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Degradable Segmented Polyurethane Elastomers for Bone Tissue Engineering: Effect of Polycaprolactone Content

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Abstract

Segmented polyurethanes (PURs) – consisting of degradable poly(α -hydroxy ester) soft segments and amino acid-derived chain extenders – are biocompatible elastomers with tunable mechanical and degradative properties suitable for a variety of tissue engineering applications. In this study, a family of linear PURs synthesized from poly(ϵ -caprolactone) (PCL) diol, 1,4-diisocyanobutane and tyramine with theoretical PCL contents of 65 to 80 wt% were processed into porous foam scaffolds and evaluated for their ability to support osteoblastic differentiation *in vitro*. Differential scanning calorimetry and mechanical testing of the foams indicated increasing polymer crystallinity and compressive modulus with increasing PCL content. Next, bone marrow stromal cells (BMSCs) were seeded into PUR scaffolds – as well as poly(lactic-co-glycolic acid) (PLGA) scaffolds – and maintained under osteogenic conditions for 14 and 21 days. Analysis of cell number indicated a systematic decrease in cell density with increasing PUR stiffness at both 14 and 21 days in culture. However, at these same time points the relative mRNA expression for the bone-specific proteins osteocalcin and the growth factors bone morphogenetic protein-2 and vascular endothelial growth factor gene expression were similar among the PURs. Finally, prostaglandin E₂ production, alkaline phosphatase activity, and osteopontin mRNA expression were highly elevated on the most-crystalline PUR scaffold as compared to the PLGA and PUR scaffolds. These results suggest that both the modulus and crystallinity of the PUR scaffolds influence cell proliferation and the expression of osteoblastic proteins.

1. Introduction

Engineered bone tissues, consisting of biologically active proteins in resorbable biomaterial scaffolds, are promising materials for the repair of large bone defects. One approach for fabricating such materials is the *in vitro* culture of bone marrow stromal cells (BMSCs)

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within porous biomaterials [1]. Under osteogenic culture conditions, the BMSCs will differentiate and synthesize an extracellular matrix (ECM) containing both osteoblastic proteins (*e.g.*, osteopontin (OPN), osteocalcin (OC), and bone sialoprotein [2]) and osteogenic growth and differentiation factors (*e.g.*, vascular endothelial growth factor (VEGF)-A, bone morphogenetic protein (BMP)-2 and -7 [3]). However, the composition of this ECM depends strongly on the properties of the cell microenvironment during the process of BMSC differentiation.

One facet of the microenvironment that can influence cell function is the biomaterial. In particular, modulus [4–7], surface chemistry [5, 8, 9], and surface roughness of the biomaterial [10–13] have all been shown to affect ECM production. With regard to modulus, a study on polyacrylimide hydrogels showed that soft (1 kPa) substrates enhance BMSC expression of BMP-2 and -7, but harder (*e.g.*, 34 kPa) surfaces stimulate expression of type I collagen, OC, and OPN [4]. Similarly, studies involving MC3T3-E1 osteoprogenitor cells on poly(ethylene glycol) hydrogels showed an increase in alkaline phosphatase (ALP) activity and expression of OC with increasing substratum modulus [6, 7]. Additionally, studies involving MG63 human osteosarcoma cells on stiff diethylene glycol dimethacrylate (DEGDMA) gels (64–507 MPa) revealed increasing OC expression with increasing modulus, but maximal ALP activity at an intermediate modulus (355 MPa) [5]. A limitation with these three studies, though, is that they were performed in model planar geometries, which deform differently in response to cellular contractile forces than porous three-dimensional scaffolds typically used in bone tissue engineering studies. Therefore, the goal of this project was to determine the effect of biomaterial modulus on ECM protein expression in three-dimensional foam scaffolds suitable for clinical studies. To accomplish this, a family of segmented polyurethane (PUR) elastomers – with similar chemistries but different mechanical properties – was synthesized and processed into foam scaffolds.

Segmented PURs are a class of polymers composed of alternating macrodiol (soft) and isocyanate (hard) segments, and are synthesized by a two-step process that involves first reacting a macrodiol with an excess of diisocyanate and then chain-extending this prepolymer with a small diamine or diol. Segmented PURs have been of increasing interest in bone tissue engineering applications – both alone [14, 15] and as a composite with ceramic fillers [16, 17]. However, the effects of the mechanical properties of 3D PUR scaffolds on osteoblastic differentiation have not been investigated. Segmented polyurethanes are excellent model systems because the synthesis process permits the tuning of material properties through judicious choice of macrodiol, diisocyanate, and chain-extender [11, 18–20]. In a previous study, we prepared a family of PURs using polycaprolactone (PCL) diols with molecular weights of 1425, 2000, and 2700 Da [21]. Chemical and mechanical analysis indicated that tensile modulus (of dry, annealed films, measured dry at 37 °C) increased from 49 to 278 MPa with increasing PCL molecular weight, and this increase correlated with increases in PCL crystallinity and the PCL melting temperature. However, cell culture of BMSCs on 2D spin-coated, annealed films revealed similar cell responses to the different PURs.

In this study, these three PURs (denoted PCL1425, PCL2000, and PCL2700) were processed into porous foam scaffolds by a process of solvent casting followed by particulate leaching.

Porosity, pore size distribution, crystallinity, and compressive modulus of the PUR scaffolds were determined. Concurrently, control scaffolds were prepared from amorphous poly(D,L-lactic-co-glycolic acid) (PLGA). Next, BMSCs were cultured in the PUR and PLGA scaffolds under osteoinductive conditions for 14 and 21 days. Cell density, prostaglandin E₂ (PGE₂) production, ALP activity, and gene expression were measured to determine how proliferation and expression of osteoblastic ECM proteins depends on scaffold modulus and crystallinity.

2. Materials and methods

2.1. Materials

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and all cell culture materials were obtained from Fisher Scientific (Pittsburgh, PA) unless otherwise specified.

2.2. Scaffold fabrication

A family of segmented polyurethane (PUR) elastomers were synthesized by end-capping 1425, 2000, and 2700 Da PCL diol with 1,4-diisocyanatobutane (BDI), and then chain extending these prepolymers with tyramine-1,4-diisocyanatobutane-tyramine (TyA.BDI.TyA) as previously described [21]. The resultant PURs are hereafter denoted by the molecular weight of the PCL segment: PCL1425, PCL2000, and PCL2700. Next, PUR elastomers were formed into foam scaffolds by the method of solvent casting and particulate leaching [22]. Briefly, 5% (w/v) PUR solutions in dimethylformamide (DMF) were prepared and cast over a sufficient mass of NaCl crystals (sieved 300–500 µm) to create 15 wt% PUR/85 wt% NaCl composites. The DMF was evaporated and the resultant PUR/NaCl composites cut into 3–5 mm pieces. These pieces were then loaded into a cylindrical Teflon-lined mold (12.7 mm internal diameter), heated to 140 °C with a band heater, and compressed axially to 1.2 MPa and held for 10 min. (Previously published data suggests that PURs do not decompose below 180 °C [21].) The cylindrical composites were dried in an oven for 72 h at 60 °C, annealed under vacuum at 80 °C for 24 h, and then cut into 2.5 mm thick discs for cell culture or into 25 mm long rods for mechanical testing. The NaCl was removed by immersion in deionized water for 72 h and the resulting porous foams were soaked in ethanol for 48 h to remove any residual DMF, dried, and stored in a desiccator until use.

Control scaffolds for these studies were prepared from 75:25 poly(D,L-lactic-co-glycolic acid) (PLGA; Lactel Biodegradable Polymers, Birmingham, AL) [22]. Briefly, a 5% solution of PLGA in dichloromethane was cast over NaCl crystals (sieved 300–500 µm) at a ratio of 15 wt% PLGA/85 wt% NaCl, dried, cut into pieces and placed in a Teflon-lined mold. Pieces were compressed at 1.2 MPa at 100 °C for 30 min, and the resultant rods were cut into 2.5 mm discs and leached for 72 h in deionized water. PLGA scaffolds were dried and stored in a desiccator.

For cell culture studies 2.5 mm thick PUR and PLGA discs were sterilized by γ -irradiation (25 kGy), incubated in 2 µg/mL fibronectin (Invitrogen) in phosphate-buffered saline (PBS) for one hour at room temperature, rinsed with PBS, and kept wet until use. (Previous studies

have shown that γ -irradiation of the PUR or the components used to synthesize the PUR at doses as high as 25kGy does not alter the mechanical, physical, or biological properties of the material [23, 24].)

2.3. Scaffold characterization

Porosity and pore size distribution were measured by mercury intrusion porosimetry (Autopore III; Micromeritics Instrument Corp., Norcross, GA) using 3 samples of approximately 0.1 g of each PUR with a mercury-filling pressure of 1.1 kPa and a maximum intrusion pressure of 345 kPa. Pore sizes were calculated from intrusion data assuming an advancing mercury contact angle on PUR of 137°.

Scanning electron microscope (SEM) images of the scaffolds were acquired and visually inspected to identify differences in pore shape, pore interconnectivity, and surface roughness. Briefly, scaffolds were mounted onto SEM studs and sputter-coated with a 5 nm thick layer of palladium as previously described [25]. Images were captured with a LEO 1550 Field Emission SEM (Carl Zeiss SMT, Thornwood, NY).

The compressive moduli of PUR scaffolds were measured using 25 mm long rods with a 2:1 length to diameter ratio. All scaffolds were soaked in PBS overnight at 37 °C and subsequently removed and tested wet (in air) with a MTS 810 (MTS Systems Corporation, Eden Prairie, MN) using a crosshead speed of 1.0 mm/min. Each sample was compressed to 10% strain and then released at the same rate. The compressive modulus was calculated from the slope of the linear elastic region of deformation for $n = 3$ samples per polymer.

Degradation of the PUR scaffolds was measured after determination of the compressive modulus. Samples were dried, cut into pieces averaging approximately 30 mg, and weighed. Pieces were then incubated in PBS at 37 °C for up to 6 months, and 3 pieces were collected every 2 weeks. Each piece was dried completely and re-weighed to determine the fraction of foam remaining. In addition, both number- and weight- average molecular weights were determined by gel permeation chromatography (GPC) using a Waters Alliance GPC 2000 (Waters Corporation, Milford, MA) with DMF as the continuous phase, toluene as an internal standard, and monodisperse polystyrene as the calibration standard.

Differential scanning calorimetry (DSC) was performed to evaluate the level of crystallinity of the processed PUR scaffolds. DSC analysis was conducted on a Q2000 (TA Instruments, New Castle, DE). Samples of porous scaffolds (3.5–5.7 mg) were heated in a nitrogen atmosphere from 20 °C to 120 °C at 10 °C min⁻¹, held at 120 °C for 2 min, and then cooled to -50 °C at 10 °C min⁻¹. The degree of crystallinity was calculated from the size of the endothermic melting peak by the following equation.

$$\% \text{ Crystallinity} = \frac{\frac{\Delta H}{g} \text{ PEUUR} \times \% \text{ PCL content}}{\frac{\Delta H}{g} \text{ 100\% PCL}}$$

Here, theoretical PCL contents of 35, 26 and 20 wt% for PCL1425, PCL2000, and PCL2700, respectively [21], were used.

2.4. Bone marrow stromal cell culture

BMSCs were developed from bone marrow explants harvested from the tibias and femurs of 125–150 g male Sprague-Dawley rats (Harlan, Dublin, VA) in accordance with the Institutional Animal Care and Use Committee at Virginia Tech [2, 26]. Briefly, bone marrow was dispersed in growth medium – consisting of α -MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini, Calabasas, CA) and 1% antibiotic/antimycotic (Invitrogen) – and expanded for approximately 10 days with medium changes every 3 or 4 days. After 10 days the cells were rinsed twice with PBS, lifted with trypsin/EDTA (Invitrogen), split 1:2, and defined as passage 1. The cells were expanded to approximately 90% confluence and split 1:2 two more times. When passage 3 cells were approximately 90% confluent they were lifted, suspended in growth medium at a density of 2×10^6 cells/mL, and seeded into scaffolds by dropwise addition of approximately 1 mL of cell suspension per scaffold. (Here, the estimated void volume of each scaffold was 300 μ L; however, an excess volume was used to ensure uniform seeding [22].) The seeded scaffolds were placed into 12 well plates and allowed to sit in a sterile biosafety cabinet for 1 hour to permit cell attachment prior to the addition of 2 mL growth media and placement into a 5% CO₂ 37 °C incubator. On the following day, denoted as day 0, growth medium was replaced with osteogenic differentiation medium (growth medium supplemented with 0.13 mM ascorbate-2-phosphate, 2 mM β -glycerophosphate, and 10 nM dexamethasone). Thereafter the culture medium was changed twice weekly.

Scaffolds and samples of conditioned culture medium were collected after 14 and 21 days of cell culture. Scaffolds were rinsed twice with PBS, cut into quarters, and the quarters were weighed. To facilitate the extraction of cellular materials, scaffolds were frozen in liquid nitrogen before being crushed and homogenized in the appropriate collection buffers for analysis of cell number, ALP activity, and gene expression.

2.5. Cell number

Cell number was determined after 14 and 21 days using PicoGreen reagent (Invitrogen). Two quarters of each sample were collected in 1 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and sonicated on ice for 10 min to release the DNA. DNA standards of 0.016 to 1 μ g/mL were prepared in TE buffer according to manufacturer's instructions. A volume of 100 μ L of samples and standards was added to each well of 96-well plates in duplicate. PicoGreen reagent was diluted 1:200 in TE buffer and then 100 μ L of the diluted PicoGreen reagent was added to each well. Fluorescence was measured with a SpectraMax fluorescent plate reader (Molecular Devices, Sunnyvale, CA) using excitation and emission frequencies of 488 and 525 nm, respectively, and a linear standard curve was plotted to correlate fluorescence to DNA concentration. Cell number was calculated from the standard curve using a constant of 8.1 pg DNA/cell (determined experimentally) and reported per gram of scaffold.

2.6. ALP activity

After 14 and 21 days of culture, ALP activity was determined using a commercially available kit (Biotron Diagnostics, Hemet, CA). One quarter of each scaffold was used for analysis of ALP activity, and quarters were homogenized in 500 μ L TGT solution (50 mM

Trizma HCL, 100 mM glycine, 0.1 % Triton X100, pH 10.5) containing 1% protease inhibitors (aprotinin, bestatin, leupeptin, E-64, and pepstatin A). A volume of 100 μ L of homogenized sample was combined with 500 μ L ALP reagent and incubated at 37°C for 15 minutes as previously described [27]. After 15 minutes the reaction was stopped with 500 μ L of 0.3 M NaOH and the absorbance of the reaction mixture was measured at 405 nm using a Spectronic Genesys 5 spectrophotometer (Spectronic Analytical Instruments, Leeds, UK). Enzyme activity, defined as the rate of conversion of *p*-nitrophenol phosphate to *p*-nitrophenol, was calculated per gram of scaffold and normalized by cell density.

2.7. mRNA expression

Expression of mRNA for OPN, OC, BSP, VEGF-A, and BMP-2 was determined at 14 and 21 days of culture by quantitative RT-PCR as described previously [21]. Briefly, RNA was isolated from one quarter of each sample using the RNeasy mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Next, 9 μ L of isolated RNA was reverse transcribed to cDNA using the Superscript kit (Invitrogen) and random hexamers as primers. Quantitative RT-PCR was performed using an ABI 7300 sequence detection system (Applied Biosciences, Foster, CA), SYBR green master mix (Applied Biosciences), and specific primers for β -actin (β A), OPN, OC, BSP, VEGF-A and BMP-2 (Integrated Technologies, Coralville, IA). Primer sequences for β A, OPN, OC, BSP and BMP-2 are listed elsewhere [2, 28]. A primer pair for detection of VEGF-A was designed using Primer Express software (ABI) and the NCBI database (Accession number: NM_031836) where the forward and reverse primers are GCT GCA CCC ACG ACA GAA and GGC AAT AGC TGC GCT GGT A respectively. Data was reported as 2^{-C_t} using β A as the internal reference and PLGA scaffolds as the control group [29].

2.8. Statistics

Data were analyzed by ANOVA with a Tukey-Kramer HSD *post-hoc* test and a 95% confidence criterion to test for differences between polymer groups. For cell culture, samples for all time points were prepared in triplicate. The study was then completely repeated with new cells, and the two replicates were combined for a total of $n=6$ samples. For analysis of mRNA data, statistical testing was performed using C_t values. All data is presented as mean $\pm$ standard deviation.

3. Results

3.1. Physical characterization of PUR foams

Low magnification SEM images of PUR and PLGA foam scaffolds revealed similar pore architectures and evidence of high pore interconnectivity for the four different scaffolds (Figure 1). Concurrently, higher magnification images of the pores indicated rougher pore walls – with micron-scale features – for foams prepared from PCL1425 and PCL2000, as compared to smoother walls for foams prepared from PCL2700 (Supplemental Figure 1). Mercury intrusion porosimetry revealed porosities of 78–86% and average pore sizes of 63–78 μ m for the three PUR scaffolds (Table 1). These properties are very similar to those reported for PLGA scaffolds fabricated in a similar manner [22], suggesting that the architectures of the four different scaffolds were similar. (Here, the authors note that

mercury intrusion porosimetry was performed above the T_g for the PURs and consequently deformation of the PUR scaffolds during the intrusion process may have occurred.)

Mechanical testing of foam scaffolds (performed in PBS at 37 °C) indicated that the compressive moduli of the PUR foams systematically increased from 0.18 MPa to 0.80 MPa with increasing PCL content (Table 1). By comparison, the PLGA controls were an order of magnitude stiffer, with a compressive modulus of 6.3 MPa. DSC analysis of PUR foams revealed systematic increases in the PCL melting (Figure 2a) and recrystallization temperatures (Figure 2b) as well as the areas of the melting and recrystallization peaks with increasing PCL diol molecular weight. Degree of crystallinity – calculated from the area of the melting peak (Figure 2a) – is summarized in Table 1.

Degradation of the foams was characterized by both percent mass loss and change in molecular weight with time. Measurement of the dry weights of PCL1425, PCL2000, and PCL2700 indicated decreases of approximately 20%, 21%, and 40%, respectively, in total mass over a 24 week time period (Figure 3a). GPC analysis of the scaffold samples indicated that PCL1425, PCL2000, and PCL2700 lost approximately 30%, 39%, and 29% of their molecular weight over this same period (Figure 3b).

3.2. Effect of PUR foams on BMSC density and markers of osteoblastic differentiation

Foam scaffolds were seeded with BMSCs and cultured for 14 and 21 days to probe for effects of scaffold properties on osteoblastic differentiation and deposition of osteogenic ECM proteins. Analysis of cell number after 14 and 21 days in osteogenic medium revealed a trend among the PUR foam scaffolds in which the one with the lowest PCL content exhibited the highest cell density whereas the one with the highest PCL content had the lowest cell density. Cell density in stiffer PLGA scaffolds was similar to that in the PUR with the intermediate PCL content, suggesting that cell proliferation is not a simple function of scaffold modulus. Finally, increases in cell density from 14 to 21 days were not observed for any polymer.

In osteogenic medium, differentiation of BMSCs is marked by a sequence of events that includes transient ALP activity and expression of collagen, BMP-2, and VEGF-A between 1 and 3 weeks of culture. This is followed by expression of bone ECM proteins such as OC, BSP, and OPN after 2 weeks, and then accumulation of a calcium phosphate mineral after 3 weeks in culture. Analysis of the ALP activity of BMSCs after 14 and 21 days in osteogenic medium indicated very similar enzyme levels (on a per cell basis) for the PCL1425, PCL2000, and PLGA scaffolds (Figure 5) despite differences in cell number. However, ALP activity in the PCL2700 foams was significantly elevated compared to all other experimental groups at both time points. Analysis of gene expression for OC, OPN, BSP, BMP-2 and VEGF-A indicated no statistically significant differences between groups. However, certain trends – with regard to different to the different scaffold materials – were noted. Expression of OPN mRNA (Figure 6a) was similar to ALP activity, with similar levels for cells cultured in PCL1425, PCL2000 and PLGA scaffolds, and elevated expression for cells in PCL2700 scaffolds. In contrast, expression of BSP (Figure 6b) was lower for cells in PCL2700 scaffolds. Finally, mRNA expression of OC (Figure 6c) and BMP-2 (Figure 6d) appeared to decrease systematically with increasing substratum modulus at day 14.

Although ALP activity and OPN expression are markers of osteoblastic differentiation, elevated levels have been observed under conditions of mechanical or chemical stress [30, 31] and have coincided with elevated production of PGE₂. Therefore, conditioned medium was examined for the accumulation of PGE₂ and OPN. Analysis of PGE₂ indicated a statistically significant higher concentration for BMSCs grown in PCL 2700 scaffolds (Figure 7a). However, the OPN concentrations were similar for all PURs, and slightly lower than that for PLGA at both 14 and 21 days.

4. Discussion

The goal of this study was to determine how the modulus and crystallinity of three dimensional porous foam scaffolds affects proliferation and expression of osteoblastic ECM proteins by BMSCs. To this end, PURs with similar compositions but different PCL contents were formed into foam scaffolds. SEM and porosimetry confirmed similar scaffold architectures, while mechanical testing and DSC demonstrated systematic increases in compressive modulus and crystallinity with increasing PCL content. Next, BMSCs were seeded into scaffolds and cultured for up to 21 days in osteogenic medium. Cell density was found to be significantly higher on the softest PUR scaffold, and a trend of decreasing cell density with increasing PCL content was observed. Concurrently, mRNA expression of OC, BSP and BMP-2 appeared to decrease with increasing scaffold modulus at day 14. Finally, ALP activity, expression of OPN, and production of PGE₂ did not vary with scaffold modulus; however, ALP activity and PGE₂ concentration were significantly elevated for cells grown on most crystalline, PCL2700 scaffolds.

Previous investigations into the effect of substratum stiffness on expression of the osteoblastic phenotype have utilized hydrogels, where the stiffness is controlled by the degree of chemical cross-linking [4, 6, 7] or by the size of the crosslinking group [5]. This approach has yielded polyacrylamide substrates with moduli of 0.1 – 39 kPa [4] and the PEG substrates with moduli of 14 – 424 kPa [6, 7] and 64–507 MPa [5]. Analysis of osteoblastic differentiation of BMSCs on polyacrylamide revealed increases in OC and OPN mRNA expression with increasing modulus, and maximal expression of BMP-2 on soft (1 kPa) gels [4]. Concurrently, analysis of MC3T3-E1 on soft PEG hydrogels indicated a systematic increase in ALP activity with increasing modulus [6], while analysis of MG63 cells on stiff PEG hydrogels revealed maximal ALP activity at an intermediate modulus (311 MPa) and maximal OC expression on the stiffest substrate (507 MPa) [5]. In contrast to those previous studies, the results presented here for BMSC behavior in PUR and PLGA foam scaffolds – with moduli of 0.18 – 6.3 MPa –revealed different trends. ALP activity (Figure 5) and OPN expression (Figure 6a) did not vary systematically with scaffold modulus, while OC expression decreased with increasing modulus at day 14 (Figure 6b). The only noted similarity in trends was the decrease in BMP-2 expression with increasing modulus at day 14 (Figure 6d), which matched the trend for BMP-2 expression at day 7 reported by Engler *et al* [4]. However, this comparison should not be over-interpreted as the experimental conditions for this study were considerably different from those used by Engler *et al*. One important difference is that in this study the modulus of the PUR foams was varied by changing the molecular weight of the PCL diol. This approach, though, may have affected the surface chemistry, crystallinity and roughness of the PUR foam scaffolds.

Previous studies have demonstrated that biomaterial surface chemistry, crystallinity and roughness can influence osteoblast proliferation and differentiation. With respect to surface chemistry, model studies have demonstrated that the rates of initial osteoblast attachment and subsequent cell proliferation are slower on hydrophobic surfaces [32, 33]. In part, this may be due to differences in the kinetics of ECM protein adsorption and the conformations that adsorbed proteins adopt on hydrophobic and hydrophilic interfaces [34, 35]. Additionally, studies with a variety of cell types have suggested that surfaces presenting amine [36] and carboxylic acid groups [9, 36, 37] promote better adhesion and proliferation of cells than those presenting methyl groups. For osteoblasts in particular, surfaces presenting phosphate and sulfate groups have been shown to enhance alkaline phosphatase (ALP) activity and mineral deposition [8]. For the study presented here, the PUR biomaterials all presented similarly hydrophobic surfaces with advancing contact angles between 74 and 83° [21]. We have previously reported that adsorption of fibronectin was similar on lysine diisocyanate/polyester triol (300 – 3000 g/mol) PUR networks with contact angles ranging from 65 to 75° [38]. Together, these observations suggest that differences in surface chemistry may not have been significant enough to affect cell adhesion.

Surface roughness and crystallinity of a material surface also influence osteoblast cell behavior. Czarnecki *et al.* examined carbon fiber substrates and found that osteoblast density increased with decreasing substrate crystallinity [39]. In addition, Webster *et al.* [12] found osteoblast adhesion increased with decreasing grain size of hydroxyapatite, alumina, and titania materials. Separately, Lim *et al.* used phase demixing of amorphous polymer films to show that cell spreading, cell proliferation and ALP activity all increase as the size of surface features decreases [40]. Finally, the research group of Boyan *et al.* employed acid-etching, sand-blasting, and micropatterning of titanium to demonstrate that ALP activity systematically increases with decreasing feature size, while the synthesis of PGE₂ and transforming growth factor β_1 (TGF- β_1) systematically decrease [10, 13, 41]. In the study presented here, elevated PGE₂ (Figure 7a) and diminished cell densities (Figure 4) were measured for cells grown within PCL2700, which would be consistent with an increased size of surface features. However, high-resolution SEM images suggested that the PCL2700 foams had smoother pore walls than those formed from PCL1425 and PCL2000 (Supplemental Figure 1). In addition, ALP activity – which has been shown to be suppressed by roughness [40, 41] – was enhanced in the PCL2700 scaffolds (Figure 5).

The elevated levels of PGE₂, ALP activity, and OPN observed with the PCL2700 materials have been reported previously for MC3T3-E1 and BMSCs, and may be indicative of chemical or mechanical trauma. Popp *et al.* [30] reported that the incorporation of zinc-stabilized amorphous calcium phosphate microparticles within PLGA scaffolds resulted in elevated ALP activity, PGE₂ accumulation, and increased mRNA expression of OPN and VEGF-A by MC3T3-E1 cells. Concurrently, they found that the microparticles suppressed mRNA expression of BSP, OC, BMP-2 and BMP-4, relative to PLGA scaffolds without microparticles. Similarly, Kavlock and Goldstein [31] showed that perfusion culture of BMSCs within PLGA foam scaffolds suppressed OC expression and increased PGE₂ and OPN synthesis, ALP activity, and expression of OPN relative to static culture. Thus, the elevated levels of PGE₂ and OPN – which are not specific markers of osteoblastic differentiation – may be indicative of cell stress, while the diminished cell density and

elevated ALP activity could be associated with decreased cell viability in the PCL2700 foam scaffolds. Two factors that may have contributed to these effects are residual solvent and byproducts of gamma irradiation. With regard to solvent, a sequence of steps, including drying, annealing, and leaching in ethanol, were undertaken to remove DMF from the PUR scaffolds. Nevertheless, residual solvent may have been present. With regard to gamma irradiation, we have previously shown that irradiation of either the reactive components of the PUR [23] or the cured PUR [24] at doses as high as 25 kGy does not affect their physical, mechanical, or biological properties. However, those studies involved different isocyanates and chain extenders than those used in this study.

Lastly, analysis of PUR degradation revealed two key features. First, the loss of scaffold mass appeared to increase systematically with increasing PCL content (Figure 3a). This observation is consistent with that of Guan *et al.* for polyurethanes synthesized from PCL, BDI and lysine ethyl ester [42], but contrasts with the findings of Skarja and Woodhouse, who reported a more rapid mass loss as the molecular weight of PCL segment is decreased [43]. However, this difference might be related to the choice of diisocyanate: Skarja and Woodhouse used the branched molecule lysine diisocyanate, whereas the aliphatic molecule BDI was used in this study and that of Guan *et al.* [42]. We recently investigated the effects of esterolytic enzymes and reactive oxygen species (ROS) secreted by macrophages on the degradation of PUR networks synthesized from either lysine triisocyanate (LTI) or HDI trimer (an aliphatic polyisocyanate synthesized from HDI) [44]. While LTI-derived PUR networks degraded six times faster in oxidative medium, HDI trimer-derived polymers did not degrade substantially faster in either esterolytic or oxidative medium compared to PBS, which underscores the effects of the polyisocyanate on polymer degradation. Second, although the molecular weight of the PCL2000 initially decreased more rapidly than that of the other PURs (Figure 3b), during the period between 5 and 24 weeks the three PURs appeared to degrade at similar rates. This is consistent with the degradation of polyurethanes synthesized from PCL, BDI and the aliphatic chain extender putrescine [42]. However, molecular weight has been shown to decrease more rapidly as the PCL segment size is reduced when either the chain extender [42] or diisocyanate [43] has a pendant group. These pendant groups may interfere with the formation crystalline PCL domains, as it has been observed in degradation studies of pure PCL that degradation of amorphous regions occurs quickly, while crystalline regions degrade very slowly [45].

5. Conclusions

A series of segmented, degradable polyurethane elastomers were processed into porous foam scaffolds with varying mechanical properties and the capacity of each material to induce osteoblastic differentiation of bone marrow stromal cells was measured. BMSCs exhibited increased ALP activity, OPN mRNA expression, and PGE₂ synthesis on the PCL2700 scaffolds as compared with the less stiff PCL1425 and PCL2000 scaffolds and the less crystalline PLGA scaffolds. While gene expression was not significantly affected, analysis of BMP-2 and OC gene expression at day 14 suggests that BMSCs differentiate faster on softer surfaces.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. SEM images of pores within foam scaffolds at $100\times$ magnification (a) PCL1425, (b) PCL2000, (c) PCL2700, and (d) PLGA. The scale bars correspond to $200\text{ }\mu\text{m}$.

Figure 2. DSC analysis of processed PUR foam scaffolds: (a) first heating curve and (b) cooling curve. Curves are offset vertically to permit visual comparison.

Figure 3. Degradation profile of PURs. Degradation of porous foam scaffolds reported as (a) percent mass remaining, and (b) weight-average molecular weight determined by GPC analysis. The lines are intended to lead the eye.

Figure 4.

Cell number in PUR and PLGA porous foams at 14 and 21 days. Data are mean $\pm$ SEM for $n = 6$ samples. Columns are arranged in order of increasing scaffold modulus. The dashed line indicates the seeding density (approximately 6×10^5 cells). An asterisk denotes a statistically significant difference in cell number with respect to PCL2700 at the same time point ($p < 0.05$).

Figure 5. ALP activity of BMSCs in PUR and PLGA foam scaffolds at 14 and 21 days. All data is normalized to PLGA foams at day 14. Data are mean $\pm$ standard deviation for $n = 6$ samples. The asterisk denotes statistically different level of ALP activity with respect to PCL2700 at the same time point ($p < 0.05$).

Figure 6.

(a) OPN mRNA expression, (b) BSP mRNA expression, (c) OC mRNA expression, (d) BMP-2 mRNA expression, and (e) VEGF-A mRNA expression of BMSCs in PUR and PLGA foam scaffolds at 14 and 21 days. β -actin was used as the housekeeping gene and data are normalized to PLGA foams at day 14. Data are mean $\pm$ standard deviation for n = 6 samples.

Figure 7.

Concentrations of (a) PGE₂ and (b) OPN in conditioned medium at days 14 and 21. Data are mean $\pm$ standard deviation for n = 6 samples for PGE₂ and n = 3 samples for OPN. An asterisk denotes a statistical difference relative to PCL2700 at the same time point (p<0.05).

Table 1

Properties of PUR foams. Percent porosity and average pore size were determined by mercury porosimetry. Compressive modulus of porous foams was determined from stress strain curves.

Polymer	Theoretical PCL Content (wt%)	Crystallinity (%)	Porosity (%)	Average Pore Size (μm)	Compressive Modulus (MPa)
PCL 1425	65	16.0	78.8 ± 3.8	77.6 ± 11.6	0.18 ± 0.07
PCL 2000	74	25.2	86.0 ± 2.4	70.2 ± 7.0	0.38 ± 0.11
PCL 2700	80	45.3	77.6 ± 3.1	63.2 ± 0.5	0.80 ± 0.40
PLGA	-	-	78.8	70	6.33 ± 2.88

* Theoretical PCL content data were taken from Kavlock *et al.* [21].

PLGA properties were taken from Goldstein *et al.* [27].