Technical Note

Evaluation of chondrogenesis within PEGT:PBT scaffolds with high PEG content

Tahir A. Mahmood,[1,2] V. Prasad Shastri,[3] Clemens A. van Blitterswijk,[1] Robert Langer,[2] Jens Riesle[1]
[1]*Institute of Biomedical Technology, University of Twente, Professor Bronkhorstlaan 10, Bilthoven 3723 MB, The Netherlands*
[2]*Department of Chemical Engineering and Division of Health Sciences and Technology, Massachusetts Institute of Technology, 45 Carleton St. E25-342, Cambridge, Massachusetts 02139*
[3]*Department of Biomedical Engineering, Vanderbilt University, 5824 Stevenson Center, Nashville, Tennessee 37232*

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Abstract: Porous poly(ethylene glycol) terephthalate:poly (butylene terephthalate) (PEGT:PBT) scaffolds with high PEG molecular weight (1000 g/mole) and PEGT content (60%) were fabricated using two different processes—paraffin templating and compression molding—for cartilage engineering applications. This polymer composition has previously been shown to enable chondrocyte adhesion and maintain differentiated phenotype in 2D monolayer culture. The influence of 3D polymer scaffold processing on the formation of cartilaginous tissue was studied by seeding primary immature bovine chondrocytes within cylindrical scaffolds in mixed flask reactors for 3 days, followed by cultivation in culture plates for a total of 10 or 24 days. Tissue–polymer constructs were evaluated morphologically by SEM and histology, and quantitatively for cellularity, total collagen, and glycosaminoglycan content, all of which remained statistically equivalent for each time point tested, irrespective of fabrication method. These data demonstrate that the polymers engineered for this study were able to support chondrogenesis independent of scaffold fabrication process, with the influence of pore architecture lessened by the highly hydrated scaffold microenvironments induced by high PEG content. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 79A: 216–222, 2006

Key words: cartilage tissue engineering; scaffolds; polymers; poly(ethylene glycol)

INTRODUCTION

Numerous materials and scaffolds have been examined for the repair of articular cartilage defects due to degenerative disease or trauma, by the transplantation of cells or tissue grown within carrier substrates into defects. The materials that have been studied include scaffolds made from natural polymers, such as collagen and hyaluronan,[1,2] as well as synthetic polymer hydrogels and porous polymer matrices such as those made of poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and their copolymers (PLGA). Despite the extensive research into using these carriers, their application has been limited by the inability of cell/tissue transplantation constructs made from these polymers to withstand sufficient mechanical load, although recent reports have demonstrated improved mechanical properties using PGA-reinforced PLGA scaffolds.[3,4] There are also concerns regarding the acidic degradation products of PLGA polymers that may have adverse local *in vivo* effects.[5,6]

In this study, poly(ethylene glycol) terephthalate: poly(butylene terephthalate) (PEGT:PBT) copolymers were engineered as novel cell and tissue transplantation materials. Overall copolymer properties are determined by the two components—the PEG segment is hydrophilic and provides soft elastomeric properties, whereas PBT imparts stiffness to the system. During polymer synthesis, the molecular weight (mw) of PEG and weight ratio of the PEGT:PBT components can be defined to allow the copolymer

Correspondence to: T. A. Mahmood, Amgen Inc., Department of Pharmaceutics, Thousand Oaks, California 91320, USA; e-mail: tmahmood@amgen.com

to be tailored for desired mechanical and surface properties. For example, reducing PEG mw from 1000 to 300 g/mole and PEGT wt % from 70 to 55% resulted in increase in tensile strength (σ) from 5.3 to 15.3 MPa, and in tensile modulus (E) from 33.9 to 187.5 MPa, respectively.[7]

Various PEGT:PBT compositions have been studied for their ability to support chondrocyte attachment, growth, and phenotype in 2D monolayer conditions.[8–10] It was observed from these studies that a balance of hydrophilic and hydrophobic segments is needed for chondrocyte attachment, while maintaining chondrogenic phenotype. Specifically, substrates with high PEG mw (>1000 g/mole) and PEGT wt (>70%) supported the differentiated phenotype, but were not conducive to cell attachment in numbers sufficient for practical application. Conversely, low PEG mw (300 g/mole) and PEGT wt (<55%) enabled cell attachment, but resulted in chondrocyte dedifferentiation to fibroblastic phenotype. Of the range of compositions evaluated, the composition with 1000 g/mole PEG and a PEGT:PBT molar ratio of 60:40 was shown to support chondrocyte adhesion and presented interfacial material-cell conditions that maintained the differentiated chondrocyte phenotype in 2D monolayer conditions, and was thus selected as the composition for studying 3D cartilaginous tissue formation. Furthermore, an orthopedic medical device made from PEGT:PBT copolymers has been approved by the US Food and Drug Administration,[11] which could facilitate the development of tissue engineered products based on these polymers.

Porous scaffolds for cell/tissue transplantation were fabricated from the high PEG content copolymer by two techniques: paraffin templating (PT) and compression molding (CM). Initial studies have demonstrated promise for the use of these scaffolding techniques for tissue engineering, although the influence of polymer composition on tissue formation within each scaffold type has yet to be fully elucidated. In the CM process, polymer resin is melted around salt crystals inside a mold under high pressure, and the salt leached out using water to form a porous structure.[12] In PT, the paraffin porogen is leached out using an aliphatic hydrocarbon solvent (porogen solvent; nonsolvent for polymer) during polymer precipitation to create porous foam blocks.[13]

Articular cartilage extracellular matrix (ECM) contains type II collagen, proteoglycan macromolecules with glycosaminoglycan (GAG) side chains, as well as other matrix molecules. The tissue is able to withstand compression loading due to interaction between collagen network and GAG, which is able to sequester water within the matrix.[2] To enhance the potential of integrating engineered tissue with ECM at the defect site, a strategy is to transplant biologic-material constructs of immature tissue. This could lead to improved integration with the host ECM, as further tissue development will occur *in vivo* within the lesion.

The objective of this study was to evaluate and compare *in vitro* chondrogenesis within high PEG content porous scaffolds fabricated by PT and CM techniques.

MATERIALS AND METHODS

Polymer synthesis

PEGT:PBT segmented copolymer resin was prepared as described previously.[14] Briefly, synthesis occurred by two-step condensation in the presence of titanium tetrabutoxide (Merck, Darmstadt, Germany) as catalyst (0.1 wt %). Vitamin E (Sigma, Uithoorn, The Netherlands) was added to prevent oxidation. The resulting copolymer can be further processed into various geometrical configurations such as films or porous scaffolds, providing a model class of substrates for studying cell–material interactions (Fig. 1a).

Scaffold processing and fabrication

The different formulations of the PEGT:PBT copolymer system are indicated as follows: *a*-PEGT *b*:*c*, where *a* is the molecular weight of PEG (g/mole), *b* is the weight percentage of PEGT, and *c* is the weight percentage of PBT. In this study, 1000-PEGT 60:40 was used.

PT scaffolds were produced by dissolving the polymer resin in methylene chloride and dispersed with spherical and irregular paraffin particles of 1 mm and 425–500 μm mean diameter, respectively, to yield a polymer–solvent–porogen putty. The theoretical average pore size was calculated to be 613 μm. The putty was packed densely into a Teflon mold, and the paraffin porogen extracted in hexane at 45–50°C for 20 min to yield a porous block of polymer foam (Fig. 1b). The foam block was dried under vacuum to remove any residual solvent.

CM scaffolds were fabricated into blocks by injection molding PEGT:PBT polymer resin with salt crystal inclusions at 220°C for 10 min under 20,000 lbs/in.^{2} pressure. These blocks were air-cooled for 20 min and immersed in demineralized H_2O to leach out the salt crystals and create 400–600 μm pores (Fig. 1b).

Cylindrical scaffolds of 4 mm diameter and 4 mm thickness were cored out from blocks of both fabrication groups, cleaned with 70% ethanol and demineralized H_2O, and gamma-sterilized under vacuum. Scaffolds were immersed overnight in culture medium prior to seeding to reach equilibrium polymer swelling.

Chondrocyte isolation and culturing

Articular cartilage was harvested from the patellar grooves of young bovine calves. Chondrocytes were isolated by type II collagenase (Worthington Biochemical, Lakewood, NJ) digestion for 16 h and transferred to a well defined culture medium containing DMEM, 10% fetal bovine serum, penicillin/streptomycin (Gibco, NJ), ascorbic acid phosphate, nonessential amino acids, and L-proline (Sigma, St. Louis, MO).

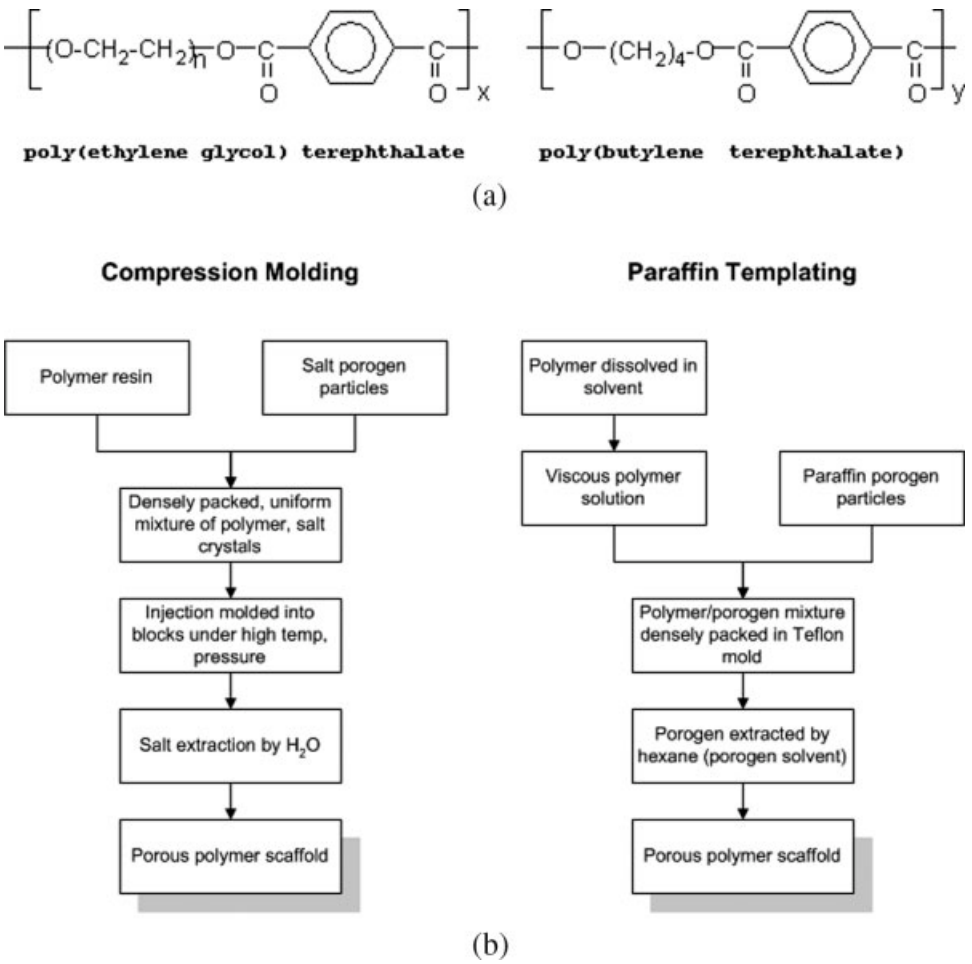

Figure 1. (a) Chemical structure of segmented poly(ether ester) [PEGT:PBT] copolymers, formed by polycondensation polymerization of hydrophilic PEG-containing segments and hydrophobic PBT segments. (b) Schematic outline of the steps involved in fabricating CM and PT scaffolds. PT scaffolds were made by dissolving the polymer resin in methylene chloride and dispersed with spherical and irregular paraffin porogen particles. The resulting viscous solution/putty was packed in a Teflon mold and porogen extracted at 45–50°C for 20 min in hexane. The foam block was dried under vacuum to remove residual solvent. CM scaffolds were fabricated into blocks by injection molding PEGT:PBT polymer resin with salt crystals (porogen) at 220°C for 10 min under 20,000 lbs/in.^{2} pressure. Blocks were air cooled for 20 min and immersed in demineralized H_2O to leach out the salt porogen. Cylindrical scaffolds used for these studies were cored out from blocks of each fabrication group, as needed.

Chondrocytes were seeded at a density of 3 million cells per scaffold in mixed flask systems, as described in detail previously.[15,16] Scaffolds were maintained in the mixed flasks for 3 days, after which they were transferred to 6-well plates (BD Falcon, Amsterdam, NL) on an orbital shaker for the remaining cultivation periods. Samples from each scaffold group were harvested for histology at day 10 and for electron microscopy at day 24. Constructs were also harvested at days 10 and 24 for total cell number, collagen, and GAG content.

Histology

Histological sections were prepared by first fixing the cell–polymer constructs in 2.5% gluteraldehyde and 10% formalin for 7 days. Specimens were then dehydrated over 1 week in a graded ethanol series, followed by embedding in glycol methacrylate and sectioning to a thickness of 5 μm on a standard microtome (Microm, Walldorf, Germany). The sections were stained by safranin O/fast green for sulfated GAG, and nuclei counterstained with hematoxylin.

Scanning electron microscopy

To examine cell–polymer construct morphology in higher detail, samples harvested at day 24 were fixed in Karnovsky's fixative, dried at the critical point of CO_2 and sputter-coated with a 12 nm layer of gold (Cressington Scientific, Watford, UK). Specimens were examined by scanning electron microscopy (SEM) (Phillips ESEM, Eindhoven, The Netherlands).

Quantitative GAG and collagen analyses

Constructs ($n = 3$) were harvested at day 10 and 24 and digested overnight at 56°C in solution containing proteinase K, pepstatin A and iodoacetamide (Sigma). Digests

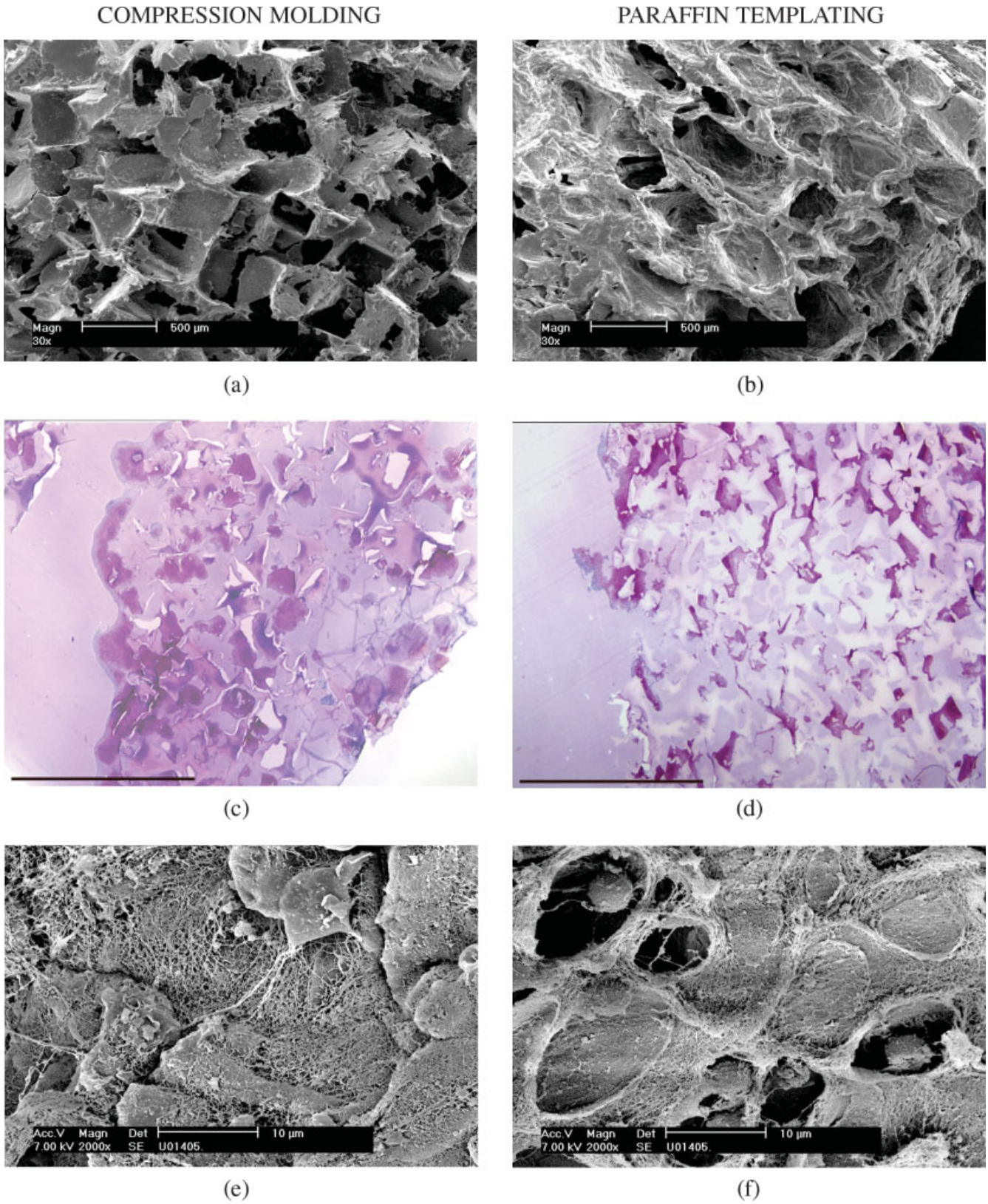

Figure 2. SEMs of high PEG content PEGT:PBT scaffolds fabricated by CM (Fig. 2a) and PT (Fig. 2b). The surface topographies that resulted from the different techniques are visible at this magnification. Histology of construct cross sections at day 10 demonstrated interconnected GAG-rich tissue formation within scaffolds [Figs. 2(c,d)]; scale bars = 2 mm. High magnification SEM examination at day 24 revealed dense ECM-like morphology with rounded chondrocytes [Figs. 2(e,f)]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

were evaluated for cellularity, GAG, and total collagen content.

For determining cell numbers, quantitation of total DNA were performed by Cyquant dye kit (Molecular Probes, Leiden, The Netherlands) using a spectrofluorometer (Perkin Elmer, IL). Cell numbers were calculated from the total DNA content by normalizing to 7.7×10^{-12} g DNA per bovine chondrocyte.[17]

GAG content was obtained by labeling with dimethylmethylene blue dye (Sigma-Aldrich) and measuring color intensity by a spectrophotometer (Bio-Tek Instruments, Neufahrn, Germany).[18]

Total collagen was determined by measuring the amount of hydroxyproline in each construct. Aliquots of proteinase K digests to be evaluated for hydroxyproline were hydrolyzed in 6*N* HCl at 110°C for 16 h. The hydrolyzate was assayed for hydroxyproline using methods that have been described in detail elsewhere, with a hydroxyproline content of 13% of collagen used to calculate final collagen quantities.[19]

Data obtained from quantitative assays were analyzed by student's t-test. $p < 0.05$ was used to determine statistical significance.

RESULTS

Histology and scanning electron microscopy

The pore structures of scaffolds produced by PT and CM are shown in Figure 2. Pores in CM scaffolds were generally cuboidal (Fig. 2a), whereas the PT process resulted in an irregular pore structure (Fig. 2b).

At day 10, histology revealed red positive staining for safranin O within the pores of constructs fabricated by both processes [Figs. 2(c,d)]. Observation of sections from both scaffold types showed regions of cartilaginous tissue interconnecting between pores.

Scanning electron micrographs of constructs cultivated for 24 days revealed a rich fibrillar extracellular-like matrix (ECM), within which chondrocytes were embedded [Figs. 2(e,f)]. In the PT constructs, the dense ECM lacunae contained cells with spheroid morphology.

In some histology sections, artifacts such as gaps were seen. Repeated sectioning from multiple samples has demonstrated that these areas contained tissue that separated from the polymer scaffold and embedding material during sectioning.

Quantitative constituent analyses (Table 1)

Cellularity evaluated by DNA assay

At day 10, PT and CM constructs contained an average 12.6 million and 13.5 million cells per construct, respectively. From 10 to 24 days cultivation, both scaffold groups displayed significant increases in cellularity (PT: $p < 0.036$, CM: $p < 0.032$). At day 24, PT and CM constructs contained 16.9 million and 20.7 million cells, respectively. At each time point, there was no significant difference in cell number between scaffold types.

TABLE I
Quantitative Evaluation of Cell Number, GAG (mg), and Total Collagen Content (mg) Per Construct in PT and CM Scaffolds at 10 and 24 Days

	Day 10		Day 24	
	Average	St. Dev	Average	St. Dev
Cell number (10^6)				
PT	12.6	1.7	16.9	0.9
CM	13.5	1.1	20.7	0.8
GAG (mg)				
PT	2.4	0.3	2.2	0.5
CM	2.2	0.3	2.1	0.2
Collagen (mg)				
PT	22.8	4.8	51.0	1.8
CM	21.7	2.2	52.5	5.1

Cell number: Both scaffold types exhibited a significant increase in cellularity from 10 to 24 days (PT, $p < 0.036$; CM, $p < 0.032$). At each time point, however, there was no significant difference in the number of cells in each scaffold type. *GAG:* There were no significant differences in GAG between both scaffold types at either time point, or between each scaffold group from 10 to 24 days cultivation. *Collagen:* Although there were no differences in total collagen content in both scaffold types at either time point, the amount of collagen increased significantly in each scaffold type from 10 to 24 days cultivation (PT, $p < 0.001$; CM, $p < 0.0035$).

GAG content

At day 10, constructs contained 2.4 mg (PT) and 2.2 mg GAG per construct (CM). Mean GAG content remained statistically the same for both scaffold types between 10 and 24 days. At day 24, mean GAG content within each PT and CM construct was 2.2 and 2.1 mg, respectively.

Collagen content

At day 10 of culture, PT constructs contained 22.8 mg collagen, whereas CM constructs contained 21.7 mg. At day 24, PT and CM constructs contained 51 and 52.5 mg collagen, respectively. Although there were no significant differences in collagen content between both scaffold types at either time point, the amount of collagen increased significantly in each scaffold type during 10–24 days culture (PT, $p < 0.001$; CM, $p < 0.0035$).

DISCUSSION

Scaffolds fabricated from 1000-PEGT 60:40 polymers using both PT and CM processes enabled the infiltration of cells within the scaffold interior, and

supported chondrogenesis. Theoretical porosities of scaffolds (determined by mass–volume) produced by both fabrication methods were similar (CM: 78%, PT: 80%), which are very similar to the porosities determined using micro-computed tomography[20] and mercury porosimetry.[13]

There were no significant differences in the number of cells, total collagen, or GAG content within scaffolds produced from this high PEG content composition. This may in part be due to the relatively high hydrophilicity of the polymer composition tested—the mass of 1000-PEGT 60:40 polymer has been shown in our laboratory to increase 1.8 times upon reaching equilibrium in water. Polymers with long chain PEG and high PEG content are likely to sequester water molecules, providing an environment similar to the hydrated conditions found in hyaline cartilage. A correlation between PEG mw, PEGT:PBT ratio and molecule diffusivity has been previously demonstrated, due to differential effects of hydration on the 50–100 Å mesh size of the hydrogel-like polymer phase.[21] Thus, it follows that the highly hydrated conditions of the 1000-PEGT 60:40 copolymer may enable greater nutrient diffusion into scaffold interiors, thereby supporting chondrogenesis throughout the volume of scaffolds, as evidenced by histology, SEM, and supporting quantitative data.

It has been reported earlier that cartilaginous tissue formation within scaffolds fabricated from hydrophobic polymer compositions (300 mw PEG, 55% PEGT) was dependent on the method of fabrication.[22] In those studies, only PT supported *in vitro* chondrogenesis, as chondrocytes seeded on CM scaffolds were unable to infiltrate the interior of the scaffolds, remained at the periphery and exhibited a dedifferentiated fibroblastic morphology. When looked at together with the earlier results, the data presented here support the notion that 3D pore architecture may be more important in scaffolds with less chondro-supportive chemistry. Our results also support a previous study that showed that a hydrophilic polymer with dense arrangement of high molecular weight PEG, as well as a baseline pore interconnectivity, are prerequisite to support chondrogenesis in static *in vitro* cultivation.[20]

Cartilaginous tissue formation has been reported in porous scaffolds with smaller pore diameters of 174 μm and 115–335 μm.[23,24] This indicates that the mean pore size in our PT and CM scaffolds was large enough to allow chondrocyte infiltration and subsequent matrix formation. Using GAG produced per cell as a comparative, positive biomarker by which to compare with the extensively studied PGA-based polymer scaffolds, the PEGT:PBT constructs evaluated in this study demonstrated up to 3-fold greater GAG per cell than PGA at similar time points.[25] High magnification SEM at 24 days revealed dense ECM and rounded cellular morphology typical for chondrocytes.

In conclusion, we have shown that scaffolds made from 1000 g/mole PEG and 60:40 PEGT:PBT ratio can be produced with different pore architectures but similar porosities using PT and CM techniques. These data demonstrate that the high PEG content PEGT:PBT copolymer polymer composition engineered for this study was able to support chondrogenesis independent of scaffold fabrication process.

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