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Porous Copolymers of ε-Caprolactone as Scaffolds for Tissue Engineering

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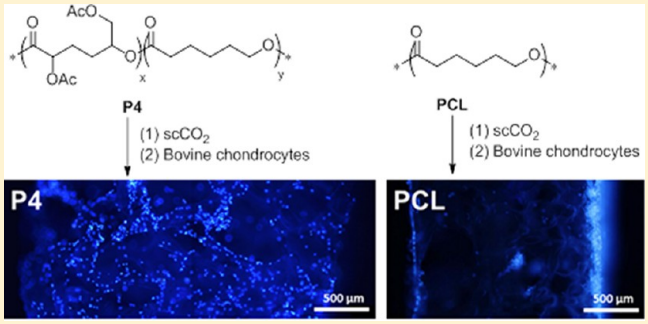
Supporting Information

ABSTRACT: A series of random copolymers were synthesized via the copolymerization of a carbohydrate lactone, acetic acid 5-acetoxy-6-oxotetrahydropyran-2-yl methyl ester (**1**), and ε-caprolactone. The copolymers were characterized by nuclear magnetic resonance (NMR) spectroscopy, size exclusion chromatography (SEC), differential scanning calorimetry (DSC), and thermal gravimetric analysis (TGA). Copolymers (**P1**−**P4**) were produced with typical carbohydrate ester compositions of 1−4 mol %. These copolymers are semicrystalline and can be processed into thin films with Young's moduli of 300−420 MPa, values that exceed that for polycaprolactone (PCL). The copolymers were processed using supercritical carbon dioxide ($scCO_2$, 35 °C, 200 bar) into foamed, porous scaffolds, which were characterized by dynamic mechanical thermal analyses (DMTA), mercury porosimetry, and scanning electron microscopy (SEM). The copolymer foams showed increased pore size with increasing carbohydrate ester content. The average pore size increased from 71 μm (PCL) to 319 μm (**P4**). The foamed scaffolds have normalized storage moduli ranging from 37 MPa $cm^3\ g^{-1}$ (**P4**) to 109 MPa $cm^3\ g^{-1}$ (**P1**). A representative copolymer foamed scaffold, tested according to ISO 10993-5 criteria, was cytocompatible for cell culture. MC3T3 cells cultured on a film of this copolymer showed increased relative metabolic activities compared to cells cultured on a PCL film. When primary bovine chondrocytes were cultured on the foamed scaffolds, increased cell penetration into the random copolymer foam was observed compared to PCL foams.



INTRODUCTION

The first steps in achieving tissue engineering are the seeding of a patient's progenitor cells onto a resorbable scaffold, often fabricated from a degradable polymer, followed by cell proliferation and growth, accompanied by the degradation of the scaffold.[1] Control of the scaffold chemistry, its internal structure, and mechanical properties are central to cell penetration into the scaffold and growth.[2] Scaffolds should ideally be porous, with a high density of interconnected pores to allow cell adhesion and colonization.[3]

Polymers used to make scaffolds must be biocompatible, and it is also beneficial if the polymer is easily prepared, blended with bioactive compounds and processed into a porous scaffold.[4−10] Aliphatic polyesters such as poly(lactic-*co*-glycolic acid) (PLGA) and poly(ε-caprolactone) (PCL) meet these criteria and have been widely investigated.[4−10] They are usually applied as copolymers, often with poly(ethylene glycol) (PEG), to increase the polymers hydrophilicity and thereby improve biocompatibility.[4−10] Although much progress has been achieved with these polymers, there is a need for new materials, especially those with hydrophilic functional groups which are appended to the polymer backbone to improve biocompati-

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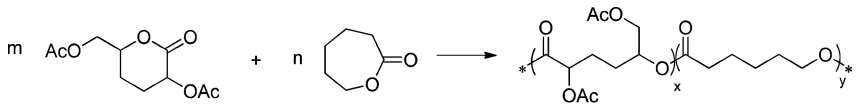
Scheme 1. Copolymerization of Monomer 1 and CL[a]



[a]Polymerization conditions: 100 °C, toluene, 0.1 mol % $Sn(OBu)_2$, isolated yields = 63%–96%. The *m*, *n*, *x*, and *y* values are listed in Table S1.

bility and better matching of the mechanical properties with those of natural tissues.[5] The central difficulty lies in the preparation of new, functionalized aliphatic polyesters. PCL and PLGA are synthesized by the ring-opening polymerization (ROP) of lactones, a process that enables accurate control of the polymers' molecular weights and chain end groups.[9,11−17] In contrast, the ROP of functionalized lactones is extremely challenging due to lactone syntheses frequently requiring multiple, low yielding steps and thermodynamic constraints limiting the degree of polymerization of these substituted lactones.[18] Recently, some of us have reported the preparation of a new carbohydrate derived lactone, **1** (Scheme 1), prepared from naturally occurring D-gluconolactone in three steps and >90% yield.[19] Monomer **1** has been copolymerized with *S,S*-lactide to produce copolyesters which are more hydrophilic and therefore more rapidly degraded than PLA.[20] Such increased hydrophilicity could be attractive for various tissue engineering applications and may obviate copolymerization with PEGs. Our attention turned to using **1** and ε-caprolactone (CL) to prepare modified polycaprolactone copolymers.

PCL is a semicrystalline aliphatic polyester (T_m = 60 °C; T_g = −60 °C), which is bioresorbable via hydrolysis of its ester linkages under physiological conditions; it has been widely applied in tissue engineering.[1,4−10,21−31] Three-dimensional porous PCL scaffolds have been fabricated using electrospinning, solvent casting and particulate leaching, gas-foaming, emulsion freeze-drying, thermally induced phase separation, and rapid prototyping technologies.[28,32−38] These techniques, however, all require elevated temperatures and/or the use of organic solvents; such conditions can denature proteins and preclude loading of the scaffold with beneficial bioactive compounds.[39] One promising alternative to avoid the aforementioned problems is the use of supercritical carbon dioxide ($scCO_2$, 74 bar, 31 °C) to produce polymer foams. $ScCO_2$ is dissolved into the polymer and allows for efficient mixing of the polymer and the preabsorbed exogenous proteins to occur. Subsequently, the CO_2 pressure is reduced which leads to nucleation of gas bubbles within the polymer, creating a porous scaffold. This method is particularly beneficial for scaffold loading with sensitive biological compounds, e.g. proteins, as it operates at low temperatures and obviates the use of organic solvents.[40,41] Despite these advantages, it remains challenging to use $scCO_2$ foaming to fabricate porous scaffolds with a high degree of pore interconnectivity; this latter aspect is important for efficient cell colonization.[28] To date, only a few investigations of PCL scaffold fabrication using $scCO_2$ have been published.[40−45] There are also some examples of PCL polymer blends, which were foamed using $scCO_2$.[46−49] A current limitation of PCL processing using $scCO_2$ is that elevated temperatures, specifically temperatures exceeding 60 °C, often need to be used.[40−45] Such high temperatures are incompatible with protein incorporation. The development of methods to modify PCL, so as to enable the production of highly interconnected porous materials at biologically compatible temperatures, is of high interest for the preparation of new scaffolds for tissue engineering. Monomer **1** has acetyl groups appended to the polyester backbone; others have shown that both acetyl functionalities and such polymer structures are CO_2-philic.[50,51] The strategy was therefore to copolymerize monomer **1** with caprolactone as this may allow the copolymer to absorb more CO_2, which should, in turn, lower its melting temperature in $scCO_2$ and, therefore, the foaming temperature.

RESULTS AND DISCUSSION

Synthesis and Characterization. We have previously described the synthesis and polymerization of a new lactone, **1**, derived from D-gluconolactone in three high yield steps.[19] A series of new random copolymers, **P1−9**, were prepared by the ROP of monomer **1** and CL (Scheme 1). The polymerizations were conducted using increasing molar ratios of **1**:CL, from 1:99 to 25:75 (Table S1); a control experiment using neat CL yielded PCL for comparison with the new copolymers. More details about the polymer synthesis are included in the Supporting Information.

Table 1 shows the copolymer compositions, as determined by integration of the ^{1}H NMR spectra; copolymers composing between 1 and 39 wt % of ring-opened **1** can be prepared. It shows a similar loading of repeat units of ring-opened **1** compared with that expected on the basis of the reaction stoichiometry (the conversions of monomer **1** and CL are listed in Table S1). SEC data show only a single peak for each of the copolymer samples, albeit with rather broad polydispersities (PDI) indicating that the polymers synthesized are copolymers and not mixtures of two homopolymers. Furthermore, it was not possible to change the composition of the copolymers by

Table 1. Characterization Data for Copolymers P1−P9[a]

copolymer	loading **1**:CL[b]	MRO **1** [%] (m/m)[c]	WRO **1**/ [%] (w/w)[d]	M_n (SEC)/ [g mol^{-1}][e]	PDI
PCL	0:100	0	0	178 300	1.37
P1	1:99	1	2	88 200	1.97
P2	2:98	2	4	82 800	2.11
P3	3:97	3	6	78 900	1.98
P4	4:96	4	7	65 900	2.03
P5	5:95	5	9	63 200	2.08
P6	10:90	9	17	40 800	1.62
P7	15:85	14	24	31 000	1.98
P8	20:80	19	32	21 300	1.81
P9	25:75	24	39	16 900	1.75

[a]Copolymerization of **1** and, initiated by $Sn(OBu)_2$ in toluene at 100 °C, [CL] + [**1**] = 2 M. [b]The loadings ratio of 1 and CL. [c]MRO **1**: the molar ratio of ring-opened **1** in the copolymer; determined by dividing the integration the peaks from 4.8 to 5.2 ppm (ring-opened **1**, H-2 and H-5) by the integration of the peaks from 3.9 to 4.3 ppm (two H_F on CL, two H_6 of ring-opened **1**). [d]WRO **1**%: the weight ratio of the ring-opened **1** in the copolymer, calculated from the molar ratio. [e]M_n determined by SEC ($CHCl_3$) using polystyrene standards.

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Table 2. Comparison of the Glass Transition (T_g) and Melting (T_m) Temperatures for the Copolymers Determined Experimentally and Theoretically,[a] the Relative Degree of Crystallinity, the Young's Moduli (E), and the Ultimate Tensile Strength (UTS)

copolymer	WRO **1** [%] (w/w)	calcd T_g[b] [°C]	T_g[c] (DSC) [°C]	T_m (DSC) [°C]	rel % crystallinity/PCL[d]	E[e] [MPa]	UTS[f] [MPa]
PCL	0		−67.0	54.3 ± 1.1	100	291 ± 12	45.3 ± 2.7
P1	2	−65.7	−63.6	54.1 ± 0.3	108.9 ± 1.3	419 ± 28	35.4 ± 4.3
P2	4	−64.2	−62.3	52.8 ± 3.0	102.1 ± 1.9	407 ± 29	28.4 ± 3.0
P3	6	−62.7	−59.8	51.6 ± 0.3	102.7 ± 2.2	346 ± 10	16.9 ± 2.5
P4	7	−62.2	−58.0	50.5 ± 0.7	96.3 ± 0.6	339 ± 12	20.7 ± 1.5
P5	9	−60.6	−57.4	48.5 ± 0.7	94.8 ± 2.4		
P6	17	−55.5	−53.8	40.3 ± 0.8	68.2 ± 1.4		
P7	24	−49.8	−51.6	31.1 ± 0.5	9.6 ± 0.4		
P8	32	−43.5	−45.7				
P9	39	−37.8	−38.5				

[a]DSC was used to determine the thermal properties for each copolymer, the experimental protocol involved measuring three heating and cooling cycles, with the samples being heated to 100 °C at 10 °C/min and cooled to −100 °C at 10 °C/min. In all cases, the second and third cycles gave reproducible results. The data used were from the second heating cycle; a representative analysis is illustrated in Figure S3. [b]T_g was calculated using the Fox equation (see Supporting Information for more details). [c]The error in the T_g values is ∼± 3 °C. [d]The relative crystallinity (i.e., % crystallinity of polymer/% crystallinity of PCL) was calculated by dividing the melting enthalpy of the copolymer by the melting enthalpy of PCL. [e]Calculated from the linear region of the stress−strain curves; five samples were measured for each polymer composition. [f]Ultimate tensile strength, maximum stress at break (see Supporting Information, Figure S7).

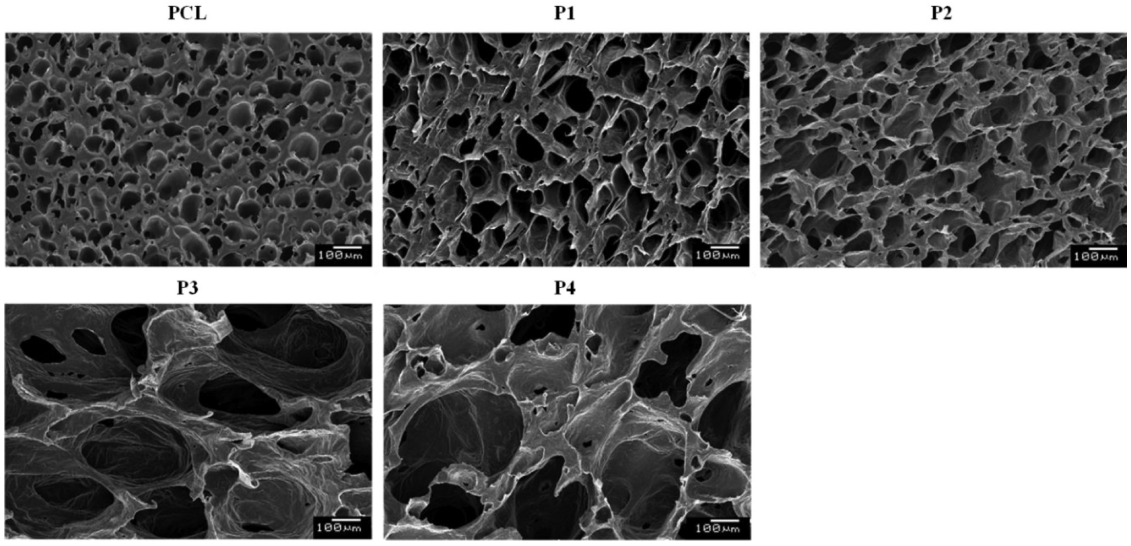

Figure 1. SEM images for PCL and **P1**−**P4** after $scCO_2$ foaming.

any solvent extraction/precipitation methods, and a single T_g was observed by DSC. SEC, in chloroform solution using narrow M_n polystyrene standards, showed M_n 16.9−178 kg/mol; there was a trend toward the polymer having higher M_n as the proportion of CL incorporation in the polymer increases. There are two probable causes for the increase in M_n; First, when the loading of CL in the feedstock is higher, there are a greater number of repeat units (equivalents of monomer reacted) overall in the copolymer (see Table S1). This is because both CL and **1** reach higher conversions when the loading of **1** is low. Second, polystyrene standards were used to calibrate the SEC instrument, and the copolymers are expected to show different correction factors vs the polystyrene standards as the compositions change. This latter effect is likely to be dominant but cannot yet be precisely determined because the Mark−Houwink−Sakurada parameters for **1** are unknown. It is therefore expected that the hydrodynamic volumes of the copolymers change with their composition. In fact, it has already been reported that PCL requires a correction factor of approximately 0.3, when polystyrene standards are used in $CHCl_3$ solutions for calibration.[52] These uncertainties mean that the M_n values should be treated only as approximate estimates.

Thermal Properties. DSC and TGA have been used to thermally characterize the random copolymers. TGA shows the degradation of the homopolymer from **1** begins at 250 °C under a nitrogen atmosphere.[19] PCL is thermally stable up to approximately 265 °C. The weight loss onset temperatures of the copolymers, **P1**−**9**, are all around 250 °C (see Figure S2). We had previously established that the glass transition temperature for the homopolymer of **1** was 29.6 °C.[19] The glass transition temperatures of the copolymers are calculated using the Fox equation (see Supporting Information for further details). The DSC results show that the glass transition temperatures of the copolymers increase as the ring-opened **1** proportion increases (Table 2). There is an excellent agreement between the experimentally determined T_g and those predicted using the Fox equation. The melting temperatures of the

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Table 3. Average Pore Size d_p and Pore Throat Size d_{PT}, Porosity (P), Degree of Pore Interconnectivity, the Absolute and Specific Storage Moduli and Mechanical T_g Determined from Dynamic Mechanical and Thermal Analyses of the Foamed Polymers

copolymer	d_p[a] (μm)	d_{PT}[a] (μm)	P[b] (%)	deg of pore interconnectivity[c] (%)	abs storage modulus at 25 °C (MPa)	specific storage modulus at 25 °C (MPa cm^3 g^{-1})[d]	mechanical T_g[e] (°C)
PCL	71 ± 17	18 ± 7	73.4	80.0	22.8	92.2	−31.7
P1	57 ± 18	23 ± 12	70.6	66.5	31.8	108.6	−29.1
P2	96 ± 63	43 ± 33	77.2	97.3	29.4	105.2	−28.8
P3	297 ± 87	112 ± 46	85.0	72.4	8.5	42.7	−27.8
P4	319 ± 91	98 ± 32	84.4	86.7	6.8	36.5	−26.4

[a]Calculated from SEM images using ImageJ 1.46. For each sample, more than 100 pores were analyzed. [b]The bulk porosity was calculated from the equation $P = (1 - \rho_{foam}/\rho_{matrix}) \times 100$, where ρ_{foam} and ρ_{matrix} denote the foam density (ratio of the mass and volume of the foam) and matrix density (taken to be 1.14 g cm^{-3}), respectively. [c]The degree of pore interconnectivity was calculated by dividing the open porosity (measured by mercury porosimetry, Figure S4) by the bulk porosity. [d]Storage modulus divided by foam density. [e]Obtained from the peak of tan δ from DMTA.

crystalline domains in the copolymers are significantly lower compared with the melting temperature of PCL (60 °C) and decrease as the proportion of **1** in the copolymer increases. This is consistent with a reduction in the degree of crystallinity (except **P1**−**P3**) in the copolymers with incorporation of **1** (for example, see Figure S3 which shows the DSC thermogram for **P7** and a T_m of 31.1 °C). When the proportion of ring-opened **1** exceeds 25 wt %, there is no crystalline region and thus no melting temperature is observed. Thus, **P7** has a T_m of 31.1 °C, while **P8** and **P9** have no melting temperatures and are viscous liquids at room temperature. Copolymers **P1**−**P3**, which have a very low proportion of **1**, show similar melting temperatures and crystallinities to PCL; the apparent increase in relative crystallinity compared to PCL was not substantiated by XRD measurements (see Supporting Information for further details, Figure S9) and is therefore likely not significant within experimental error. For tissue engineering scaffold applications, it is important that the T_m of the materials is higher than the highest temperature the human body can reach (~40 °C). Polymers **P1**−**P5** meet this criterion and were foamed using $scCO_2$ for further investigation.

$scCO_2$ Foaming. The copolymers were foamed using $scCO_2$ at 35 °C, 200 bar. Sample **P7** has a melting temperature less than 35 °C, and **P8** and **P9** are viscous liquids at room temperature so the foaming process was not applicable for these samples. Therefore, the method was suitable only to foam **P1**−**P5** and PCL. Representative SEM images of the copolymer foams are shown in Figure 1. As the scaffold produced from **P5** had large cavity sites (multiple mm size), it was not used for further investigation.

PCL and **P1**−**P4** formed both closed and interconnected pores during $scCO_2$ foaming. The bulk porosity was determined by measuring the mass and volume of the foams (using the density of pure PCL for the calculations, as the proportion of the monomer **1** was always less than 5 mol %). All the foams have a high porosity, ranging from 70.6% to 85.0% (Table 3). The foamed PCL had a porosity of 73.4%. For the copolymers **P1**−**P4**, the porosity increased with the proportion of **1** present, leveling off at proportions >3%; i.e., **P3** had porosity of 85.0% and **P4** of 84.4%. The interconnectivity of these foams is also high; more than 70% of the pores are interconnected for all copolymers, except **P1** (Table 3), as indicated by mercury porosimetry measurements (see Figure S4). The pore and pore throat sizes of these foams were determined from the SEM images; more than 100 pores were measured for the calculation. The average pore size of PCL was 71 ± 17 μm. By increasing the proportion of **1** in the copolymer, the pore size in the foam also increased: for **P1** the average pore size was 57 ± 18 μm, for **P2** 96 ± 63 μm, for **P3** 297 ± 87 μm, and for **P4** 319 ± 91 μm. The pore throat size followed a similar trend as the pore size. For PCL, the average pore throat size was 18 ± 7 μm. By increasing the proportion of **1** in the copolymer, the pore throat size in the foam also increased: for **P1** the average pore throat size was 23 ± 12 μm, for **P2** 43 ± 33 μm, for **P3** 112 ± 48 μm, and for **P4** 98 ± 32 μm. The pore throat size determined from the SEM images using ImageJ (ImageJ 1.46) program is in good agreement with the mercury intrusion porosimetry data (see Figure S4). For scaffold applications, maximizing the pore size and interconnectivity is important to ensure a high degree of cell penetration. It has been reported that pore sizes exceeding 300 μm are required for vascularization of scaffolds and bone in-growth.[53−55] Here, the targets were bone and cartilage cells (MC3T3 preosteoblasts and primary bovine chondrocytes); therefore, **P3** and **P4** are the best candidates for meeting the pore size requirement.

Thermomechanical Properties of the Foams. The absolute storage moduli at room temperature and the mechanical T_g of the foams measured in compression mode are shown in Table 3. Both the absolute storage and the specific storage moduli (storage modulus divided by the foam density) are reported to allow comparison between the scaffolds with varying porosity. It can be seen from Table 3 that PCL, **P1**, and **P2** have similar specific storage moduli, while **P3** and **P4** have lower specific storage moduli. The Young's moduli of polymer **P1**−**P4** films were all in the range of 300−420 MPa. These values exceed that for a PCL film of 291 MPa (Table 2; further details of the tensile mechanical measurements can be found in the Supporting Information, Figure S7), a value which is in agreement with published literature data for PCL.[56,57] Thus, the variable absolute storage moduli between the foams was not caused by intrinsic differences in the polymers' mechanical properties but rather was dependent on the pore sizes and structures. PCL, **P1**, and **P2** have smaller pores, resulting in stiffer foams compared to **P3** and **P4**, which have much larger pores. Similar observations have been reported by Liu et al. using other polymer foams.[58] The mechanical T_g of the foams corroborate the T_g values measured by DSC, whereby the increase in the content of **1** increases the glass transition temperature of the resulting polymer. The graph of absolute storage moduli as a function of temperatures for various foams is shown in Figure S6 (see Supporting Information).

The storage modulus for **P4** is comparable with that of the bovine articular cartilage at 25 °C, highlighting the potential for

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this material as a scaffold in cartilage tissue engineering. Therefore, **P4** foam was selected for cell culture with primary bovine chondrocytes, after the successful outcome of the ISO 10993-5 (2009) cytocompatibility test and 2D *in vitro* assessment with MC3T3 preosteoblasts.[59]

Cell Culture and Testing. The cytotoxicity of neat PCL and **P4** foams was tested according to ISO 10993-5 (2009). The test detects cytotoxic leachates by preconditioning growth medium with polymer samples under *in vitro* culture conditions for 24 h. Cytotoxicity is detected by transferring the conditioned growth media to a culture of MC3T3 preosteoblasts, a well-characterized cell line. Viability is assessed at 72 h relative to known cytotoxic and cytocompatible control polymers. The results of the viability test indicated that the PCL and **P4** foams are both cytocompatible; neither of the samples were statistically different from the negative control, as shown in Figure 2, indicating their potential suitability as

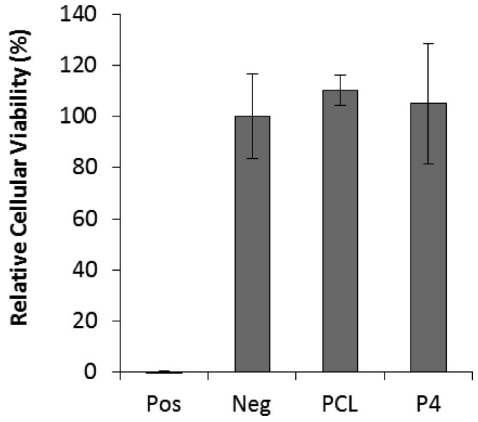

Figure 2. ISO 10993-5 cytotoxicity test on PCL and **P4** foams. Media was preconditioned with the polymer then applied to subconfluent MC3T3 preosteoblast cells for 72 h. Viability was measured by an MTT assay normalized to the negative control. There was no significant difference between the negative control and the foam samples ($P < 0.05$), indicating that both samples are cytocompatible. ($n = 3$).

scaffold materials. Tin compounds can be cytotoxic, and a tin catalyst was used to prepare the copolymers (including PCL). The residual tin concentration in the copolymers was determined using ICP-OES, which showed that both PCL and **P4** have similarly low concentration of tin residues in the samples (9.98 and 8.12 ppm for the testing samples, respectively; more information about ICP-OES is provided in the Supporting Information).

The impact of the different copolymer chemistries on the cellular viability and morphology, independent of their foaming properties and resulting surface areas, was assessed using hot-pressed films. Previous reports have shown that the incorporation of **1** increases the hydrophilicity of polyester copolymers.[20,60] The water contact angle was measured by the captive bubble method (Figure S8). Although there was no dramatic difference between samples, there was a trend of decreasing water contact angle with increasing proportion of monomer **1** in the copolymer (see Supporting Information for further details). The rather modest change in hydrophilicity is likely caused by the low content of monomer **1** in these copolymers (<5% in every case). **P4** comprises only ~4% of monomer **1**, which is insufficient to dramatically alter the thermal or mechanical properties of the copolymers. Therefore, it is expected that **P4** and PCL will behave similarly in the hot-pressing process and result in films of close surface roughness. MC3T3 preosteoblasts, cultured for 14 days on **P4** films, exhibited statistically greater viability and increased cellular spreading compared to the neat PCL control (Figure 3). This is tentatively attributed to the slightly increased hydrophilicity and functional group density of **P4** vs PCL.[61]

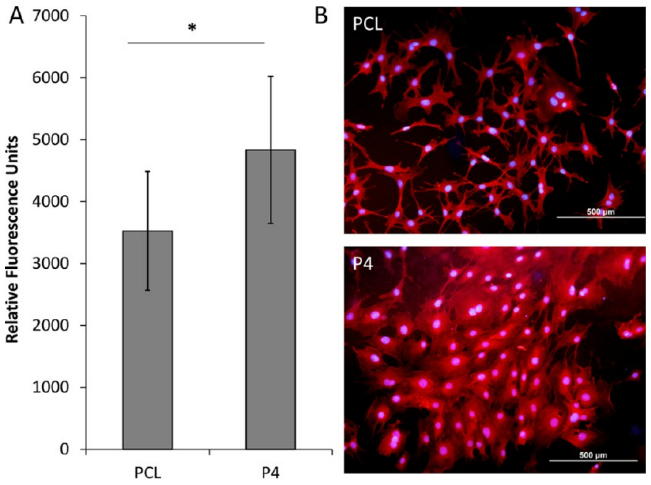

Figure 3. MC3T3 cells cultured on neat PCL and **P4** films for 14 days. (A) Plot of alamar blue metabolic activity at day 14, showing increased relative activity on **P4** films ($n = 6$, $^*p < 0.05$). (B) Representative images of MC3T3 cells on PCL and **P4** films at 14 days. The actin cytoskeleton (red) is highlighted to visualize the differences in cellular morphology attributed to the polymer chemistry of the surfaces.

P4 is cytocompatible and, when foamed, has an absolute storage modulus (in compression) close to that of natural cartilage. Therefore, PCL and **P4** foams were seeded with primary bovine chondrocytes to assess their applicability as scaffolds. Figure 4 shows representative cross sections, demonstrating the homogeneous cell distribution and cell adhesion throughout the **P4** foam. In contrast, the PCL foam shows significant surface localization and only isolated clusters

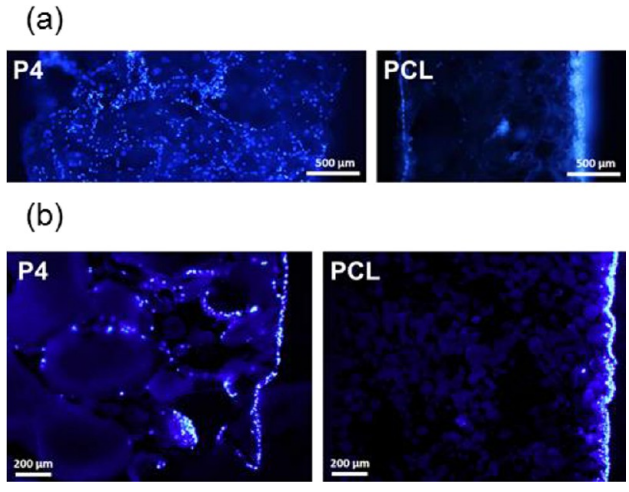

Figure 4. Representative cross sections: (a) full-thickness scans; (b) 20 μm thick sections of PCL and **P4** foams cultured with primary bovine chondrocytes for 7 days, imaged with DAPI nuclear stain. The top surface of the scaffold is on the right.

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of chondrocytes within the bulk. The differing distributions were attributed to the increased pore and pore throat size of the **P4** foam relative to the PCL foam, while the increased cell adhesion was due to the surface chemistries.[20,60] The difference in the pore and pore throat sizes between **P4** and PCL foams is likely to be mainly due to the different chemical compositions of the copolymers. The incorporation of **1** into the copolymer seems to result in a greater proportion of CO_2 being absorbed into the polymer during foaming and leads to larger pore sizes and high porosity. The CO_2 also acts as a plasticizer for the copolymer, lowering its lower melting temperature and enabling the low temperature foaming process.

As mentioned in the Introduction, it is beneficial to be able to blend the copolymers with bioactive compounds, prior to processing into a porous scaffold composite. Here, it has been discovered that copolymers of PCL, with monomer **1**, can be foamed under lower temperatures than usual. This low-temperature fabrication offers the potential to blend bioactive compounds into the copolymer foams. A preliminary assessment of the protein-loading and release properties of the **P4** foams, relative to a PCL control, was undertaken. For this study, bovine serum albumin (BSA) was used as a model protein, and foams containing 5 wt % BSA were prepared using the $scCO_2$ foaming method. The release of BSA, under physiological conditions, was monitored for 7 days (see Figure S5). The release characteristics of BSA from the two foams were significantly different. The PCL control exhibited a burst release of 67 ± 7 wt % of the total theoretical BSA content within the first 3 h, whereas the **P4** foam only released 17 ± 2 wt % over the same interval. Both polymers adopted linear releasing profiles beyond 2 days, resulting in a final 7 day release of 82 ± 15 and 57 ± 5 wt % for PCL and **P4**, respectively. Surface-exposed BSA is readily dissolved, followed by the more controlled release of BSA from the polymer bulk in a linear fashion. The differences in release are tentatively attributed to BSA localization primarily on the surface of the PCL foam, while BSA is more evenly distributed throughout foams of **P4**. These preliminary release data indicate the potential for using the copolymers to produce scaffolds loaded with suitable bioactive compounds, such as growth factors, so as to enhance tissue regeneration.[40,62]

■ CONCLUSIONS

A series of new copolymers, prepared from CL and a carbohydrate derived lactone **1**, were synthesized, characterized, and processed into porous scaffolds using $scCO_2$ foaming. These porous scaffolds were analyzed for their porosity, interconnectivity, and mechanical properties. The inclusion of **1** into the copolymers, particularly at 1−4 mol % loading, was sufficient to completely change their behaviors in the $scCO_2$ foaming process compared to neat PCL. Thus, copolymers with 1−4 mol % **1** loading were easily processed, using $scCO_2$, into foams with high degrees of pore interconnectivity. Furthermore, $scCO_2$ foaming occurs at sufficiently low temperatures (<35 °C) so as to be compatible with bioactive protein incorporation into the porous scaffold. All the new copolymers were cytocompatible; thin film studies showed that the new materials were additionally beneficial to stimulate cell adhesion (MC3T3) and spreading (vs neat PCL). A preliminary tissue engineering assessment, using primary bovine chondrocytes, showed a uniform distribution of cells throughout the foam prepared from the copolymers. This is in stark contrast to neat PCL, where most cells were surface localized likely as a result of the small pore throat size. Thus, these new PCL copolymers are easily synthesized and processed using benign media ($scCO_2$) to produce foamed scaffolds, which show significant promise for cartilage tissue engineering. The detailed biomedical profiles of the materials will be the subject of future reports.

■ EXPERIMENTAL SECTION

Materials. Compound **1** and the initiator $Sn(OBu)_2$ were prepared and purified according to the literature methods.[19,63] ε-Caprolactone was purchased from Sigma-Aldrich; it was purified by distillation from calcium hydride under under reduced pressure (2.3×10^{-1} mbar) and stored under molecular sieves in a nitrogen-filled glovebox prior to use. Toluene was distilled from sodium and stored under nitrogen. Chloroform-d_1 was dried by distilling it from calcium hydride, three freeze−thaw cycles were performed, and it was stored under nitrogen.

Characterization. NMR spectra were carried out on a Bruker AV400 instrument; ^{1}H NMR spectra were collected at 400 MHz. $CDCl_3$ was used as the NMR solvent and reference compound. The SEC measurements were performed on a Polymer Laboratories SEC 50 instrument with two Polymer Laboratories mixed D columns and $CHCl_3$, at a flow rate of 1 mL/min, as the eluent. Narrow molecular weight polystyrene standards (Polymer laboratories, Mixed A and B) were used to calibrate the instrument.

Thermal Analyses. The thermal properties were measured using DSC Q2000 (TA Instruments, UK). Scans were performed from −100 to 100 °C, at a controlled heating and cooling rate of 10 °C/min. A sealed empty crucible was used as a reference, and the DSC was calibrated using indium. Each sample was run for three heating−cooling cycles. After the first cycle, the DSC traces of the second and third cycles were identical. The glass transtion temperatures reported are taken from the second cycle. Thermogravimetric analysis was carried out on TGA Q500 (TA Instruments, UK), under nitrogen flow (60.0 mL/min), at a heating rate of 10 °C/min from room temperature to 450 °C. The reported data are the onset degradation temperatures of the samples.

Mechanical Testing. The mechanical properties of the PCL and copolymer films and the foams were tested in tension, using an Instron 5540 testing machine equipped with a 500 N load cell (Norwood, MA). The tensile modulus was determined from 220 ± 60 μm thick hot-pressed films at 0.5% strain s^{-1}. The modulus was extrapolated from the linear portion of the curve. The viscoelastic properties of $scCO_2$ foams were characterized in compression mode using DMTA (Tritec 2000, Triton Technology Ltd., Keyworth, UK). The foams have an average dimension of 5 × 5 × 5 mm^3. The storage modulus, loss modulus, and energy dissipation factor (tan δ) were measured from −65 to 40 °C at a heating rate of 5 °C min^{-1} and a frequency of 1 Hz.

Copolymer Syntheses (Representative Example: Synthesis of P5). Compound **1** (1.15 g, 5.0 mmol) and the initiator $Sn(OBu)_2$ (0.0265 g, 0.1 mmol) were weighed and added to an oven-dried Schlenk tube with a stirrer, followed by addition of ε-caprolactone (10.5 mL, 95.0 mmol) and toluene (20 mL). The closed Schlenk tube was taken out of the glovebox and stirred at 100 °C for 20 h. The reaction was quenched by addition of wet $CHCl_3$ (100 mL). The polymer was purified by repeated dissolution and precipitation ($CHCl_3$ and diethyl ether, 3×) and dried under vacuum to yield a white solid (11.5 g, 96.0%). Please refer to the labels for NMR characterization in Figure S1. ^{1}H NMR (400 Hz, $CDCl_3$, δ): 5.14 (bs, H-5); 5.00 (H-2); 4.0−4.3 (m, H-6, H-6′; H_F); 2.33 (m, H_B); 2.16 (s, $COCH_3$); 2.09 (s, $COCH_3$); 1.89 (m, H-3, H-3′); 1.60−1.80 (m, H-4, H-4′, H_E, H_C); 1.40 (m, H_D). $^{13}C\{^1H\}$ NMR (100 Hz, $CDCl_3$, δ): 173.5 (C═O); 64.1 (C_F); 34.1 (C_B); 28.3 (C_C); 25.5 (C_D); 24.6 (C_E). SEC($CHCl_3$, 1 mL/min), M_n = 63 200, PDI = 2.08.

The same general procedure was used for the preparation of **P1**−**P9** and PCL. More details are listed in Table S1.

$scCO_2$ Foaming. The bulk polymer was cryo-ground into a coarse powder. 300 mg of this powder was placed into individual cylindrical wells (11.2 mm × 10.4 mm), for each polymer, in a custom-made PTFE mold (University of Nottingham, School of Chemistry). The

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PTFE mold was placed into a 60 mL stainless steel autoclave, which was clamp-sealed, heated to 35 °C, filled with CO_2, and pressurized to 20 MPa over a period of 20 min. The pressure was held for 60 min and then reduced over a period of 30 min back to ambient conditions; heating then ceased, and the mold was removed from the autoclave. A BSA loaded polymer scaffold was foamed in the similar way, except BSA concentrated water solution (75 mg/mL, 0.2 mL) was added with polymer to PTFE molds such that 5 wt % loading of BSA in the copolymer was achieved. The polymer/BSA was then foamed in $scCO_2$ as previously described.

ISO 10993-5 Cytotoxicity Test. MC3T3-E1 cells were used to assess cytotoxicity in accordance with ISO 10993-5. Cells were cultured at 37 °C, 5% CO_2, in complete medium composed of Minimum Essential Medium α (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 50 μg/mL gentamycin (Invitrogen, Paisley, UK). Cells were plated in 96-well tissue culture plates at 20 000 cells/cm^2 and incubated for 48 h to form a subconfluent monolayer. PCL and **P4** foam samples, a nontoxic PVC (Med7539 noDop) tubing negative control, and organo-tin stabilized PVC sheet (both kindly supplied by Raumedic, Munchberg, Germany) were sterilized with 70% ethanol for 30 min, washed three times with sterile PBS, and pretreated with complete medium for 24 h. Fresh medium was then substituted at 5 mL/g polymer and conditioned for 24 h at 37 °C, 5% CO_2. The subconfluent MC3T3 monolayers were then cultured in the conditioned medium, and viability was assessed at 72 h with the 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT product, solubilized in DMSO, was quantified by the difference in absorbance between 570 and 650 nm on a Spectra Max M5 (Molecular Devices, UK), with values normalized to the negative control.

Cellular Viability and Morphology. MC3T3 preosteoblasts were seeded at 20 000 cells/cm^2 onto PCL and **P4** films and cultured in nonadherent agarose-coated wells following the same culture conditions, sterilization, and pretreatment as outlined for the ISO 10993-5 test. Following 14 days of culture, the metabolic activity of the samples was assessed by Alamar Blue (Invitrogen, Paisley, UK) which was diluted 1:10 in growth medium, incubated for 4 h, and analyzed at 540 nm excitation, 580 nm emission on a Spectra Max M5 (Molecular Devices, UK). Primary bovine chondrocytes were isolated from the lower leg joint of young calves as described by McCullen et al.[64] Chondrocytes were expanded to passage 2 in Dulbecco's Modified Eagles Medium supplemented with 10% FBS and 50 μg/mL gentamycin (Invitrogen, Paisley, UK). PCL and **P4** scaffolds were sterilized with 70% ethanol for 30 min, washed three times with sterile PBS, and pretreated with complete medium for 24 h. Scaffolds were placed on nonadherent agarose-lined 24-well plates and seeded with aliquots of 2 million chondrocytes in 100 μL of growth medium. Following 2 h of adhesion, additional growth medium was added and the scaffolds were cultured for 7 days at 37 °C, 5% CO_2. Samples were fixed in 3.7% w/v formalin for 15 min and washed with PBS. Actin fibers were stained with 1/300 Alexa Fluor 568 phalloidin. Nuclei were stained simultaneously with 1/10 000 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen, Paisley, UK) for 15 min. Samples were washed with PBS and visualized on an Olympus BX51 fluorescent microscope equipped with an Olympus DP70 camera.

Protein Release. PCL and **P4** foams were produced containing 5% w/w bovine serum albumin (BSA). Protein release from the foams was assessed by incubation in PBS at 37 °C. The 10 mL volume of PBS was replaced at 3 h, 24 h, and every day thereafter to 7 days. Aliquots were frozen at −20 °C, for later analysis by a Bradford assay (Sigma-Aldrich, Dorset, UK) calibrated to a BSA. Protein release was summed over the week of experiments and expressed relative to the theoretical BSA content of the scaffold.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details of the polymerization reactions, ^{1}H NMR spectrum of the copolymer, TGA and DSC analyses, mecury porisometry tests, protein release studies, absolute storage moduli, stress-strain plots and water contact angle measurements, and stress−strain curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

TLC, thin layer chromatography; NMR, nuclear magnetic resonance spectroscopy; SEC, size exclusion chromatography; DSC, differential scanning calorimetry; TGA, thermal gravimetric analysis; $scCO_2$, supercritical carbon dioxide; DMTA, dynamic mechanical thermal analysis; SEM, scanning electron microscopy; PLGA, poly(lactic-*co*-glycolic acid); PCL, poly(ε-caprolactone); PEG, poly(ethylene glycol); ROP, ring-opening polymerization; CL, ε-caprolactone; FBS, fetal bovine serum; MTT, tetrazolium bromide assay; BSA, bovine serum albumin.

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