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Poly(limonene thioether) Scaffold for Tissue Engineering

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Abstract

A photocurable thiol-ene network polymer, poly(limonene thioether) (PLT32o), was synthesized, characterized, fabricated into tissue engineering scaffolds, and demonstrated in vitro and in vivo. Micromolded PLT32o grids exhibited compliant, elastomeric mechanical behavior similar to grids made of poly(glycerol sebacate) (PGS), an established biomaterial. Multilayered PL32o scaffolds with regular, geometrically defined pore architectures supported heart cell seeding and culture in a manner similar to multilayered PGS scaffolds. Subcutaneous implantation of multilayered PLT32o scaffolds with cultured heart cells provided long term 3D structural support and retained the exogenous cells, whereas PGS scaffolds lost both their structural integrity and the exogenous cells over 31 days in vivo. PLT32o membrane implants retained their dry mass, whereas PGS implants lost 70 percent of their dry mass by day 31. Macrophages were initially recruited to PLT32o and PGS membrane implants but were no longer present by day 31. Facile synthesis and processing in combination with the capability to support heart cells in vitro and in vivo suggest that PLT32o can offer advantages for tissue engineering applications where prolonged in vivo maintenance of 3D structural integrity and elastomeric mechanical behavior are required.

Keywords

thiol-ene polymer; biomaterial; heart cell; in vivo

1. Introduction

Important specifications for tissue engineering templates were recently summarized to include: (i) capability to recapitulate the architecture of the niche of the target cells, (ii) elastic properties, particularly stiffness, (iii) surface properties to facilitate cell adhesion and function and (iv) minimal immunogenicity.[1] While numerous materials are being used for tissue engineering research,[2] in the present study we investigate synthetic polymers which, as compared with naturally occurring materials, can be more precisely processed into the 3D pore architectures that can guide cellular organization, and also afford a wider range of

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thermomechanical properties.[3-6] Materials-based therapies have been applied to cardiac disease [7], which causes significant morbidity and mortality worldwide.[8] These approaches have focused on porous elastomeric structural templates that can enable cell adhesion and assembly into functional muscle-like tissue.[3, 9, 10-16]

Wang et al.[17] envisioned poly(glycerol sebacate) (PGS) as a synthetic, biocompatible, biodegradable elastomer with mechanical properties suitable for medical applications. Solid PGS implants degraded by surface erosion, lost >70 % of their dry mass 5 weeks after subcutaneous implantation in rats, and did not induce persistent inflammation or scar tissue.[18] However, microfabricated PGS degraded quickly, within 2 to 4 weeks in vivo,[14, 15] which can be attributed to high surface area per unit mass of thin porous scaffolds[4]. These studies, and others,[19-21] demonstrated that PGS degraded too quickly to provide long term 3D structural support that could be useful for the regenerative repair of soft tissues that are slow to heal, such as infarcted myocardium.[22] Although more slowly biodegradable versions of PGS have been invented,[23-26] a photocurable thiol-ene network polymer could offer more facile prepolymer synthesis and processing and thereby enable more rapid and higher throughput microfabrication into scaffolds with defined 3D pore architectures.

The thiol-ene “click reaction” is a powerful synthetic tool that proceeds in the presence of oxygen at ambient temperature and in the presence of UV light and a photoinitiator. Hubbell and co-workers first described photopolymerizable degradable thiolacrylate systems,[27] and Anseth and co-workers custom synthesized and functionalized thiolacrylates to control degradation rate and cell-material interactions.[28, 29] Hearon et al.[30] demonstrated a one-pot synthesis of a thiol-ene polymers wherein brief heating and/or UV irradiation of a mixture of trimethylol-propane tris(3-mercaptopropionate) (TMPTMP) and the naturally occurring citrus-derived extract D-limonene afforded a homogenous solution that could be transferred to a mold without further polymer network formation occurring until subsequent exposure to UV irradiation. Synthetic, processing and environmental health and safety advantages of PLT32o make this material an attractive candidate for biomedical engineering applications where recapitulation of the niche of the target cells through fabrication of complex templates with patterned 3D pore microstructures is desired.

The goal of the present study was to investigate whether PLT32o could enable the photopolymerization and microfabrication of elastomeric polymer scaffolds with organized 3D pore microstructures, and provide long in vivo half-life. We reasoned that scaffolds based on PLT32o might be of relevance to tissue engineering, and consequently we characterized PLT32o with respect to physicochemical properties (wettability, swelling, mechanical behavior after microfabrication) and performance as a biomaterial (capability to provide heart cells with 3D structural support for 7 days in vitro and 31 days in vivo). PGS, an established tissue engineering biomaterial, was selected to serve as a control.

2. Results

2.1 Polymer chemical structures and analyses

The chemical structures of PLT32o and PGS are respectively shown in **Figure 1A** and Figure 1B. PLT32o is the network polymerization product of a trifunctional alkene macromonomer, TMPTMP, end-capped with D-limonene, and a difunctional thiol comonomer, 1,10-decanedithiol (1,10 DDT). PGS is the branched polyester polymerization product of glycerol and sebacic acid. A photoreactor for carrying out a solvent-free 100 g scale synthesis of the PLT32o macromonomer was developed, and after addition of 1,10 DDT to this macromonomer, PLT32o was photocured by exposure to 365 nm UV light under ambient conditions for 2 h. By contrast, PGS preparation was more complex, requiring a nitrogen atmosphere for pre-polymer synthesis, heat (≥120°C), vacuum (<40 mTorr) and an extended time (72 h) for curing. Proton NMR data were recorded for D-limonene, TMPTMP, and three batches of the unpurified macromonomer shown in Figure 1A. The three batches of macromonomer exhibited similar chemical shifts and signals, suggesting reproducibility of the described synthetic method. Fourier transform infrared (FTIR) spectra were also recorded for the aforementioned species. The FTIR spectra showed: a C=C stretch at ~1640 cm^{-1} for D-limonene, C=O functionality at ~1725 cm^{-1}, thiol functionality at ~2560 cm^{-1} for TMPTMP, and both the C=C stretch and the C=O stretch for the macromonomer (**Figure S1**). Additionally, the macromonmer lacked the thiol stretch that was present in the TMPTMP starting material. Gel fractions approached 100% for both PLT32o and PGS, demonstrating high degrees of network formation and minimal sol content after ethanol/water ($EtOH/H_2O$) leaching process used prior to cell culture and additional swelling in dichloromethane (DCM) (Figure 1C). Uptakes of phosphate buffered saline (PBS) were very low (≤3%) for both PLT32o and PGS, with PLT32o swelling more slowly than PGS (Figure 1D). Substrate preconditioning by incubation at 37°C for 48 h in serum-supplemented medium markedly improved wettability of PLT32o and PGS, as shown by significant reductions in water contact angles (Figure 1E).

2.2 Polymer micromolding

Polymer micromolding by hot embossing[31] was selected as a rapid, high throughput, low cost method to create thin layers with rectangular grid-like through-pores and 1:1 aspect ratio (height:width) microfeatures. The rectangular through-pore with intervening solid strut design was selected because rectangles are structurally anisotropic and are capable of aligning collagen fibers,[32] as well as C2C12 myoblasts and heart cells.[10, 12, 13] The resolution of this method, determined by the smallest dimension (strut width) of PLT32o and PGS grids that could be readily demolded, was ~125 μm. There was no significant difference between PLT32o grids and PGS grids with respect to strut width, pore dimensions, or thickness (**Table 1**), and the final grid feature dimensions very closely matched those of the master mold.

2.3 Mechanical characterization

To determine mechanical behavior of PLT32o and PGS grids, dynamic mechanical analysis and tensile testing until material failure were performed; bulk membranes were also tested to provide controls. Dry PLT32o and PGS grids behaved mechanically as compliant

elastomers, with high strain-to-failure values exceeding 0.5 mm/mm and low moduli. Young's moduli of PLT32o and PGS grids, which did not differ significantly from one another, were respectively 410 ± 75.5 kPa and 379 ± 72.6 kPa. Effects of geometry (grid versus solid membrane) and material (PLT32o versus PGS) are shown in **Figure 2**. Geometry had a significant effect on all four parameters tested, with lower values obtained for grids than for membranes. For the subset of solid membranes, material had a significant effect on all four mechanical properties measured, with higher values for PLT32o than PGS.

2.4 Heart cell cultivation on polymer scaffolds

Heart cells were tested on multilayered scaffolds that were fabricated by combining two grids made of either PLT32o or PGS with an underlying porous PGS membrane (**Figure 3** A,B). Briefly, the grids were manually aligned and stacked with short strut offset (SSoff), solvent bonded together and then the pair was bonded onto a PGS membrane with ~30 μm diameter sphere templated pores, as previously described.[15] Heart ventricles were harvested from 1 to 2 day old neonatal rats following a protocol according to an Institutional Animal Care and Use Committee (IACUC), and heart cells were isolated using a GentleMACS Dissociator.[13] Heart cells adhered to and formed multilayered tissue-like structures on multilayered scaffolds, as shown by DNA assay of cell number and actin-phalloidin staining of cell morphology (Figure 3 C-E). Cell number, determined after 2 days of culture, showed that heart cells adhered in similar quantities to PLT32o and PGS grids at a time point just before the planned implantation in vivo. Cell morphology, determined after 7 days in vitro, showed similar degrees of heart cell spreading and multicellular tissue formation on PLT32o and PGS grids. Heart cells also attached to and remained viable on solid PLT32o and PGS membranes with no differences observed between the two materials (**Figure S1** A,B,G). A murine cell line (C2C12 myoblasts) also showed similar attachment to PLT32o and PGS membranes and remained viable on both materials (Figure S1 C-F,H). Earlier time points of 1 and 4 days were examined for C2C12 cells which, unlike the heart cells, had reached confluence by culture day 4.

2.5 Polymer scaffold degradation and heart cell retention in vivo

To investigate whether multilayered PLT32o or PGS scaffolds with 3D anisotropic pore architectures could provide structural support and retain exogenous heart cells upon implantation in vivo, the multilayered scaffolds shown in Figure 3 A-B were seeded with neonatal rat heart cells and implanted subcutaneously in NIH homozygous RNU nude rats according to an IACUC-approved protocol; scaffolds without added heart cells were implanted as controls. Explants were harvested after 7 or 31 days and histologically evaluated for general appearance by hematoxylin & eosin (H&E) staining and for cardiomyocytes by immunostaining for the specific cardiac isoform troponin T ($cTnT^+$). PLT32o scaffolds maintained their original 3D pore architectures over the entire 31 day study (**Figure 4** A-F), whereas PGS scaffolds lost their structural integrity by day 31 (Figure 4 G-I). Cardiomyocytes were detected at both the 7 and 31 day time points in the group of PLT32o scaffolds implanted with exogenous heart cells (Figure 4 E-F). No cardiomyocytes were detected at either time point in any of the other three groups, i.e., PLT32o scaffolds implanted without exogenous heart cells (Figure 4 B-C), PGS scaffolds implanted with exogenous heart cells (Figure 4 H-I) or PGS scaffolds implanted without exogenous cells

(not shown). Also, the PGS scaffolds completely lost their structural integrity by day 31 (Figure 4G).

2.6 Time histories of polymer appearance and mass in vivo

Solid polymer membranes were selected for in vivo degradation studies in order to accurately assess remaining dry mass, which would not have been feasible after implantation of scaffolds due to an inability to separate the polymer from the ingrown tissue. To assess polymer degradation kinetics in vivo, solid PLT32o and PGS membranes were implanted subcutaneously in Sprague Dawley rats according to an IACUC-approved protocol. Explants were evaluated histologically and by dry mass loss after 7, 14, and 31 days in vivo. The PLT32o membranes retained their initial dry mass for the entire 31 day study, whereas the PGS membranes degraded rapidly after day 14 with a loss of ~70 % of the initial dry mass by day 31 (**Figure 5A**). As a preliminary investigation of host immunological response to PLT32o, explanted polymers were stained for the macrophage marker CD68. Macrophages that were initially observed to surround the PLT32o and PGS membranes on days 7 and 14 (Figure 5 B,C,E,F) were essentially no longer present on day 31 (Figure 5 D,G), suggesting transient inflammatory responses to both materials as previously described for PGS.[33]

3. Discussion

The present work introduces, characterizes and demonstrates PLT32o, an elastomeric thiol-ene network polymer, for a soft tissue engineering application. The photoreactor for carrying out one-pot, 100 g scale syntheses of PLT32o was a robust system that required no harsh chemicals. PLT32o grids were rapidly microfabricated by photocuring in low cost hot-embossed molds and assembled into multilayered scaffolds with 3D pore architectures. With respect to mechanical behavior and compatibility with cultured heart cells, microfabricated PLT32o grids performed on par with PGS, a polymer that has been widely used as a tissue engineering scaffold for more than a decade.[4, 17-20] PLT32o can potentially advance to the field of tissue engineering because, like PGS, PLT32o has physicochemical properties that are compatible with cells and tissues but, unlike PGS, PLT32o offers synthetic and processing advantages that enable implanted scaffolds with 3D pore architectures to maintain their structural integrity and retain exogenous heart cells for 31 days in vivo.

Chemical structures and properties, including a balance of surface hydrophobicity and hydrophilicity, wettability and functional groups, contribute importantly to the biocompatibility of polymeric materials.[34] PLT32o contains ester and thio-ether linkages in its backbone and PGS also contains ester linkages, although PLT32o has substantially fewer ester linkages per network repeat unit as compared with PGS. Gel fractions approached 100% for both PLT32o and PGS, demonstrating high degrees of network formation and <2% sol content after the EtOH/H_2O leaching process used prior to cell culture. The finding that EtOH removed less sol from PLT32o than from PGS and subsequent swelling in DCM removed more sol from PLT32o than from PGS could indicate more hydrophilic sol content for the PGS (i.e., residual glycerol or sebacic acid monomers and oligomers with pendant hydroxyl or carboxylic acid groups) and more hydrophobic sol content for the PLT32o (i.e., residual limonene or limonene-capped polythiol species). The wettability of PLT32o and

PGS was markedly enhanced by preconditioning substrates in serum-supplemented culture medium for 48 h at 37°C, as determined by reduction in water contact angle. This finding, which can be attributed to nonspecific adsorption of serum biomolecules, has been previously used to precondition polymers by using either serum,[12, 13, 35] or extracellular matrix proteins,[36] and has been selected for previous cardiac tissue engineering studies based on the reasoning that heart cells should interact with scaffolds only to the degree required for retention during surgical handling and localization at the implant site, but not to a degree that would inhibit cell-to-cell interaction and multicellular tissue formation either in vitro or in vivo. Neither PLT32o nor PGS provides specific functional groups for cell-receptor binding, and consequently cell adhesion to both polymers is expected to occur nonspecifically. An alternative approach others have taken to control cell-polymer interactions has been to functionalize polymers, such as by adding RGD to thiol-acrylates,[29] or YIGSR to PGS.[37]

PLT32o, which is curable by UV irradiation under ambient conditions over 2 h, can be rapidly microfabricated at high throughput into scaffolds with defined 3D pore architectures as compared with PGS, which requires curing under extreme conditions of heat and vacuum for an extended time periods of up to 72 h.[4, 17, 19] By using master molds that were laser cut in a few minutes and hot embossed plastic molds that were likewise fabricated in a matter of minutes, PLT32o and PGS grids were fabricated with dimensions (125 μm strut width, 125 μm layer thickness) suitable for stacking and bonding into multilayered tissue engineering scaffolds. The finding of low swelling (≤3%) is expected to facilitate micromolding of PLT32o and PGS as compared with hydrogels, such as methacrylated gelatin[38] and thiolacrylates.[28] Although silicon wafer molding can be used to fabricate PGS grids with smaller strut width (~30 μm) and higher aspect ratio features (~3:1),[12, 13] hot embossed molding offers other advantages of rapid prototyping and reduced scaffold manufacturing time and cost.[31] Microfabricated PLT32o and PGS grids exhibited elastomeric mechanical behaviors and Young's moduli of ~400 kPa; for comparison, Young's moduli for human myocardium are reportedly ~10 to 20 kPa at the start of diastole and 200 to 500 kPa at the end of diastole.[2, 4] One drawback common to both PLT32o and PGS grids is that their stiffnesses were too high to permit macroscopic heart cell-mediated contractility. Future studies can explore alternative pore designs, such as accordion-like honeycombs, and lower polymer volume fractions than the rectangular grids tested herein in an effort to reduce scaffold stiffness.[10, 11, 16] Future studies can also investigate the mechanical properties of the grids under physiological conditions, as hydration was shown to lower the stiffness of electrospun acrylated PGS in vitro,[25] and can address the time history of scaffold mechanical properties following implantation.

Multilayered scaffolds based on PLT32o or PGS grids supported the in vitro cultivation of heart cells, which attached in similar numbers to both materials and remained attached for at least 7 days. Future in vitro studies will be needed to address cytotoxicity over a longer time period than 7 days since the findings of the in vivo degradation study suggest that PLT32o degradation products would only be produced over an extended time period. As part of an ongoing effort to develop thick, viable myocardial grafts by creating a rapidly degradable interface to a perfusable microchannel network, two offset grids were bonded to a porous PGS membrane in the present study and our previous study,[15] and macroscopic

contractions were not observed in either study. Future studies can use more sensitive methods to assess contractility, such as force transducers[39] or optical mapping of intracellular calcium transients.[40]

Unlike PGS, PLT32o offered synthetic and processing advantages that enable implanted scaffolds with 3D pore architectures to maintain their structural integrity and retain exogenous heart cells for 31 days in vivo, which can be concluded from the combination of the pre-implantation data showing similar initial heart cell numbers and morphologies for scaffolds based on either PLT32o grids or PGS grids, and the endpoint histological analyses showing retention of structural integrity and cardiomyocytes only for the PLT32o-based scaffolds. The finding that PGS scaffolds degraded rapidly could be mitigated to some degree by increasing the volume fraction of polymer in the scaffold, but this change would also decrease the volume fraction of pores in the scaffold thereby reducing the volume available as a niche for heart cell seeding and multicellular tissue assembly. PGS modified by acrylation degraded more slowly than PGS [23, 25] but, like other acrylated polymer systems,[27, 28] required highly toxic reagents (e.g., acryloyl chloride) for synthesis. PGS modified either by introducing amide linkages [24] or by adding silica to form nanocomposites [26] also degraded more slowly than PGS, but, like PGS, required extended times (24-72 h) and extreme conditions of heat (>120°C) and vacuum (<40 mTorr) for polymerization. As such, PLT32o provides synthetic, processing, and/or environmental health and safety advantages as compared with slowly degradable versions of PGS that were reported previously.

Implanted PLT32o membranes retained their dry mass whereas PGS membranes lost ~70% of their initial mass over 31 days in vivo which was similar to previous reports.[4, 18, 19, 21] The finding that PLT32o did not biodegrade within 31 days can be explained by its low PBS uptake and low average ester composition per network repeat unit as compared with PGS. One drawback of PLT32o for tissue engineering applications is that its degradation rate may be too slow, as no degradation was measured in the present 31 day in vivo study. Future work can include longer in vivo studies, and can explore new PLT formulations with degradation rates in-between PGS and PLT32o. The finding that multilayered scaffolds based on PLT32o grids retained exogenous cardiomyocytes for 31 days warrants further investigation. It is reasonable to expect that heart cell retention can be improved by seeding the cells in association with a hydrogel such as fibrin or Matrigel.[16, 41, 42] While PLT32o scaffold testing in a cardiac implantation model was beyond the scope of the present work, future testing on the myocardium and in the setting of an infarction will be necessary because differences in implantation site and local conditions are known to affect the degradation rate and performance of biomaterial implants.[43] In a cardiac implant model it will be important to determine whether PLT32o scaffolds can support MI healing over its typical time course of 4 to 12 weeks,[22] and to quantify vascularization of the scaffold, cardiomyocyte survival and retention, and host immune responses.

4. Conclusion

PLT32o, a photocurable elastomeric thiol-ene network polymer, was synthesized, characterized, microfabricated and demonstrated in vitro and in vivo in the context of a

cardiac tissue engineering scaffold. Micromolded PLT32o grids exhibited compliant elastomeric mechanical behavior, and multilayered PLT32o-based scaffolds with 3D pore architectures supported heart cell seeding and culture. Subcutaneous implants of multilayered PLT32o scaffolds with cultured heart cells provided long term 3D structural support for retention of exogenous cells, whereas PGS scaffolds lost both their structural integrity and the exogenous cells over 31 days in vivo. PLT32o membrane implants retained their dry mass, whereas PGS implants lost 70 percent of their dry mass by day 31. Facile synthesis, processing and capabilities to support heart cells in vitro and in vivo suggest that PLT32o can offer advantages in tissue engineering or other biomedical engineering applications where prolonged in vivo maintenance of 3D structural integrity and elastomeric mechanical behavior are required.

5. Experimental Section

Polymer synthesis, micromolding and mechanical characterization

To synthesize PLT32o pre-polymer, a one-pot synthesis,[30] was carried out using a D-limonene-capped TMPTMP macromonomer similar to the M1 Macromonomer reported in Scheme 4 by Claudino et al.[5] and the co-monomer 1,10 DDT. To create a photoreactor, a UVP CL-1000 cross-linking chamber with 365 nm bulbs was positioned on top of a magnetic stir plate in which UV irradiation of monomers could be carried out under vigorous stirring conditions. Step 1 reagents (1.0 molar equivalents limonene (f=2 C=C), 0.25 molar equivalents of TMPTMP (f = 3 -SH) and 0.25 % (w w^{-1}) 2,2-dimethoxy-2-phenylacetephenone (DMPA) with respect to Step 1 constituents) were massed in 100 mL glass bottles and magnetic stirrers were added to each bottle. For Step 1, each prepolymer mixture in 100 mL glass bottles was subjected to ~365 nm UV irradiation for 3 h with stirring. After a 3 h photoreaction, Step 2 reagents (0.25 molar equivalents TMPTMP and 0.25 % (w w^{-1}) DMPA with respect to added Step 2 constituents) were then added to the reaction mixtures in the same 100 mL glass bottles and subjected to an additional 3 h of irradiation with stirring. For Step 3, 0.575 molar equivalents of 1,10 DDT were added to the reaction mixture to afford a prepolymer mixture with a 0.93:1.00 ratio of C=C to SH groups. Additional DMPA was also added to the reaction mixtures after Step 3 to afford an overall 1 % (w w^{-1}) DMPA concentration. PLT32o prepolymer mixtures were heated to 80°C for 20 min after Steps 1 and 2, and for 1 h after Step 3 to enable DMPA dissolution. PLT32o prepolymer mixtures were stored in the dark until needed. To synthesize PGS pre-polymer, a previously described method was used with minor modifications.[17] 1.0 mol glycerol and 1.333 mol sebacic acid were stirred vigorously in a 500 mL round bottom flask under a nitrogen atmosphere with a constant nitrogen purge at 140°C for 24 h. After 24 h, PGS prepolymer, which was a molten liquid, was poured from the flask in which it was synthesized into a 250 mL glass bottle, and 200 proof ethanol was added to the PGS prepolymer to afford a working solution of 33 % (w w^{-1}) PGS prepolymer.

Proton NMR data were recorded with a Varian inverse probe INOVA-500 spectrometer with a Magnex Scientific superconducting actively shielded magnet, reported in parts per million on the δ scale, and referenced from the residual protium in the solvent ($CDCl_3$: δ 7.24),[44] displaying a window range of 9 to −0.5 ppm. Infrared data were obtained for the same three

materials with a Bruker Alpha FTIR spectrometer. Samples were collected neat on a ZnSe ATR crystal, and spectra were reported as percent absorbance as a function of frequency of absorption (cm^{-1}). Sol/gel analysis included determining initial dry mass (M_0) of cured PLT32o or PGS membranes, leaching with shaking at room temperature (RT) for 24 h in 200 Proof EtOH (>10 mL solvent per 50 mg polymer) leaching with shaking at RT for another 24 h in dIH_2O, for the EtOH/H_2O group drying for 18 h at 60°C and 40 mTorr and obtaining final mass (M_f), and, for the DCM group, immersion with shaking at RT for another 24 h in DCM and then drying and obtaining M_f as described for the EtOH/H_2O group. Gel fraction was expressed as the ratio of final mass to initial mass (M_f/M_0). Swelling was quantified measuring intial mass of PLT32o and PGS membranes that were dried for 24 h at 60°C and 40 mTorr, incubated at RT in PBS at 37°C while shaking, and measuring final mass of specimens at timed intervals. PBS uptake was expressed as a percentage ($100*M_f/M_0$). Contact angles were measured for PLT32o and PGS membranes after drying for 24 h at 60°C and 40 mTorr and then again after preconditioning by incubation at 37°C for 48 h in serum-supplemented heart cell culture medium. A sessile drop (5 μL) of Ultra-pure water was placed on the substrate and the drop profile was recorded at 1 s intervals for 60 s using a DSA 100 contact angle system (KRÜSS GmbH, Germany).

PLT32o and PGS grids were fabricated in hot embossed molds. Master molds was made from 125 ± 10.2 μm thick poly(imide) (PI) sheets (product #2271K6, McMaster-Carr, Robbinsville, NJ) and molds were made either from poly(methyl methacrylate) (PMMA, 5 cm × 5 cm × 0.599 cm, Interstate Plastics high molecular weight ACRCLCP) or polycarbonate (PC, 5 cm × 5 cm × 0.159 cm, McMaster-Carr, Robbinsville, NJ). Master molds were fabricated using a programmable Protolaser U3 (LPKF Laser & Electronics, Tualatin, OR). Molds were initially embossed at 198°C and 15 psi and 5 min using a custom-built laminator and subsequently embossed batch-wise using a Carver Press (AutoFour/3012H, Wabash, IN) at 170°C, 16,000 lb and 5 min for PMMA or at 170°C, 8,000 lb and 20 min for PC.

PLT32o grids were micromolded made by adding prepolymer (20 μL) to a PC mold, photocuring at 365 nm for 2 h, and post-curing at 120°C and 15-40 mTorr for 20 h, whereas PGS grids were micromolded by pipetting 33 % (w w^{-1}) PGS prepolymer in EtOH (80 μL) into a PMMA mold, heating on a 55°C hot plate for 2 h, adding additional PGS prepolymer solution (80 μL) into the same mold, and vacuum curing at 120°C and 15-40 mTorr for 72 h. Grids were demolded from PMMA by immersion in acetone for ~15 to 40 min or demolded from PC by immersion in dioxane for ~10 min. Solid PLT32o membranes were made by injecting prepolymer into a chamber between two glass slides (5.08 × 7.62 × 0.1 cm) separated by glass spacers (5.08 × 1.0 × 0.1 cm) after coating the glass with Rain-X® original to facilitate demolding, and photocuring as described above for PLT32o grids, whereas PGS membranes were prepared by casting 33 % (w w^{-1}) PGS prepolymer in EtOH on a PMMA surface and evaporating the ethanol on a 55°C hot plate for 4 h, and vacuum curing as described above for PGS grids. Grids and membranes were first immersed in 200 proof EtOH for ~24 h with shaking to remove sol, and then rinsed in dIH2O for ~24 h with shaking, and dried at RT. Specimen thicknesses were measured with a dial gauge (accuracy 0.01 mm; L.S. Starrett Co., Athol, MA). Master molds were imaged with a 200M Axiovert microscope (Carl Zeiss, Thornwood, NY) and grids were imaged by SEM (JSM-5600OV,

JEOL, Peabody, MA). ImageJ software was used to quantify feature sizes for at least 2 specimens and 3 fields with 5 to 10 measurements per field.

Mechanical assessments of PLT32o and PGS grids included storage modulus at 1 Hz, Young's modulus, ultimate tensile strength (UTS), toughness and strain-to-failure. Grids were cut into test strips and tested using a DMA Q800 (TA Instruments, New Castle, DE) with an 18 N load cell, in the Multi-Frequency/Strain mode using a preload force of 0.01 N, a strain of 0.1 % and a Force Track of 150. On average, the specimens (n ≥7 per group) had dimensions of 8.7 mm long x 6.3 mm wide x 0.087 mm thick (for PLT32o) and 8.2 × 6.2 × 0.091 mm (for PGS). Storage moduli of PLT32o and PGS membranes were assessed as described above for grids. Young's moduli, UTS, toughness and strain-to-failure were assessed for polymer membrane specimens cut with an ASTM D638 Type V dog bone die on model 5943 Instron with a 10 N load cell at a strain rate of 1% s^{-1}. Young's moduli were determined by linear regression of the stress strain curve over a strain range of 0.005 to 0.10 mm mm^{-1} (R^2>0.98); UTS, toughness and strain-to-failure were respectively taken as the maximum stress and strain measured prior to the onset of failure and the area under the stress-strain curve.[10]

Heart cell studies in vitro and in vivo

In vitro studies and in vivo studies of heart cells were carried out on multilayered scaffolds based on PLT32o and PGS grids; solid membrane of PLT32o and PGS provided controls. Two geometries were compared for the multi-layered scaffolds: two short strut offset (SSoff) PLT32o on a PGS porous membrane versus two SSoff PGS grids on a PGS porous membrane. Multilayered scaffolds were made as previously described,[15] by aligning, stacking and solvent bonding the 1L grids on a PGS porous membrane, itself made from a sintered templates of ~30 μm diameter PMMA microspheres, and curing a weight at 120°C and 40 mTorr for 20 h.[15] Substrates were autoclave-sterilized and incubated at 37°C for 48 h in heart cell culture medium (Dulbecco's Modified Eagle Media supplemented with 2 % fetal bovine serum, 10 % horse serum and 1 % penicillin/streptomyocin)[42] prior to cell culture. Immediately prior to cell seeding, the substrates were blotted dry and placed within polydimethylsiloxane gaskets in 24-well plates.[12] Freshly isolated heart cells were seeded at an initial density of ~10 million cells cm^{-2} (for multilayered scaffolds) or ~0.3 million cells cm^{-2} (for membranes). The number of cells per specimen was assessed by the Quant-iT Picogreen assay, assuming 8 pg of DNA per cell as previously described.[15] Heart cell appearance and viability of cells were assessed by actin-phalloidin staining and Live/Dead staining. C2C12 cells (ATCC, Manassas, VA) were also tested on PLT32o and PGS membranes and tissue culture polystyrene (TCPS) well-plates using the same methods of substrate preparation, tissue culture and analyses that were described for heart cells. The C2C12 cells became confluent over 4 days in association with a significant increase in cell number between day 1 and 4 as determined by DNA assay.

In vivo studies of multilayered scaffolds with cultured heart cells were carried out in nude rats; acellular scaffold implants provided controls. Scaffolds were leached in $EtOH/H_2O$, dried, die punched into 6 mm diameter disks, autoclave-sterilized, preconditioned in medium, seeded with neonatal rat heart cells at an initial density of ~10 million cells cm^{-2},

cultured for 3 to 4 days in vitro, and implanted into surgically created dorsal subcutaneous pouches in nude rats (n=9 male NIH homozygous RNU, 7 to 9 weeks old weighing between 170 and 323 g). Each rat received implanted samples from multiple experimental groups. Specimens were oriented with the grids facing the skin and were loosely fixed to the underlying muscle using 6-0 Prolene suture (product# 8697G, Ethicon™). Specimens for histological analyses (n=3 with heart cells or n=2 without heart cells per group per time point) were harvested en-bloc, fixed in neutral buffered formalin (NBF) for 24 h and stored in 70 % EtOH after 7 or 31 days.

In vivo studies of macrophage recruitment and polymer degradation were carried out in immunocompetent rats. PLT32o and PGS membranes were leached in EtOH/H_2O, dried, die-punched into disks (8 mm diameter and 0.5 to 1.0 mm thick), and initial dry mass of each disk was recorded for the degradation analysis. Implants were autoclave-sterilized and inserted into surgically created dorsal subcutaneous pouches in Sprague-Dawley rats (n=18 males weighing between 250 and 290 g), with each rat receiving specimens from both groups. After 7, 14, or 31 days, specimens designated for degradation analysis (n=4 per group per time point) were explanted, denuded of surrounding host tissue, dried at 80°C for 24 h, and weighed to determine final dry mass. Specimens for histological analyses (n=2 or 3 per group per time point) were harvested en-bloc, fixed in NBF and stored in 70 % EtOH.

Explanted specimens were embedded in paraffin, sectioned to 5 μm, and stained with H&E or immunostained. The primary antibodies and dilution factors were: anti-cardiac troponin T (cTnT) (1:400) (MA5-12960, Thermo Fisher, Rockford, IL) and anti-CD68 (1:100) (MAB1435, EMD Millipore, Temecula, CA). Briefly, sections were exposed to antigen retrieval in citrate buffer antigen at 90°C for 20 min, hydrogen peroxide for 5 min, rodent block for 30 min (RBM961, Biocare Medical, Concord, CA), primary antibody for 60 min, rat probe for 15 min, rat polymer (RT517, Biocare Medical) for 15 min, and 3,3’-Diaminobenzidine 10 min prior to coverslipping[45].

Statistical analyses

Statistical significance was assessed by ANOVA with Tukey's *post hoc* analysis using Prism version (v6.0c, Graphpad Software Inc.) Data were reported as average +/− standard deviation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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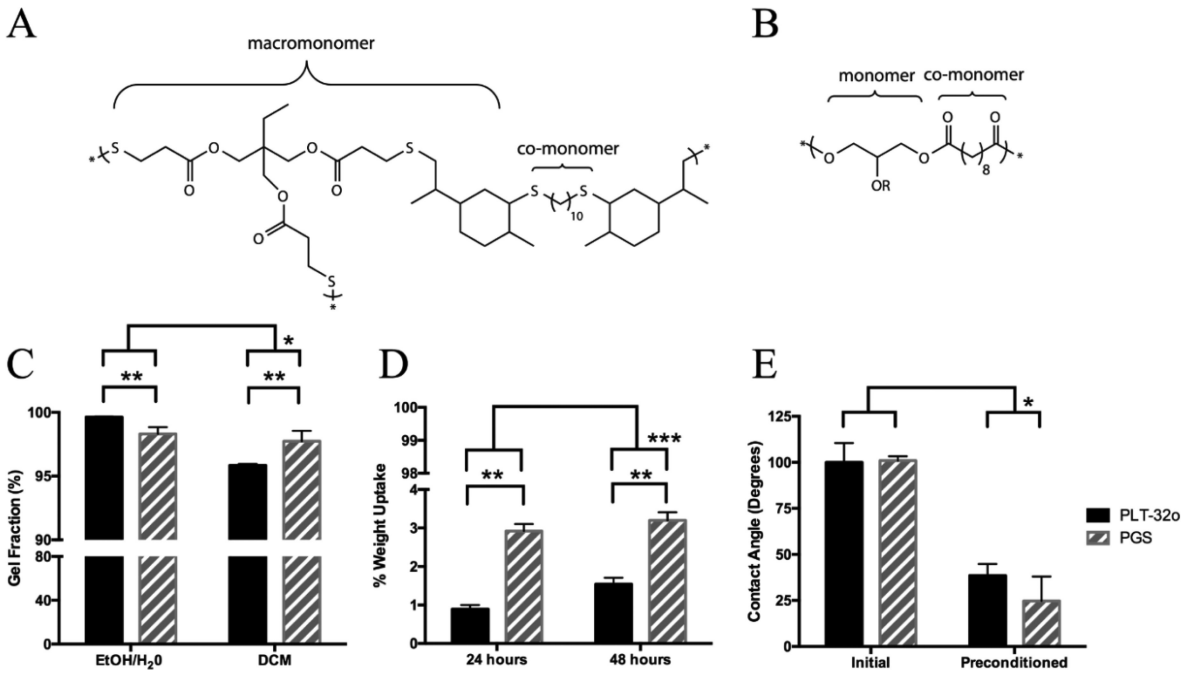

Figure 1. Chemical structures and properties of PLT32o and PGS

(A) PLT32o, the network polymerization product of a trifunctional alkene macromonomer based on TMPTMP end-capped with D-limonene and the difunctional thiol co-monomer 1,10 DDT, (B) PGS, the branched polyester polymerization product of glycerol and sebacic acid, (C) Gel fractions after leaching in EtOH/H_2O and after additional swelling in DCM, (D) PBS uptake after incubation at 37°C for 24 or 48 h. (E) Water contact angle initially and after preconditioning at 37°C for 48 h in serum-supplemented culture medium. Data show AV ± SD for 5 to 8 samples per group; *Significant effects of solvent (C, $p<0.0001$) and preconditioning (E, $p<0.0001$); **Significant effect of material (C, $p<0.005$; D, $p<0.0001$); ***Significant effect of time (D, $p<0.0001$).

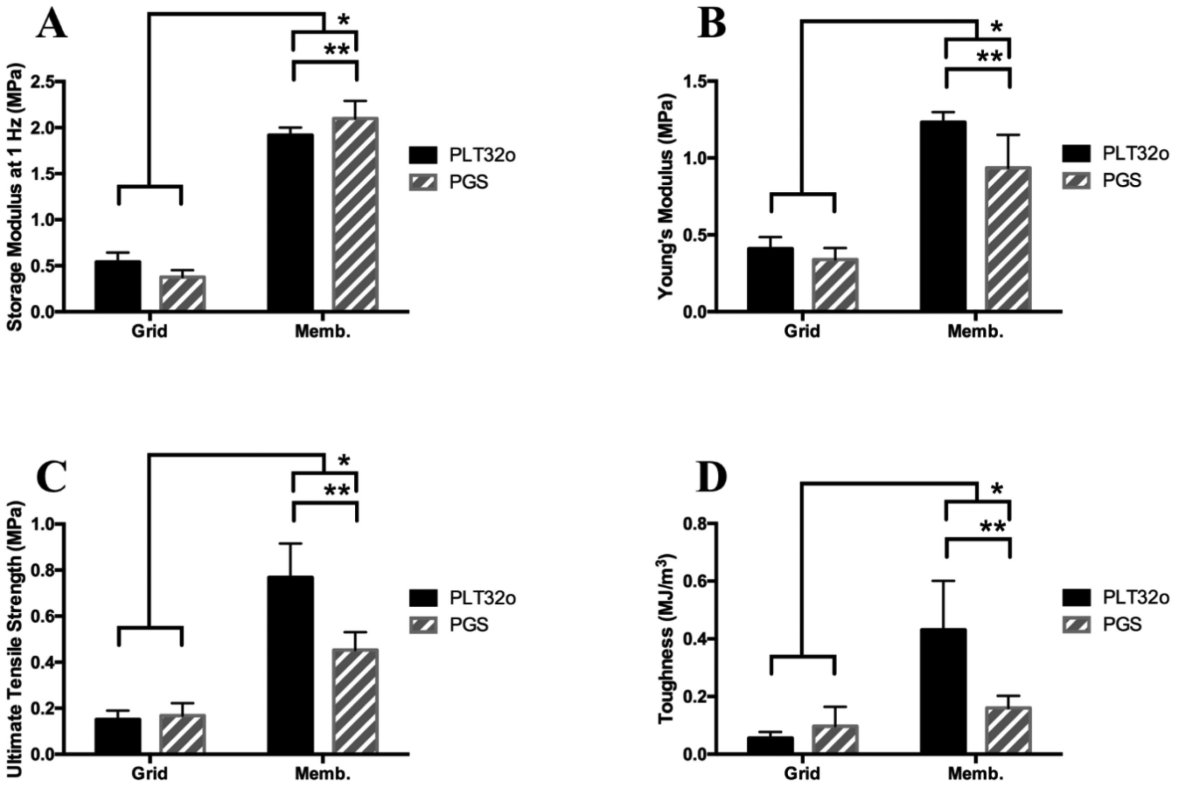

Figure 2. Mechanical behaviors of PLT32o and PGS grids and membranes

(A) Storage modulus at 1 Hz, (B) Young's modulus, (C) ultimate tensile strength, (D) toughness. Data show AV ± SD for replicates of (A) 7 to 9 or (B-D) 7 to 18. *Significant effect of geometry (grid vs. membrane, $p<0.0001$); **Significant effect of material (PLT32o vs. PGS, $p<0.0001$).

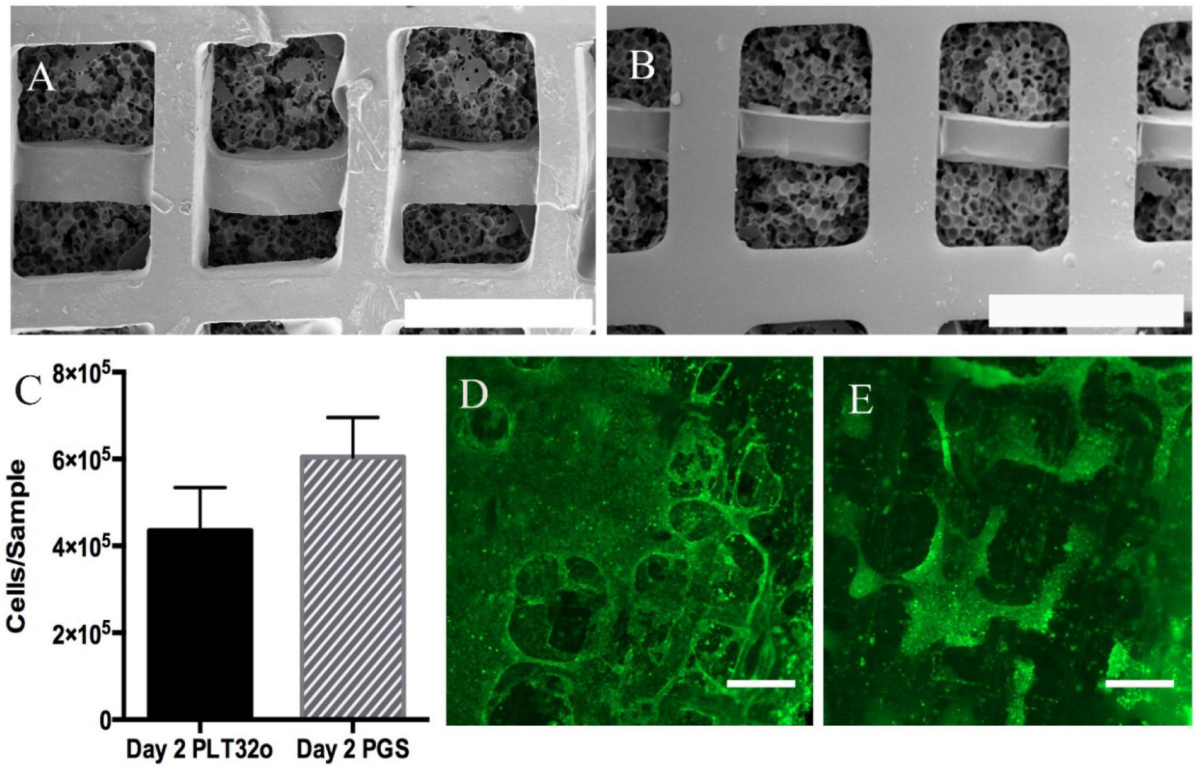

Figure 3. Multilayered scaffolds for heart cell culture

Scaffolds comprised of (A,C,D) two SS_{off} PLT32o grids or (B,C,E) two SS_{off} PGS grids and (A-E) an underlying PGS porous membrane. (A,B) SEM images, (C) DNA analyses after heart cell culture for 2 days, (D,E) confocal micrographs after heart cell culture for 7 days and actin-phalloidin staining. Data show AV ± SD of n=4. Scale bars: 500 μm.

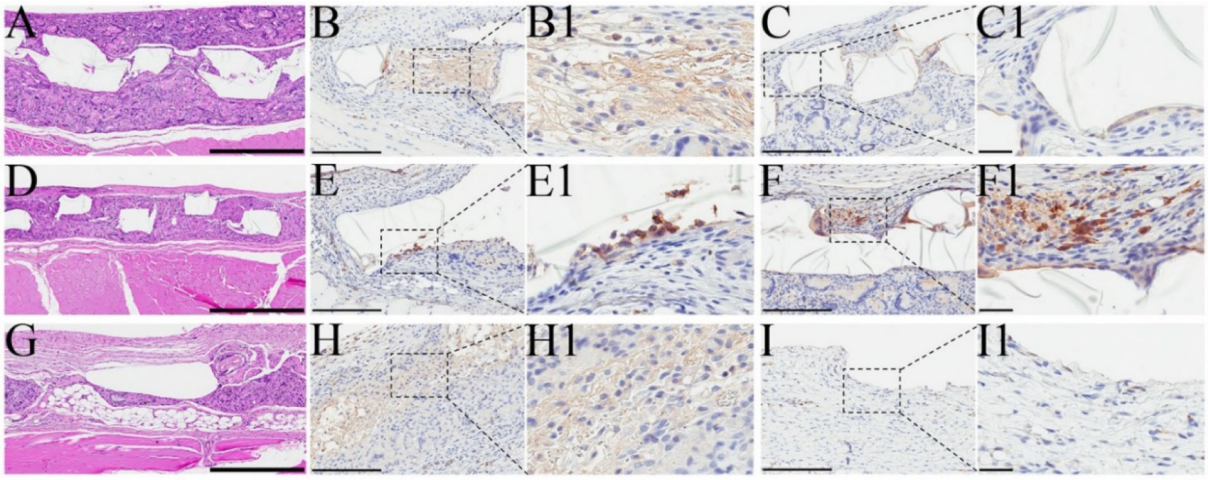

Figure 4. Multilayered scaffolds based on PLT32o grids maintain their 3D structure and retain exogenous heart cells in vivo

(A-C) Scaffolds based on SS_{off} grids of PLT32o implanted without exogenous heart cells; (D-I) Scaffolds based on SS_{off} grids of (D-F) PLT32o or (G-I) PGS implanted (D-I) with exogenous heart cells. Explants were harvested after (A,C,D,F,G,I) 31 days or (B,E,H) 7 days. Stains: (A,D,G) H&E; (B,C,E,F,H,I) cardiac troponin T (cTnT). Cytoplasmic cTnT appears dark brown; nuclei appear blue; polymer appears white. Scale bars: 500 μm (A, D, G); 200 μm (B, C, E, F, H, I); 30 μm (B1, C1, E1, F1, H1, I1).

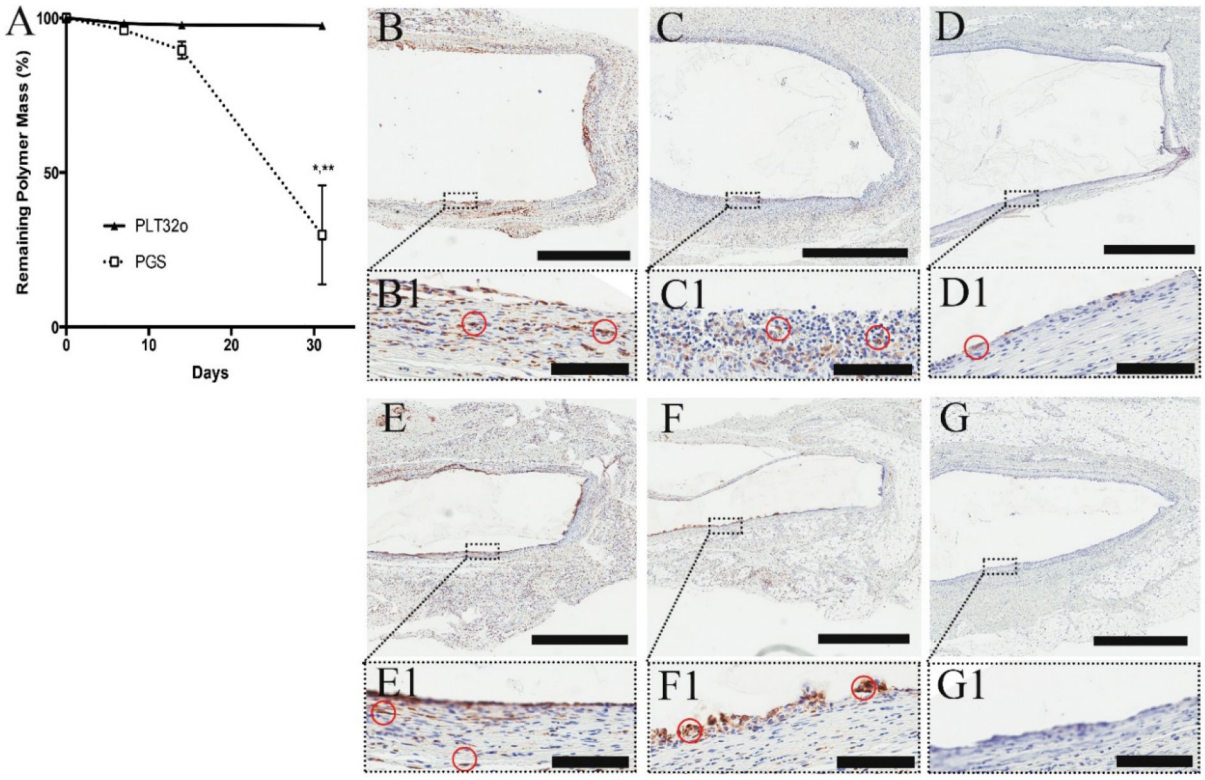

Figure 5. Time histories of polymer membrane degradation in vivo

(A) Remaining dry mass of PLT32o and PGS membranes (% of initial). *Significantly different from the corresponding PLT32o sample (*p<0.0002)*; **Significantly different from the corresponding 7 and 14 day PGS samples (*p<0.0002)*. (B-G) Cross sections of (B-D) PLT32o and (E-G) PGS explants harvested after (B,E) 7 days, (C,F) 14 days, or (D,G) 31 days. Stain: CD68 for macrophages (brown, encircled in Insets); nuclei counterstained blue; polymer appears white. Scale bars: (B-G) 1 mm; (Insets B1-G1) 100 μm.

Table 1

Feature sizes of master molds and grids[a]

Component	Strut width (μm)	Short Pore Length (μm)	Long Pore Length (μm)	Thickness (μm)
PI Master mold	111.8 ± 7.8	393.0 ± 5.7	601.1 ± 15.9	
PLT32o Grid	123.3 ± 10.6	361.7 ± 18.5 [b]	563.6 ± 25.8 [b]	87.2 ± 10.7
PGS Grid	126.4 ± 39.4	348.8 ± 14.9 [b]	560.4 ± 19.7 [b]	91.0 ± 14.6

[a] Data show the AV ± SD of 30 measurements for all data except grid thicknesses where data show the AV ± SD of at least 10 measurements.

[b] Statistically different from corresponding value measured for the PI Master mold.