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Synthesis and Characterization of Poly(caprolactone triol succinate) Elastomer for Tissue Engineering Application

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ABSTRACT: A novel polymer poly(caprolactone triol succinate) (PPCLSu) was synthesized from monomers polycaprolactone triol and succinic acid by direct polycondensation. The tensile strength of PPCLSu was found to be 0.33 ± 0.03 MPa with an elongation of $47.8 \pm 1.9\%$. These elastomers lost about 7% of their original mass in an *in vitro* degradation study conducted in phosphate-buffered saline (PBS) at 37°C up to 10 weeks. Three-dimensional (3D) porous scaffolds were created by a porogen-leaching method and these constructs were evaluated for primary rat osteoblast (PRO) proliferation and phenotype development *in vitro*. This elastomer promoted primary rat osteoblast adhesion, proliferation and increased expression of alkaline phosphatase, an early marker of osteoblastic phenotype. These preliminary results suggest that PPCLSu may be a good candidate material for scaffolding applications in tissue regeneration. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 130: 3770–3777, 2013

KEYWORDS: biodegradable; polycondensation; biomedical applications; biomaterials; elastomers

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INTRODUCTION

In the past decade, much advancement has been made in the tissue engineering field fueled by the application of traditional biomaterials, and by the development of novel biomaterials. For example, elastic tendon,[1] blood vessel,[2] fibrocartilage,[3] functional islets,[4] bone,[5] and ligament[6] have been engineered using poly(lactic acid) (PLA), poly(glycolide) (PGA) and their copolymer poly(lactide-*co*-glycolide) (PLGA). In recent years, polyester elastomers derived from poly(acids) and poly(ols) by polycondensation including poly(glycerol sebacic acid) (PGS),[7–9] poly(-diol citrate),[10] poly(polyol sebacate) (PPS),[11] and poly(glycerol-dodecanoate) (PGD)[12] have become attractive for a variety of biomedical applications. This material platform allows for the synthesis of novel, biocompatible elastomer materials with controlled mechanical and degradation properties at low costs. These elastomers have been developed for blood vessel,[13,14] cartilage,[15] and heart muscle tissue engineering.[16] These flexible materials have also been successfully applied for bone tissue engineering.[17] The mechanobiology of bone and cartilage tissue has revealed that cells are highly responsive to mechanical stimuli, for example, tissue strain and shear stress is, associated with cell deformation. These stimuli may excite different signaling pathways related to the growth and remodeling of both bone and cartilage.[18] Cytoskeletal modification allows bone and cartilage to adapt to novel mechanical environments.[19] Cell deformation is believed to regulate proliferation, differentiation and extracellular matrix synthesis.[20] Currently, scaffolds amenable to dynamic mechanical environments are collagen constructs such as collagen hydrogels,[20,21] and collagen-GAG scaffolds.[22,23] Polycondensation-derived polyester elastomers are among the most suitable candidate biomaterial for exerting deformation on skeletal cells. It is thus desirable to evaluate their application in bone and cartilage tissue engineering.

Polycaprolactone (PCL) has been widely used for a variety of biomedical applications including scaffolds for cartilage,[24] and bone[25] because of its excellent biocompatibility and ease of processing. Poly(butylene succinate), a polycondensation derived polymer, has been successfully applied for bone tissue engineering.[26] Polycaprolactone triol is a modified PCL that has been used to design biodegradable block copolymers either by polycondensation[27] or reaction with diisocyanate.[28] Succinic acid, or butanedioic acid, is one of the intermediate products of the Krebs cycle, and is currently in widespread use in the food and beverage industry. We hypothesized that a polycondensation

polymer obtained using polycaprolactone triol and succinic acid may yield a biocompatible and useful elastomer material for tissue engineering. The purpose of this study is to synthesize and characterize poly(caprolactone triol succinate) for its structure, tensile properties, degradation and human osteoblast compatibility and establish its suitability for tissue engineering applications.

MATERIALS AND METHODS

Materials

PCL triol ($\bar{M}n$=300), succinic acid and sodium chloride crystals were procured from Sigma-Aldrich (St. Louis, MO). Gluteraldehyde and 10% formalin in phosphate buffered saline were purchased from Fisher Scientific (Fair Lawn, NJ). BioRad alkaline phosphatase (ALP) substrate kit (Hercules, CA) and Invitrogen Quant-iT PicoGreen dsDNA assay kit (Eugene, OR) were used in this study. Cell culture media was prepared by mixing Ham's F-12 media supplemented with 12% FBS, 1% penicillin–streptomycin (PS) (Invitrogen, Greens Island, NJ), 3 m*M* of β-glycerophosphate (Sigma-Aldrich, St. Louis, MO), and 10 μg/mL of ascorbic acid. Media was replenished every 2 days (Invitrogen, Greens Island, NJ). RNAse and DNAse free water and 7.4 pH PBS buffer used were purchased from Invitrogen (Greens Island, NJ).

Synthesis of Poly(polycaprolactone triol succinate)

The synthetic protocol for PPCLSu polycondensation is presented in Scheme 1. In brief, PCL triol and succinic acid were mixed at 1:1 molar ratio and charged into a 100 mL single-neck round-bottom flask. This flask was immersed in an oil bath maintained at 160°C and contents were stirred with the help of a magnetic bar for one hour. The resulting viscous pre-polymer was poured into a teflon mold and post-polymerized in an oven at 120°C for 6 days to obtain PPCLSu polymer.

3D Porous Scaffold Fabrication

The PPCLSu prepolymer was mixed with 15 wt % (dry weight of monomers) of sodium chloride (700–1000 μm) crystals and poured into a Teflon mold for post-polymerization in an oven held at 120°C. Following complete polymerization, scaffolds were repeatedly washed with deionized water to remove salts. These porous scaffolds were dried overnight in vacuum and kept desiccated until further use.

Attenuated Total Reflectance FTIR Spectroscopy

FTIR spectra on thin PPCLSu films were recorded using a Thermo Scientific Smart FTIR at room temperature in ATR mode. The PPCLSu film was kept in direct contact with the probe and spectra was scanned in the range of 4000–500 cm^{-1}.

Tensile Properties of PPCLSu Film

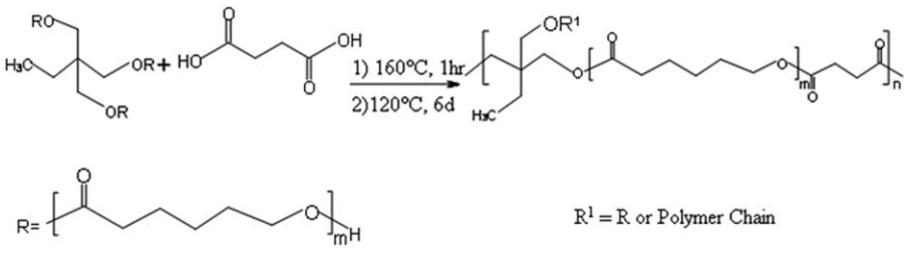
Films of PPCLSu having thickness of 1.2 ± 0.1 mm thick were cut into dogbone shapes with a length to breadth measurement of 2:1 as per the ASTM standard. In brief, samples measuring 25 × 12.5 mm (n = 6) were pulled at a rate of 10 mm/min using an Instron® tester (model 5544; Instron, Canton, MA) equipped with a 500*N* load cell until sample failure. From the load and displacement values a stress-strain curve was constructed. This curve was used to calculate tensile strength, percent elongation, and modulus.

Field Emission Scanning Electron Microscopy

Surface morphology of the thin films and 3D porous scaffolds were characterized by using JEOL 6335F FESEM operated at an accelerating voltage of 2 kV at various magnifications. Scaffold surfaces were sputter coated with Au/Pd using a Hummer V sputtering system (Technics Inc., Baltimore, MD) prior to imaging. Scaffold pore sizes were measured using Image J, NIH software. For each fiber diameter measurement, three different samples at three different locations were considered (average of about 100 fibers). FESEM was also used to qualitatively characterize cell adhesion, morphology, and proliferation. In brief, at various time points cellular constructs were harvested and fixed with 2% glutaraldehyde overnight. Fixed samples were dehydrated through a series of ethanol gradients and kept desiccated. Dried samples were sputter coated with Au/Pd prior to imaging.

In Vitro Degradation

Two sets of circular PPCLSu discs (10 mm in diameter, 1.2 ± 0.1 mm thick) (n = 6) were placed in 15 mL polypropylene tubes containing either 10 mL phosphate buffer saline (PBS, pH 7.4) or DI water up to 10 weeks under agitation at 100 rpm and 37°C to study degradation profiles. Degradation studies in water were included to study the change in the pH of the degradation media because of polymer hydrolysis in non-buffered media.[29] Every week a fresh 10 mL of PBS was added to the samples undergoing degradation in PBS while the water remained unchanged for the whole process. Every week changes in the degradation media pH were recorded using a Fisher Scientific AB15 pH meter. At each week degradation samples were isolated (n = 6) and washed repeatedly with DI water and completely dried. The dried samples were weighed (W_t) using a digital Mettler Toledo analytical balance. The percent mass loss was calculated using the equation percent mass loss = (W_t–W_0)/



Scheme 1. Polycondensation protocol for the synthesis of poly(caprolactone triol succinate) elastomer. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

$W_0 \times 100$, where W_0 is mass prior to degradation. Optical and SEM micrographs were used to grossly characterize surface morphology following degradation.

Isolation of Primary Rat Osteoblasts

Primary rat osteoblasts were isolated from 2 to 3-day old neonatal Sprague–Dawley rat calvaria.[30] The cell isolation protocol was approved by the University of Connecticut Health Center, Animal Care and Use Committee. Under sterile conditions soft tissue attached to the calvaria were removed by gently scraping and washing with PBS using 1% penicillin/streptomycin. Leftover calvarial tissue was minced and incubated with collagenase and trypsin digestion solution for 45 min. The cell suspension was filtered and the first digestion was discarded to minimize fibroblast populations. The supernatant from the next three digestions were collected and diluted in excess of cell culture media. The entire contents were centrifuged at 1500 rpm for 10 min. The obtained cell pellet was resuspended in a fresh cell culture media and plated in a T-25 flask. Cells at passage 3 were used for seeding scaffolds. The cell culture media comprised of Ham's F-12 media supplemented with 12 % FBS and 1 % PS.

In Vitro PRO Culture on PPCLSu 3D Porous Scaffolds

PPCLSu porous samples were cut into circular discs measuring ~8.6 mm in diameter. These discs were sterilized by immersing in 70% ethanol for 30 min inside the cell hood and exposed to UV radiation for 15 min on each side. Scaffolds were placed in 48 well plates and seeded with 60,000 PRO on each scaffold. Cell-seeded scaffolds were incubated for 3 h to promote cell adhesion prior to adding 1 mL cell culture media. Osteogenic media comprised of Ham's F-12 media supplemented with 12% FBS, 1% PS, 3 m*M* of β-glycerophosphate, and 10 μg/mL of ascorbic acid was used throughout the study with media replenished every 2 days. The culture was maintained for 7, 14, and 21 days in an incubator at 37°C, 5% CO_2, and 95% humidified air.

Cell Proliferation (PicoGreenDNA Assay)

PRO proliferation in osteogenic media at different culture times of 7, 14, and 21 days was quantified by measuring the amount of cellular DNA content using PicoGreen® dsDNA assay.[30,31] The cellular constructs were washed twice with PBS, transferred to new well plates and 1 mL of 1% Triton X-100 solution was added to lyse the cells. The well plates underwent three freeze-thaw cycles and the contents were thoroughly mixed with the aid of a pipette to extract cell lysate. One hundred and twenty-five microliter of sample DNA was transferred into a new well plate to which 375 μL component B and 500 μL Component A were added. Well plates were covered with aluminum foil to prevent light exposure and incubated for 5 min. A BioTek plate reader was used to measure fluorescence (485 nm/535 nm). Optical readings were converted to DNA concentration using a standard curve.[32]

Alkaline Phosphatase Activity

ALP, an early osteoblast phenotypic marker, expression by PRO cultured on scaffolds at 7, 14, and 21 days was evaluated using an ALP substrate kit.[33] In brief, a 100 μL of cell lysate was transferred into a well plate to which 400 μL of P-NPP (*para*-nitro phenol phosphate) substrate and buffer solution were added and incubated at 37°C for 30 min. After 30 min, 500 μL of 0.4 *N* of sodium hydroxide was added to stop the reaction. The intensity of the color produced though the reaction is proportional to ALP activity. The optical density of the solution was measured at 405 nm using a BioTek plate reader. The results for ALP activity optical density were normalized to DNA content determined in a companion DNA assay.[32]

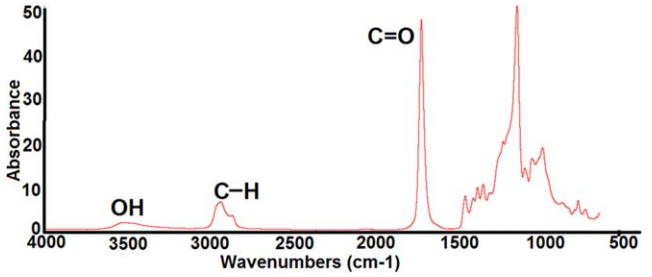


Figure 1. FTIR spectrum of poly(polycaprolactone triol succinate) thin film obtained by condensation polymerization. The characteristic frequency bands present at ~1728, ~2939, and ~3500 cm^{-1} confirms the proposed polymer structure in Scheme 1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Statistical Analysis

In vitro cellular characterization used a sample size of $n = 4$ for all the characterization while $n = 6$ were used for mechanical testing. The quantitative data were reported in the form of mean ± standard deviation. Statistical analysis was performed using a one-way analysis of variance (ANOVA) using Tukey test to determine the statistical significance between the two means evaluated at $p < 0.05$.

RESULTS AND DISCUSSION

Synthesis and Characterization of PPCLSu

The ATR-FTIR spectra of PPCLSu obtained by polycondensation of poly(ε-caprolactone) triol and succinic acid is presented in Figure 1. The characteristic bands present at ~1728 and ~2939 cm^{-1} were assigned to carbonyl groups and aliphatic C-H stretching frequencies present on the anhydride type polymer.

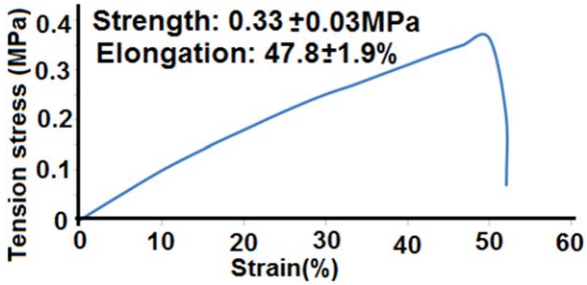


Figure 2. A representative stress–strain curve obtained for the dogbone-shaped PPCLSu thin sheet under tensile testing. The modulus, found by the slope of the linear increase, was found to be approximately 7.3 kPa. These tensile properties are in the range of a variety of human soft tissues. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

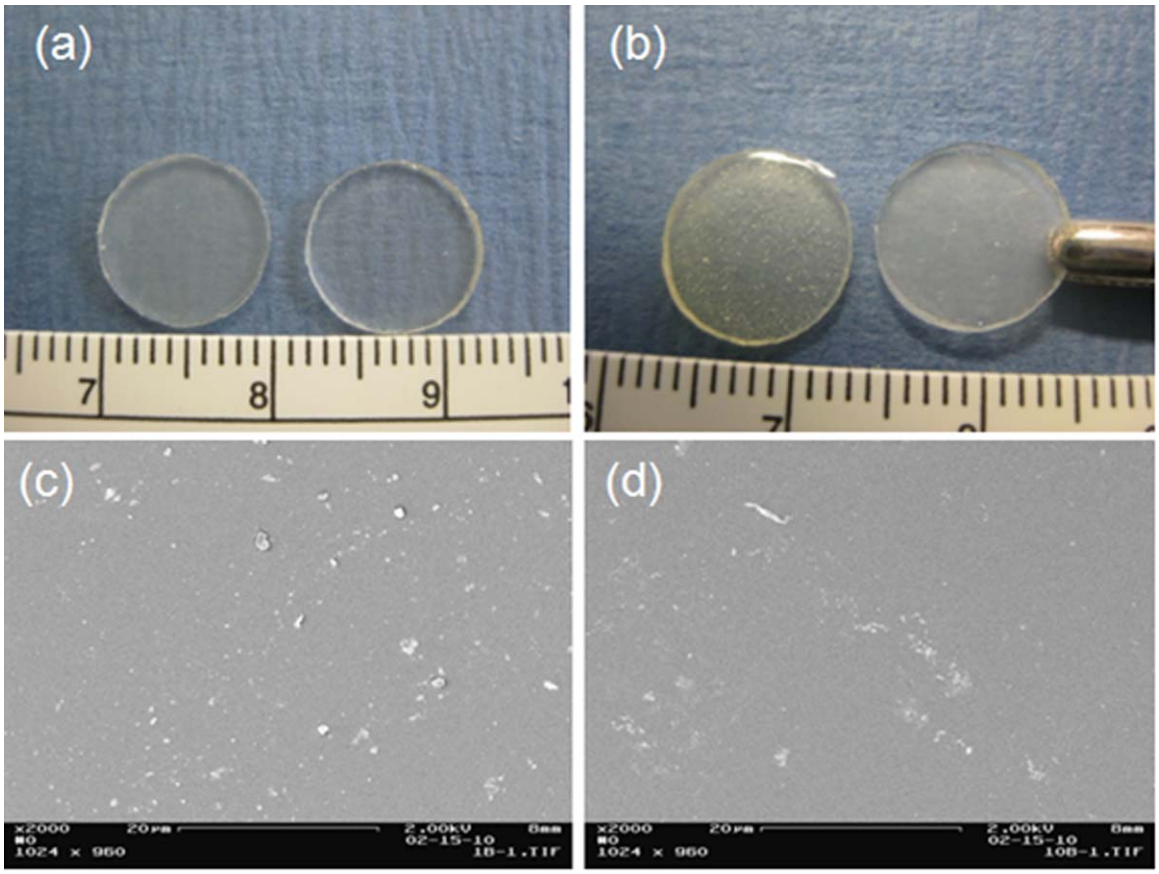


Figure 3. Representative optical micrographs of PPCLSu elastomer showing the gross thin film morphology following degradation both in PBS and water. The circular film presented first was incubated in PBS while the next one was in water. The morphology of the samples at week 1 (a) and week 10 (b) did not show any noticeable changes in the gross morphology as evidenced through high magnification SEM images (c and d). PPCLSu samples incubated in PBS turned yellow while samples in water became "sticky" following degradation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The broad band ~3500 cm^{-1} corresponds to OH stretching involving hydrogen bonding.

The stress-stain behavior of PPCLSu thin films tested under tension is presented in Figure 2. These elastic materials have a tensile strength of 0.33 ± 0.03 MPa, which is comparable to soft tissues such as human bladder, pig bladder and rat abdominal aorta.[30] The modulus, calculated from the slope of the stress versus strain curve, was found to be 7.3 KPa. This modulus is

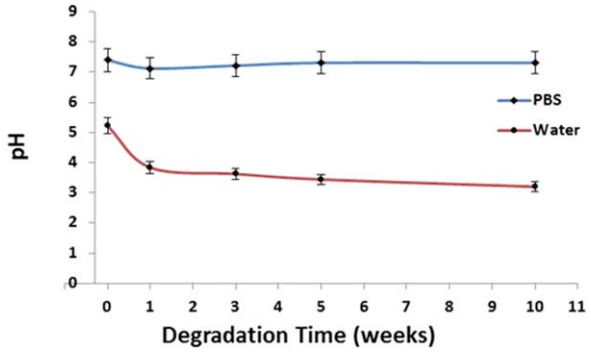


Figure 4. Changes in the pH profile of the degradation media following PPCLSu *in vitro* degradation both in DI water and PBS at 37°C up to 10 weeks. The initial pH drop is due to leaching of residual succinate or low molecular weight oligomers resulting from polycondensation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

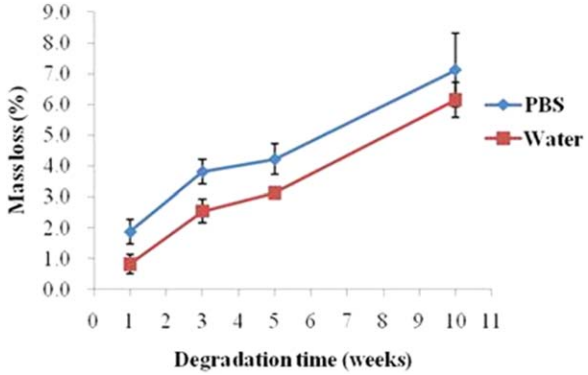


Figure 5. Weight loss profile of PPCLSu thin films following *in vitro* degradation in PBS and water at 37°C up to 10 weeks. These polymers underwent hydrolysis and *in vitro* weight loss was slow. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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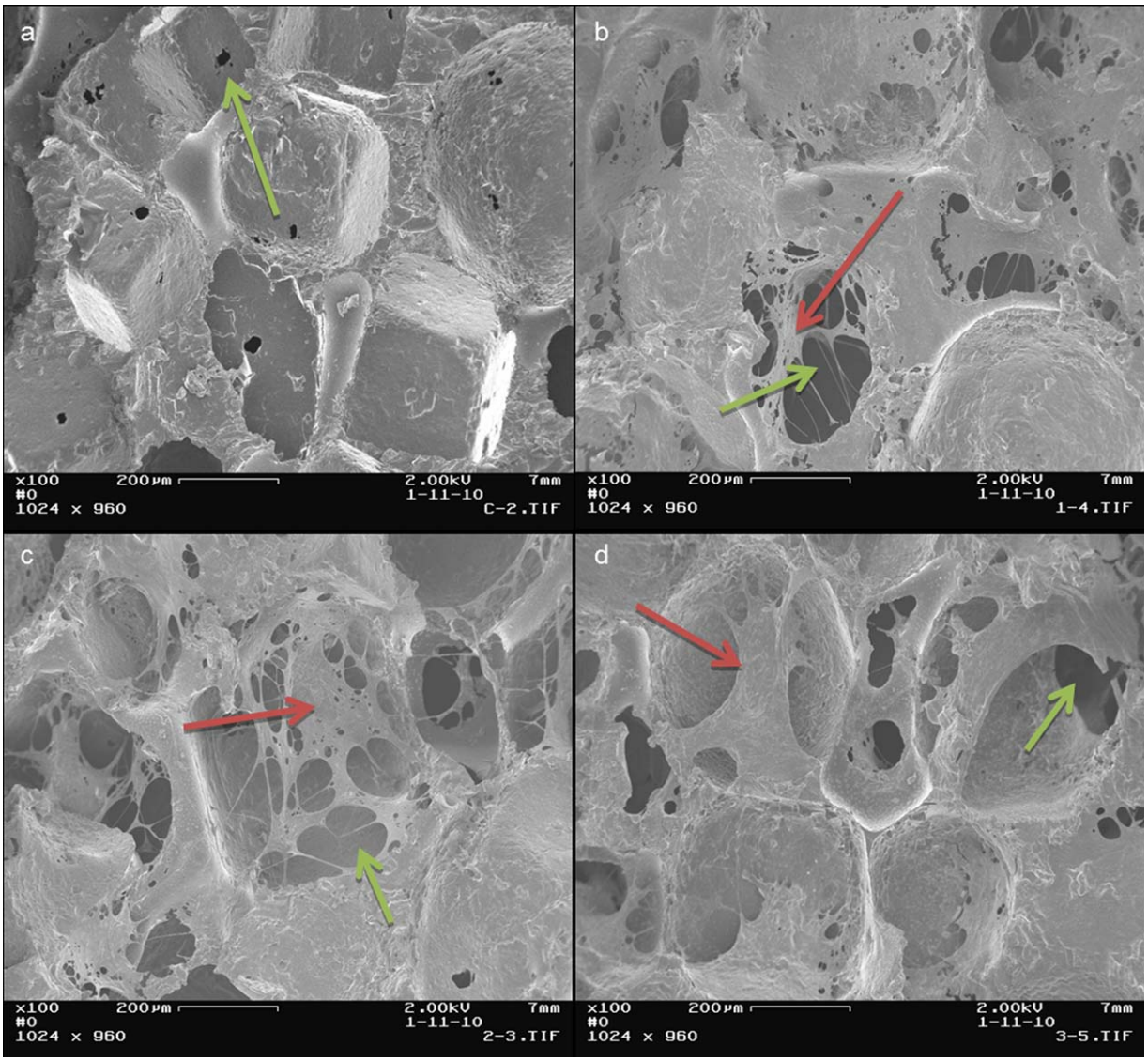

Figure 6. Representative SEM micrographs illustrating the PRO morphology (attachment and proliferation) on the 3D porous PPCLSu scaffold at different culture points of (b) 1, (c) 2, and (d) 3 weeks where (a) is the control scaffold morphology without any cells. Well spread PRO morphology and progressive cell growth with culture time is evident. (Green arrows indicate pores within the scaffold created by salt leaching technique and red arrows indicate PRO exhibiting spread morphology within the pores.) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

well within the range of a number of soft tissues. The samples have an elongation of 47.8 ± 1.9%, which is similar to porcine aortic heart valve (circumferential) and collagen fibers.[34] A number of researchers have demonstrated that the mechanical properties of scaffolds can be enhanced significantly following cell seeding and growth. For example, the compressive modulus of poly(1,8-octanediol citrate)-chondrocyte constructs cultured *in vitro* for 28 days was significantly higher than the control scaffold.[15] In addition, our laboratory has previously reported increased mechanical strength following cell culture possibly because of the increased deposition of ECM by cultured cells.[35]

The surface morphology of the PPCLSu following 10 weeks of degradation is presented in Figure 3. Samples subjected to degradation in PBS developed a translucent yellow color and became more opaque following 10 weeks. In contrast, samples in water remained transparent over the degradation period and developed an adhesive surface texture after 10 weeks of degradation. These variations in polymer properties are suggestive of PPCLSu hydrolysis both in PBS and DI water. The SEM images presented in Figure C and D did not show any noticeable changes in the gross surface morphology of the film and the surface remained smooth throughout degradation time of 10 weeks both in PBS and water. Previous studies concerning the degradation of PCL have shown a minimal change in morphology.[36] Over a 12-month period, PCL degraded slowly resulting in large surface pore formation. Degradation of succinic acid-based copolymers proceeds fairly quickly and is catalyzed by hydrolysis. The increased susceptibility of succinic acid to

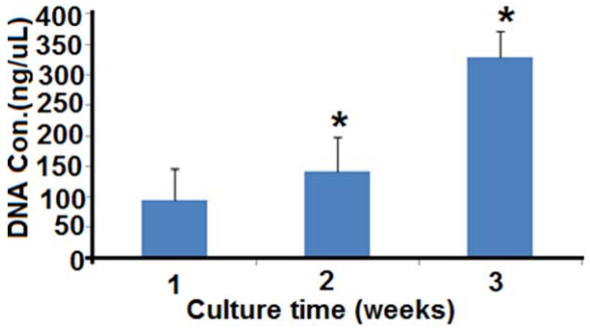

Figure 7. Proliferation of PRO seeded on PPCLSu scaffolds in osteogenic media. At each time point triplicate samples were measured for DNA content and comparisons were made within the group. *Indicates the statistical significance within the group at $p < 0.05$. Cell proliferation was significantly higher at weeks 2 and 3 as compared to week 1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

hydrolysis enables control of PPCLSu degradation properties. This extremely versatile characteristic of the elastomer occurs mainly through a chain scission mechanism.

The variations in the pH following PPCLSu degradation up to 10 weeks is presented in Figure 4. As depicted in the graph, the pH values of the samples in PBS at 7.4 dropped to 7.1 at week 1 and then gradually increased to a pH of 7.3. The initial pH drop may be because of the leaching of residual succinic acid or low molecular weight prepolymer into the degradation media. The drop in pH at 1 week when incubated in PBS was not statistically significant. Similar observations were also made with samples subjected to degradation in DI water at pH 5.2, which dropped to 3.8 with significance at week 1. The cumulative pH changes of the water samples over 10 weeks dropped to a pH of 3.2. The water degradation media was not changed throughout the 10-week study.

The weight loss profile for the PPCLSu water and PBS over 10 weeks is presented in Figure 5. In general, these polymers lost about 6–7% of their original mass without any visual changes in their surface morphology. The samples in PBS appeared to lose more weight than the samples in water over 10 days. The PBS degradation media was replaced with fresh PBS each week throughout the 10-week degradation period. Because of media replacement, the PBS degradation environment may have assisted the diffusion of degradation byproducts and could account for increased sample weight loss. Because the water degradation studies were conducted without replacement of media, the possible saturation of the media with degradation byproducts may have slowed diffusion and mass loss. This accumulation of the degradation byproducts in the water media may have decreased the pH of the media while hastening the polymer degradation rate. The adhesive nature of the polymer surface after degradation may be indicative of this enhanced degradation. Furthermore, these polyester polymers tend to degrade much faster in the presence of an acidic environment, as their degradation is acid catalyzed.[37] These observations are consistent with earlier reports where poly(glycerol sebacic acid) lost $17 \pm 6\%$ weight in 60 days *in vitro* in PBS.[7] Interestingly the same polymer underwent complete degradation *in vivo* within 60 days.[7] Such an observed rate of degradation was attributed to the possible involvement of biological enzymes and macrophage activity in polymer hydrolysis.[7] The PPCLSu degradation rate can be altered by using the differing molecular weight PCL triols and fabricating scaffolds of varying porosity for *in vivo* tissue healing applications. Further *in vivo* degradation studies will provide a detailed understanding of polymer degradation rates. Such studies are currently underway.

In Vitro Cell Culture

Primary rat osteoblast morphology and spreading is presented in Figure 6. It is evident from the SEM micrographs that PROs attached to the PPCLSu porous scaffolds and show a well spread morphology extending to the edges of the pores (red arrows). Cells exhibited progressive growth from weeks 1 to 3, identified by an increased dsDNA content and area of coverage on the scaffold surface. In general, cell attachment onto a scaffold, its proliferation and differentiation into a particular phenotype are governed by a balance of scaffold characteristics such as material chemistry, hydrophilicity/hydrophobicity, softness/hardness, topography, and charge.[38] The presence of hydroxyl groups and porous structures on PPCLSu provided a suitable environment for cell attachment and spreading. The salt leached PPCLSu scaffolds have an average pore diameter of 210 ± 15 μm (green arrows). This pore diameter has been previously established to be beneficial for cell migration.[32,39] Smaller pore sizes inhibit media diffusion and infiltration of cells into the scaffold and may lead to a necrotic core. The quantitative cell proliferation rate obtained by measuring DNA concentration is presented in Figure 7. A significantly higher number of cells was present on the scaffold at weeks 2 and 3. These results are consistent with microscopic observations presented in Figure 6 where increased cell spreading and increasing cell density was observed. Alkaline phophatase, the early mature osteoblast phenotype marker expression by PRO cultured on salt leached 3D PPCLSu is presented in Figure 8. Increasing amounts of ALP expression were observed with an increase in culture time. These scaffolds supported PRO adhesion,

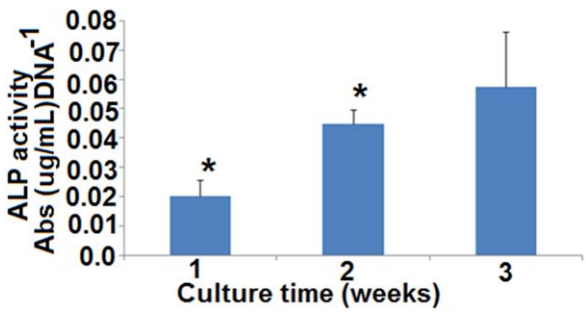

Figure 8. Alkaline phosphatase activity expressed by PRO cultured on PPCLSu porous scaffolds in osteogenic media. At each time point triplicate samples were measured and comparisons were made within the group. *Indicates the statistical significance within the group at $p < 0.05$. These scaffolds supported the PRO maturation by expressing higher level of ALP expression with increased culture time. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

proliferation and mature osteoblast phenotype expression *in vitro*. The observed *in vitro* cell proliferation and differentiation trends are consistent with earlier reported PCL-based scaffolds.[40,41]

CONCLUSIONS

The focus of this preliminary study was to establish the synthesis and characterization of poly(caprolactone triol succinate) as a scaffolding material for tissue engineering applications. We have successfully synthesized and characterized PPCLSu for its structure, mechanical stability, degradation and cell compatibility using various analytical techniques and *in vitro* cell culture experiments. This PCL copolymer was found to degrade *in vitro* at a much slower rate than other polyesters such as PLGA and PGA as reported in literature. The tensile properties of this material are comparable to several human soft tissues and the elongation properties are comparable to collagen fibers. This elastomer promoted primary rat osteoblast adhesion, proliferation, and expression of alkaline phosphatase, an early marker of osteoblastic phenotype. These preliminary results suggest that PPCLSu may serve as a good candidate for numerous tissue engineering applications including the repair and regeneration of elastic tissues such as skin, blood vessels, skeletal muscle, heart muscle, cartilage, and tendon. The mechanical properties of these scaffolds can be fine-tuned by varying polymer composition and unit ratios, thereby satisfying the requirements of the desired application. Studies are currently underway to establish the rate of *in vivo* polymer degradation and polymer biocompatibility. These ongoing studies are aimed at fine-tuning the material and scaffold properties for a variety of tissue engineering and drug delivery applications.

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