrated.[44] The properties of these injectable foams can be tuned by altering pore size, porosity, composition, and macromer/polymer chemistry.

This study details the fabrication and characterization of poly(ester urethane urea) based polyMIPEs as candidates for soft tissue scaffolds. The compositional effects on polyMIPE pore architecture and mechanical properties were determined to illustrate the range and control of scaffold properties. The porous media properties, permeability and form factor, were also measured in order to better compare pore interconnectivity of several compositions. Finally, human mesenchymal stem cell (hMSC) viability and morphology was studied as an early indicator of whether these scaffolds can support tissue regeneration.

II. Methods

Materials

Polyglycerol polyricinoleate (PGPR 4125) was donated by Paalsgard. All other chemicals were purchased from Sigma-Aldrich and used as received.

PCL-Isocyanate Synthesis

Isocyanate functionalized prepolymers were synthesized in bulk. Briefly, polycaprolactone (PCL) diol or triol (530, 900 Mn respectively) was heated with 50°C air and added dropwise to a reaction flask charged with hexamethylene diisocyanate (HDI, $2.02 \times \text{mol diol}$, $9.00 \times \text{mol triol}$), Figure 1. The reaction was heated to 80 °C for 90–120 minutes and reaction progress was monitored with FTIR spectroscopy every 30 minutes until complete. The product was washed with hexane to remove excess HDI and dried under vacuum overnight. The final products were colorless liquids with a viscosity similar to honey. The prepolymer structure was confirmed using ^1H NMR (300 MHz, CdCl_3): PCL-diisocyanate (PCL-DI) δ 1.33–1.47 (m, 8H), 1.52 (q, 4H), 1.60–1.75 (m, 8H), 2.30–2.40 (m, 4H), 3.18 (s, 2H), 3.33 (q, 2H), 3.70–3.73 (m, 2H), 4.09 (t, 4H), 4.24–4.27 (m, 2H), 4.77 (s, 1H). PCL-triisocyanate (PCL-TI) δ 0.91(t, 3H), 1.33–1.52 (m, 8H), 1.60–1.75 (m, 8H), 2.31–2.38 (m, 4H), 3.18 (s, 2H), 3.33 (m, 2H), 4.04–4.11 (m, 4H), 4.74 (s, 1H).

MIPE Fabrication

PCL-DI and -TI were mixed with the surfactant PGPR in a 3-necked round bottom flask prior to emulsification. This mixture was stirred with a glass stirring rod fitted with a D-shaped PTFE paddle connected to an overhead stirrer motor for 10 minutes. The aqueous solution, comprised of varied amounts of 1,4-diazabicyclo[2.2.2]octane (DABCO) and calcium chloride (1% v/v) in reverse osmosis water, was added at a rate of 1 mL/min using a syringe until it totaled 50% v/v, while constant stirring at 300 rpm was maintained. The emulsion was stirred for 10 additional minutes to ensure emulsion homogeneity. The MIPE was then transferred to a 50 mL polypropylene tube and immersed in a bead bath at 37 °C for 48 h. All specimens were dried *in vacuo* at room temperature for 24 h prior to characterization, Figure 2.

Gravimetric Analysis

PolyMIPE porosity was measured gravimetrically. Briefly, dried MIPE samples were cut into cubic sections ($9 \times 9 \times 3$ mm) and weighed. Following eq (1), the MIPE porosity can be calculated by comparing MIPE density (ρ_M) with the neat polymer density (ρ_P). Neat

polymer density was assumed to equal 1 g/cm³, a reasonable value for polymers. Reported values are an average of nine sections per polyMIPE composition.

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

Characterization of Scaffold Architecture

Scanning electron microscopy (SEM) was utilized to characterize polyMIPE pore architecture. Circular specimens were sectioned into quarters, fractured at the center of the quarter, sputter-coated with gold, and imaged using SEM (JEOL NeoScope JCM-5000). Images at 20× were utilized to determine the average void diameter and 50× to measure water template pores, Figure 3. Three specimens of each composition were generated and then three sections were cut from each monolith. Three horizontal lines were drawn across the image, dissecting it into four equal rectangles. Starting with the topmost mark, measurements were made on the first 10 voids crossed by the lines to minimize user bias. Averages void sizes for each polyMIPE composition are reported ($n = 90$). Water pores/interconnects were measured similarly except that 30 pores/openings were measured per image, average interconnects per composition are reported ($n = 270$). A statistical correction was calculated to account for the random fracture plane through spherical voids and pores, $2/\sqrt{3}$.^[45] The average diameter values were multiplied by this correction factor resulting in a more accurate description of pore diameter. These values are listed in Figure 5.

Compression Testing

The foams were tested in compression with an RSA III (TA, Delaware) dynamic mechanical analyzer, equipped with a 30 N load cell. Four specimens were taken from each sample. The test specimens were cut into flat rectangular shapes (10 × 10 × 4 mm) and compressed at 50 μm/s. Tangent modulus was calculated at 5% strain and strength at 20%. Reported moduli data were an average of the three sections for each sample tested ($n = 12$).

Cyclic Loading and Strain Recovery

Samples were subjected to three compressive loading–unloading cycles from 0 to 50% strain and back to 0% at 50 μm/s (~1 %/s). The dimensions (diameter and height) of the samples were measured with calipers after each cycle to ensure that the stress–strain curves always started from the new unloaded specimen height. The samples rested for 2–3 minutes between cycles as dimensions were measured and samples re-positioned on the compression platen. New sample height was divided by the previous cycle height to calculate strain recovery.

Permeability Testing

Scaffold interconnectivity was characterized by measuring their respective intrinsic/Darcy's permeability using the Forchheimer-Hazen-Dupuit-Darcy equation:

$$-\frac{\partial P}{\partial x} = \frac{\mu}{K} v_0 + \rho C v_0^2 \quad (2)$$

where $-\frac{\partial P}{\partial x}$ is the pressure gradient along the sample in the direction of flow (Pa/m), μ is the dynamic viscosity of the fluid (Pa·s), K is the intrinsic permeability of the (scaffold, sample) (m²), v_0 is the Darcy velocity (flow rate divided by cross-sectional area of the (scaffold, sample)) (m/s), ρ is the density of the fluid (kg/m³), and C is the form factor of the

(scaffold, sample) (m^{-1}). The experimental details for measuring the permeability and form factor of porous polymers have been described elsewhere by Muschenborn et al.; briefly, MIPes were polymerized inside 3D printed sample holders (inner diameter = 15.1 mm, outer diameter = 18.9 mm) containing three 4×4 mm pressure port openings, 15 mm apart from one another.[46] The sample holders had o-rings to isolate each pressure port. Each sample holder was then inserted into a custom-built measuring chamber, and two pressure transducers (PX429-2.5G5V, Omegadyne Inc) were connected at the upstream and downstream pressure port locations. Water flow at room temperature was enabled via a gear pump (Chemsteel R106, Oberdorfer), a servo motor (750 W M-series, Applied Motion Products), and a motor controller (BLuAC5-Q, Applied Motion Products). The output voltage of the pressure transducers was recorded at 1 Hz through a data acquisition system (USB6251, National Instruments) for 120 seconds. The flow rate was measured by hand using a stop watch and a graduated cylinder. A second-order least squares fit to the data of pressure gradient plotted against Darcy velocity was implemented for nine values to calculate the samples' porous media properties, permeability and form factor.[46] The Reynolds number at each velocity point was calculated as the ratio of the second term to the first term on the right side of Equation 2.[47]

Cytocompatibility

Initial hMSC viability and morphology studies were conducted on 2:1 PCL-DI:PCL-TI films and 50% PCL-DI: 50% PCL-TI polyMIPes to assess cytocompatibility. Cell viability was determined using the Live/Dead Viability/Cytotoxicity Kit (Molecular Probes) and morphology accessed by imaging cells stained with CellTracker™ Orange (Molecular Probes). The hMSCs were obtained from the Center for the Preparation and Distribution of Adult Stem Cells at Texas A&M Health Science Center College of Medicine and cultured *in vitro* with Minimal Essential Medium α (MEM α , Gibco) supplemented with 16.5% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals), and 1% L-Glutamine (200 mM, Gibco). Cells were cultured to 80% confluency and utilized at Passages 4–5. Samples were prepared for cell seeding as follows: 3 hour 70% ethanol sterilization, ethanol wetting ladder and progressive solvent extraction, and overnight media incubation supplemented with 40% v/v FBS in MEM α . Following overnight incubation in 37 °C, 5% CO₂, medium was removed, specimens were dried in the hood for 15 min, washed 1× with PBS, and preconditioned with growth medium for 15 min. Cells were seeded at 5,000 cells/film and 100,000 cells/foam. Live/Dead staining was conducted at 3, 24, and 72 hours and cell adhesion and morphology on foams was assessed at 3 and 24 hours. For viability analysis, images of each of the four specimens were obtained through Raster patterning ($n = 20$) for each timepoint using a fluorescence microscope (Nikon Eclipse TE2000-S). Observation of hMSC morphology was completed with one sample imaged with raster patterning ($n = 5$) at each timepoint.

Statistical Analysis

The data are displayed as mean $\pm$ 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 ($p < 0.05$).

III. Results and Discussion

Pore Architecture

All of the scaffolds exhibited a bimodal pore structure composed of discrete 1–2 mm voids surrounded by 30–400 μm pores, Figure 4 and 5. The pore size remained relatively unchanged despite large differences in void diameter between compositions. Increased PCL-TI concentration significantly increased void size once it exceeded 50%, from 1 to 1.3 mm.

This phenomena was observed by David and Silverstein in a similar system and hypothesized to be caused by side products of urea formation.[23] Briefly, the isocyanate functional groups react with the encapsulated water to form amine end groups with the liberation of carbon dioxide. These amine end groups react rapidly with remaining isocyanates to form urea linkages in the resulting polymer network. David and Silverstein determined that the large pores were caused by carbon dioxide and the smaller pores formed by water droplets.[23] They hypothesized that carbon dioxide would separate from both the organic and aqueous phases, merge, and form the observed voids.[23] This is consistent with the present study, except the water-templated pores are not as clearly defined. Instead, the walls of the voids are porous with irregular interconnects between them, Figure 4D, E, F. The oblong shapes of these interconnects indicate that the emulsion is relatively unstable and is undergoing droplet coalescence, whereas stable emulsions would result in spherical pores.[23, 30] Additionally, some interconnects appear to be torn open. This is most likely the result of carbon dioxide escaping from the polymerizing emulsion after the pore walls had mostly gelled.

The effect of catalyst concentration on pore structure was also evaluated. Void size decreased from ~ 0.9 to 0.7 mm with increased DABCO concentration. The increased cure rate from higher DABCO amounts was hypothesized to cause rapid polymer wall strengthening that better resisted expansion from carbon dioxide production and forced the gas to find existing exit paths and thus form smaller voids. Foaming in polyurethane foams has long been studied for industrial and commercial uses. Anti-foamers (or pore openers) have been used to prevent or control foam cell production and size.[48–50] The Guelcher group has used calcium stearate pore openers in their injectable bone grafts to form interconnects and tune pore size.[48, 49, 51, 52] Although it was not studied here, a biocompatible pore opener could potentially be used to homogenize void sizes in this injectable system.

Scaffold Porosity

A benefit of the carbon dioxide blowing process is increased porosity, as evidenced by polyMIPE expansion during polymerization. In traditional emulsion templating, porosity is limited by the template volume fraction, 50% in this study. However, due to foaming, these polyMIPE porosities ranged from 64–76%, Figure 5C, while higher porosity scaffolds could be fabricated they often exhibited poor mechanical properties. Porosity increased with PCL-TI content due to the increase in total isocyanate groups, and decreased with increased catalyst concentration. For example, a MIPE with 100% PCL-TI would have approximately 1.25x the number of isocyanates of a 100% PCL-DI emulsion. Increasing catalyst concentration, from 0 to 1 wt%, for a 50% PCL-TI polyMIPE decreased porosity from 70 to 64%. This corresponded with a decrease in void size from approximately 881 μm to 721 μm while water pore size did not follow any trend. This decrease in porosity was not attributed to reduced carbon dioxide generation given that the total number of isocyanates was constant which indicates that the carbon dioxide generation was most likely unchanged. The increase in catalyst was hypothesized to increase polymer film strength more rapidly, thus resisting carbon dioxide void expansion and decreasing total porosity. These results indicate that changing isocyanate content or reaction rate with catalyst could be used to modulate scaffold porosity and expansion *in situ* through control of the carbon dioxide blowing mechanism. Researchers have shown that increasing scaffold porosity increases cell viability and growth.[3, 43, 53–57] Also, the expansion could help the graft “space fill” the defect, improving tissue integration.[28, 29, 39, 41, 49, 52] There is concern over the safety of carbon dioxide blowing *in vivo* resulting from reacting isocyanates into the human body. Recently, researchers have safely injected foaming isocyanate-functionalized prepolymers for a variety of applications and no deleterious side effects were noted in relation to the

presence of free-isocyanates or the blowing process.[37, 38, 48, 51] Similar to pore size, porosity of polyurethane foams has been studied extensively and can be further controlled using methods beyond the scope of this study.[50]

Mechanical Properties

PolyMIPE compressive modulus and strength decreased with decreasing PCL-DI content. The 100 and 75% PCL-DI with 0.1 wt% DABCO were the stiffest and strongest compositions tested, with a modulus and strength approximately 215 and 55 kPa, respectively (Figure 6). These values decreased steadily to 48 and 8 kPa as PCL-TI was increased to 100%. The greatest change occurred when 75% TI was reached, afterwards modulus and strength decreased by nearly half of the 50% TI values. Catalyst concentration did not have a significant effect on the properties of the 25, 50, and 75% PCL-TI scaffolds, with the 0 (Figure 6C) and D)) and 0.1% (Figure 6A) and B)) compositions behaving near identically. However, the uncatalyzed 25% PCL-TI polyMIPEs were less consistent than their catalyzed counterparts, as evidenced by the large standard deviation. Furthermore, the 100% PCL-TI and 100% PCL-DI polyMIPEs were too macroporous to make mechanical specimens, represented by 'X' in Figure 6.

The reductions in compressive modulus and strength with increasing PCL-TI content was not anticipated given the increase in crosslink density that is typically observed with higher macromer functionality. The observed trend corresponded with increases in pore size and porosity. However, the difference between the strongest and weakest scaffolds was a five-fold change with only a 13% increase in porosity. Therefore, the change in porosity is unlikely to be solely responsible for the reduction in compressive properties. It was hypothesized that the PCL-TI disrupted secondary chain interactions within the polymer network, specifically changes in crystallinity and hydrogen bonding. The PCL-diol and -triol are semicrystalline with melting temperatures of 7 (37% crystalline) and 21 °C (29% crystalline) respectively, as determined with differential scanning calorimetry. Upon endcapping the macromers with isocyanate groups, PCL-DI becomes more crystalline as evidenced by increased viscosity and opacity, whereas the PCL-TI displayed a reduction in crystallinity becoming less viscous and transparent. Hydrogen bonding between the newly formed urethane linkage is also more favorable in the linear PCL-DI.[58] The steric hindrance of the branched PCL-TI structure most likely prevents intra-chain hydrogen bonding and impedes inter-chain hydrogen bonding and crystal formation. Additional physical crosslinking occurs after polymerization due to the formation of urea linkages that can undergo bidentate hydrogen bonding.[15] Bidentate bonds form between the two active hydrogens and the nitrogen of the urea linkages and thus result in stronger hydrogen bonding when compared to urethane linkages with only one active site. This hypothesis was supported by FTIR spectral analysis of hydrogen bonding of the urethane and urea carbonyls of the polyMIPE scaffolds. Peaks assigned to the free, hydrogen bonded, and bidentate hydrogen-bonded urea carbonyls (1680, 1654, 1625 cm^{-1} respectively) were used to assess relative levels of hydrogen bonding. PCL-TI rich polyMIPEs displayed a reduction of 1625 cm^{-1} peak indicating a reduced level of hydrogen bonding.

Initial strain recovery increased with PCL-TI content, and approached 100% upon subsequent loading, Figure 7. These properties allowed the scaffolds to recover from large strains, as can be seen in the video file included with supplementary information. The 100% PCL-DI samples exhibited the lowest initial recovery of the catalyzed scaffolds, approximately 90%, and the highest recovery was found with the 50% PCL-DI scaffolds, approximately 95%. All initial strain recoveries were equal to or greater than 90% for all scaffolds tested. Subsequent strain recoveries were always higher than the initial values, commonly approaching 100%. Subsequent recovery, like initial recovery, improved with

increased PCL-TI content. Likewise, hysteresis between the loading and unloading curves decreased with subsequent loading and PCL-TI content. This behavior is similar to conditioning seen in other polyurethane materials and characteristic of the “Mullin’s effect”. [59–62]

The observed decrease in hysteresis and increased strain recovery after the first cycle is most likely due to chain reorganization during compression. Strain first breaks physical crosslinks in the network, predominantly hydrogen bonding between urethane and ureas in these foams, prior to loss of chemical crosslinks. In a series of loading cycles, the first conditioning cycles generates a new network with permanent set associated with the loss of these physical bonds. Subsequent cycles do not exceed the strain needed to cause further loss of netpoints and therefore have a high level of strain recovery. Similar phenomena have been observed in polyurethanes under tensile strain.[59, 62–64] This behavior was most pronounced in the PCL-DI rich compositions, which exhibited the lowest initial strain recovery and largest hysteresis, due to the increased hydrogen bonding observed in these chains. PCL-TI also exhibited this behavior to a lesser extent, with almost no hysteresis, due to the increase in covalent crosslinking and reduced hydrogen bonding of the PCL-TI network. Overall, these properties are similar to many soft tissues in the body and can be tuned to closely match them by changing the ratio of branched crosslinkers to linear chain extenders.[6, 65] Furthermore, these properties mimic those of injectable hydrogels with the advantage of being porous and elastomeric.[2, 5, 6, 33, 66]

Scaffold Permeability

Several researchers have attempted to determine the ideal pore size for tissue engineered scaffolds by comparing scaffold properties with *in vivo* and *in vitro* studies. The majority found that porosities between 57 and 75% with 80–500 μm pores regenerated tissue *in vivo*. [3, 43, 53–57, 67–72] However, results are often contradictory and may be specific to cell type. For example, a more metabolically active cell may need a higher nutrient flux that corresponds with a more permeable scaffold. As such, researchers have been maximizing porosity with the assumption that fluid permeability and nutrient flux will increase beyond the limits of diffusion and allow larger defects to be treated. Pore interconnectivity and fluid permeability are inherently linked and can be quantified in many ways, including Darcy’s permeability constant.[73, 74] Permeability is mathematically related to pore size, porosity, and tortuosity. Tortuosity is a unit-less measure of the fluid path length through the porous medium relative to the end-to-end path length, with lower values correlating to an increase in pore interconnectivity.[75] Several researchers have studied permeability in tissue engineered scaffolds to find relationships between pore structures and cell/tissue viability. [20] Darcy’s permeability constant can be related to solute diffusion, and thus cell viability for similar scaffold geometries and types.[20, 73, 74]

Permeability and form factor are constitutive geometrical parameters of the porous medium and depend on the energy losses that arise when a fluid flows through it.[76–78] These losses are due to viscous forces and inertial drag that result from the fluid’s resistance to motion and friction as it flows over the porous scaffold. Thus, permeability is inversely proportional to the surface area of contact between the solid and the fluid, and form factor is proportional to the projected cross-sectional area of the obstructing solid perpendicular to the direction of the flow. Consequently, a scaffold with highly interconnected pores would have higher permeability and a lower form factor.

In this study, the Reynolds numbers ranged between 0.088 and 1.250, but were mostly below 1, indicating that flow was primarily in the Darcian regime.[77] The Darcian regime is dominated by viscous energy losses, which are inversely proportional to permeability. Permeability increased four-fold with PCL-TI content, and form factor decreased by an

order of magnitude, Figure 8. These changes are most likely due to the void, pore, and porosity differences noted earlier. The 100, 75, and 50% PCL-DI compositions have similar pore sizes and porosities, therefore it follows that their permeabilities are almost equal ($\sim 1.60 \times 10^{-10} \text{ m}^2$). Permeability begins to increase with the 25 and 0% PCL-DI polyMIPEs to 3 and $4 \times 10^{-10} \text{ m}^2$ respectively, which had significantly higher average pore diameters and porosities than the lower concentrations. Overall, these results confirm that all of the tested compositions possess interconnected porous networks, despite having emulsion volume fractions of only 50%. The blowing process of the isocyanate reaction with water allows these materials to not only increase their porosity above volume fraction but is also hypothesized to drive interconnect formation as carbon dioxide tries to escape the scaffold.

Cytocompatibility

As an initial test of cytocompatibility, percent cell viability was tested on neat polymer films of similar composition to the porous scaffold but without any catalyst or surfactant. These films exhibited greater than 95% viability after 72 hours with no significant difference to the tissue culture polystyrene (TCPS) control at any time point. These results indicate that the polymer chemistry is cytocompatible and support the use of this system for biomedical applications, Figure 9. Neat films were used to isolate the effect of polymer chemistry on cell viability and due to the complications associated with testing viability on porous specimens. Subsequent analysis of hMSCs seeded on polyMIPE specimens (75% PCL-DI: 25% PCL-TI, 0.1 wt% DABCO) indicated that hMSCs were able to adhere and spread on the foam as characterized by the numerous pseudopodia extending from the bright red cell nuclei. The results indicate that the cells thrived for up to 24 hours on the polyMIPE despite the presence of DABCO (0.1 wt%). Of the handful of catalysts typically used in polyurethane and polyurea syntheses, DABCO is considered the most cytocompatible but still regarded as a health hazard by many.[79] Tanzi et al. found the IC50 of DABCO in solution to be 0.013 and 0.071 wt% for human endothelial cells and Swiss 3T3 fibroblasts after 72 hours, respectively.[79] A key difference between the studies is how the cells were exposed to the catalyst. In Tanzi et al., the cells were cultured in solutions containing DABCO, but in this study DABCO had to leach out of the scaffold before contacting cells. [79] This leaching mechanism could explain the cell spreading at 24 hours when very little DABCO had escaped the polymer, but longer time points need to be investigated before any conclusions on cytocompatibility can be drawn. The isocyanate chemistry of this system is advantageous in this regard, since the presence of water will result in conversion to amines that react with remaining isocyanates, without catalyst. Utilizing these reactions could allow for certain polyMIPEs to retain their biocompatibility, be injected into patients, and cured *in situ* with similar final properties to their catalyzed counterparts. Previously, isocyanates had been found to be carcinogenic but those harmful isocyanates were very low-molecular weight aromatic monomers. Novel injectable polyurethane foams have been safely deployed in animal studies without serious adverse reactions.[37, 38, 48, 51] The improved biocompatibility was hypothetically linked to the increased molecular weights of the isocyanate prepolymers and are similar to the macromers described here.[37, 48, 51, 52] Therefore, these macromers are not expected to cause adverse reactions *in vivo*. Additionally, the surfactant layer is hypothesized to act as a barrier between the prepolymers and cells, further mitigating potential side effects prior to polymerization. Future studies will examine the effects of catalyst concentration and polyMIPE composition on cell viability and spreading, as well as test the cytocompatibility of unpolymerized PCL-isocyanate macromers.

IV. Summary and Conclusions

Emulsion templating of tissue engineering scaffolds is a relatively new area of research that can be used to generate injectable foams with tunable mechanical properties. In this study, elastomeric polyMIPs were fabricated at body temperature without toxic solvents and with viscosities appropriate for *in situ* deployment through a syringe. In addition to the porosity generated from the water droplet phase of the emulsion, a chemical blowing mechanism generated additional porosity through carbon dioxide generation. The interconnected porous structure of the resulting foams yielded high graft permeability that is expected to enable improved mass transport and regeneration *in vivo*. Finally, initial human mesenchymal stem cell (hMSC) cytocompatibility testing supported the use of these candidate scaffolds in regenerative applications. Overall, these new scaffolds have potential application as biodegradable and injectable tissue engineered grafts for soft tissue regeneration and offer unique advantages in deployment and tissue integration over current methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PCL-polyols are functionalized with HDI in bulk to synthesize isocyanate terminated macromers. A) PCL-diisocyanate (PCL-DI) and B) PCL-triisocyanate (PCL-TI)

Figure 2.

A) The isocyanate macromers were mixed with surfactant while water was added to form medium internal phase emulsions. The MIPEs viscosity allows them to be injected through a syringe prior to polymerizing in the body. B) The isocyanates react with water to produce primary amines and carbon dioxide, increasing porosity beyond the aqueous volume fraction, a limiting factor for typical styrene-based polyMIPEs.

Figure 3.
PolyMIPE scaffolds exhibited a bimodal pore structure with large voids and smaller interconnected pores.

Figure 4. Scanning electron micrographs of the polyMIPE scaffolds indicated an open-pore interconnected structure. A bimodal pore distribution was also observed due to the difference in pores formed by A, B, C) carbon dioxide generation and those resulting from D, E, F) water templating.

Figure 5.

A) Average CO₂ void diameters, B) average pore diameters, and C) average porosities of polyMIPEs with varied PCL-DI: PCL-TI ratio and constant water volume fraction.

*: $p < 0.05$ compared to 100% PCL-DI

†: $p < 0.05$ compared to 75% PCL-DI

‡: $p < 0.05$ compared to 50% PCL-DI

•: $p < 0.05$ compared to 25% PCL-DI

○: $p < 0.05$ compared to 0% PCL-DI

Figure 6.

A) Compressive modulus and B) strength of polyMIPes with increasing PCL-TI content (0.1 wt% DABCO). C) Compressive modulus and D) strength of polyMIPes without DABCO.

*: $p < 0.05$ compared to all other compositions

†: $p < 0.05$ compared to 25% PCL-TI, 0.1 wt% DABCO

‡: $p < 0.05$ compared to 75% PCL-TI, 0.1 wt% DABCO

Figure 7.

Representative loading-unloading curves for A) 0%, B) 50%, and D) 100% PCL-TI polyMIPes with 0.1 wt% DABCO.

Figure 8.

Darcy permeability “K” and form factor “C” of polyMIPes with varied PCL-DI: PCL-TI ratio and constant water volume fraction.

*: $p < 0.05$ compared to 100% PCL-DI

†: $p < 0.05$ compared to 75% PCL-DI

‡: $p < 0.05$ compared to 50% PCL-DI

ˆ: $p < 0.05$ compared to 25% PCL-DI

○: $p < 0.05$ compared to 0% PCL-DI

Figure 9.

A) Cell viability of neat PUU films, B) hMSCs spread on polyMIPE scaffold

Table 1

Effect of catalyst and PCL-TI crosslinker on cure time, pore sizes, and porosity.

DABCO (wt %)	PCL-DI (wt %)	PCL-TI (wt %)	Tack Free Time (hours)	Void Size (μm)	Water Pore Size (μm)	Porosity (%)
0	75	25	5.5	1021 ± 583 <i>O</i>	181 ± 230 [†] <i>†</i> [•] <i>O</i>	70 ± 6 <i>O</i>
0	50	50	5.0	881 ± 454 [•] <i>O</i>	101 ± 60 [•] <i>†</i> [•] <i>O</i>	70 ± 2 [†] [•] <i>O</i>
0	25	75	5.5	919 ± 386 [•] <i>O</i>	131 ± 99 [•] <i>†</i> [•]	73 ± 4 [†] [•] <i>O</i>
0.1	50	50	4.5	1086 ± 454 [†] <i>O</i>	62 ± 28 [•] <i>†</i> [†] <i>O</i>	67 ± 2 [†] [†] <i>O</i>
1	50	50	4.0	721 ± 264 [•] <i>†</i> [†] [•]	127 ± 71 [•] <i>†</i> [•]	64 ± 2 [•] <i>†</i> [†] [•]

* $p < 0.05$ compared to 75% PCL-DI, 0% DABCO

[†] $p < 0.05$ compared to 50% PCL-DI, 0% DABCO

[•] $p < 0.05$ compared to 25% PCL-DI, 0% DABCO

[•] $p < 0.05$ compared to 50% PCL-DI, 0.1% DABCO

O $p < 0.05$ compared to 50% PCL-DI, 1% DABCO