Electronic Supplementary Information

ROS-cleavable proline oligomer crosslinking of polycaprolactone for pro-angiogenic host response

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Materials and Methods

Materials: PCL 70-90K MW, Lithium Diisopropylamide (LDA), tetrahydrofuran (THF), diethyl ether, dichloromethane (DCM), triethylamine (TEA), dimethylformamide (DMF), N-hydroxysuccinimide (NHS), N,N'-dicyclohexylcarbodiimide (DCC), KBr, NaCl, lipopolysaccharide (LPS), RIPA buffer were used as purchased from Sigma-Aldrich. PEG (2000 MW)-dihydrazide was purchased from Laysan Bio, and KP7K peptides were synthesized on PS3 (Protein Technologies) and characterized in our previous study.[1]

Synthesis of PCL-CPCL: In a 500 mL round-bottom flask, 5 g of PCL was dissolved in 400 mL anhydrous THF, and 50 mL of 2 M LDA was added dropwise at -78 °C. Then 50 g of dry CO_2 gas was added into the flask while stirring via subliming dry ice for 50 minutes. After the temperature was raised to 0 °C, 20 g of NH_4Cl in 100 mL H_2O was added to the mixture. PCL-CPCL was filtered, precipitated in cold diethyl ether and dried under vacuum. Chemical composition was determined by ^{1}H-NMR. Molecular weight properties were determined by gel permeation chromatography against PMMA standards (Agilent Technologies) using a Phenogel 10E3A column (Phenomenex Inc.) in THF.

Synthesis of PCL-(NHS)CPCL: 3 g of 70% PCL-30% CPCL and 2.09 g of NHS were dissolved in 30 mL of dry DCM. 3.74 g of DCC dissolved in 9 mL DCM was added drop by drop, and the reaction continued for 8 hours at 0 °C. After filtration through 2 μm Whatman filter paper, PCL-(NHS)CPCL was precipitated in excess of 1:1 mixture of cold ethyl ether and methanol, and dried under vacuum.

Crosslinking of PCL-(NHS)CPCL and Scaffold Fabrication: 0.8 mmol of PCL-(NHS)CPCL was dissolved in 10 mL DCM, and 0.2 mmol of crosslinkers (KP7K or PEG-dihydrazides) dissolved in 10 mL DCM, 5 mL DMF and 500 μL TEA was added drop by drop. The reaction continued for 8 hours in room temp, then precipitated in excess of 1:1 mixture of cold ethyl ether and methanol. After dissolving in 10 mL DCM,

the mixture was poured over 10 g of NaCl (sieved to 245 – 410 μm) bed in a 100 mm Teflon dish. After drying overnight in vacuum, scaffolds were punched with a 6 mm punch, NaCl was leached in daily changes of excess H_2O over 4 days.

Fourier Transform Infrared Spectroscopy: 2 mg of polymer scaffold samples and 100 mg of KBr were ground and pressed to form pellets. Samples were subjected to FTIR spectroscopy using a Bruker PMA 50 spectrophotometer. Scanning was conducted from 4000 to 400 cm^{-1} with 64 scans averaged and 4 cm^{-1} resolution.

Swelling Ratio Measurement: Scaffolds were incubated in PBS at 37 °C overnight, and wet masses of the scaffolds were measured. Swollen scaffolds were blotted dry to remove excess buffer before weighing. Swelling ratio was calculated according to the formula: swelling ratio = (wet mass)/(dry mass).

Gel Content Measurement: 5-10 mg scaffolds were washed three times with 3 mL THF. Remaining polymer was dried in vacuum and weighed, and gel content was calculated according to the formula: gel content (%) = (remaining polymer mass after washes)/(dry mass before washes) x 100.

Differential Scanning Calorimetry: All polymeric scaffolds were analyzed for thermal transitions and heat capacity via on a TA Instruments Q2000 DSC. Samples were weighed (2−5 mg) and pressed in aluminum sample pans with tops. The measurement procedure included two temperature sweeps from −50 to 100 °C at a ramp rate of 10 °C/min. The values from the second sweep were reported such that thermal history was erased.

Dynamic Mechanical Analysis: To determine mechanical properties, rectangular strips (~20mm x ~4.0mm x ~0.1mm) were loaded onto a tension clamp and subjected to a stress ramp of 1.0 MPa min^{-1} until break at 37 °C using a TA Instruments Q2000 dynamic mechanical analyzer (DMA).

Oxidation Experiments: To investigate oxidative degradation, scaffolds were incubated in PBS with or without 1 mM SIN-1 (Invitrogen) for 28 days at 37 °C. Buffers were changed daily owing to the relatively short half-life of SIN-1 in aqueous environments (<10 h). At 30 days post incubation, scaffolds were dried in vacuum.

Cell Studies: For cell studies involving bone marrow-derived macrophages (BMDMs), 4 week old C57/bl6 mice were used. Briefly, after euthanasia, femurs and tibia were collected and flushed with RPMI 1640 media (Invitrogen), and collected cells were plated on non-tissue culture treated plates in RPMI 1640 containing 10% FBS, 1% penicillin-streptomycin, and 20% L929-conditioned DMEM medium for 7 days. On day 7, proper differentiation into BMDM was confirmed via immunostaining against CD11b and F4/80.[2] Cells were directly seeded onto scaffolds in 24-well plate at a density of 300,000 cells/cm^2, and cultured with or without 50 μg LPS for inflammatory activation with ROS overproduction for 7 days. Cells were removed by washing the scaffolds with RIPA buffer and H_2O.

Scanning Electron Microscopy: SEM was performed on a Hitachi S-4200 system. An accelerating voltage of 2.5 kV was used for all images. To prepare scaffolds for imaging, scaffolds were sputter-coated with gold (Cressington Sputter Coater 1080) at a plasma current of 30 mA for 120 s.

Subcutaneous Implantation: All animal work and related protocols were approved and performed in accordance with Vanderbilt IACUC. Wild type C57/bl6 mice were anesthetized with 1.5 L/min oxygen and 1.5% isoflurane on a warm water blanket, and shaved. A small 1.5 cm longitudinal incision was made on the ventral side, and scaffolds were inserted into individual subcutaneous pockets. The skin incision was closed with sutures.

H&E Staining and Cell infiltration Analysis: Scaffolds were collected and fixed in 4% paraformaldehyde overnight, and embedded in OCT compound (TissueTek) for cryosectioning. Sections were then submitted to Vanderbilt Translational Pathology Shared Resources Core for H&E staining. Brightfield microscope images were acquired using a Nikon Eclipse Ti microscope (Nikon, Japan), and color deconvolution plugin in ImageJ (National Institutes of Health, USA) was used for quantifying the density of nuclei in tissue/scaffold sections.

Perfusion and Microangiography: At 2 weeks post implantation, mice were perfused under heavy, near-lethal level of anesthesia with 4% isoflurane in 2 L/min oxygen. First, PBS containing 0.1 mg/ml heparin sulfate was injected into the left ventricle to exsanguinate via the cut inferior vena cava. Then mice were perfused with PBS containing fluorescent microbeads (Invitrogen) for micro-angiography.[3] Micro-angiograms were then acquired using a Zeiss 710 confocal laser microscope. ImageJ (National Institutes of Health, USA) was used for all image preparation and analysis, including z-stacking fluorescence images.

Gene Expression Analysis via Quantitative Polymerase Chain Reaction (qRT-PCR): Samples were homogenized in Trizol (Invitrogen), and RNA was collected using RNeasy kit (Qiagen). RNA concentration and 260/280 ratios were measured on a TECAN M1000 plate reader. RNA was treated with DNAse to eliminate genomic contamination, and reverse-transcribed using High Capacity cDNA Synthesis Kit (ABiosystems). SYBR Green PCR mix (Biorad) was used for quantitative PCR. Each sample containing at least 40 ng cDNA and 500 nM of each primer with annealing temperature at 55°C was run in technical triplicates, followed by melting curve analysis. Raw data were analyzed using CFX Manager (Biorad), and biological replicates from different animals were combined.[4] GAPDH expression was used as a reference gene, where the GAPDH expression level divides each gene expression level for normalization. This relative gene expression to GAPDH is then normalized to that of the control PCL condition. Primers used in this study include: GAPDH primers 5' TGAAGCAGGCATCTGAGGG 3' and 5'CGAAGGTGGAAGAGTGGGAG 3', CD31 primers 5' TCCCTGGGAGGTCG TCCAT 3' and 5' GAACAAGGCAGCGGGGTTTA 3', and VEGFA 5' ATGCGGATCAAACCTCACCA 3' and 5' CCGCTCTGAACAAGGCTCAC 3'.

Statistical Analysis: Results are presented as means ± standard deviation (SD) or standard error mean (SEM) as indicated. Comparisons among different conditions were performed via ANOVA, followed by Tukey's HSD test in Prism 6 (Graphpad). For all statistics, $p < 0.05$ was considered statistically significant, and such significance is indicated where appropriate.
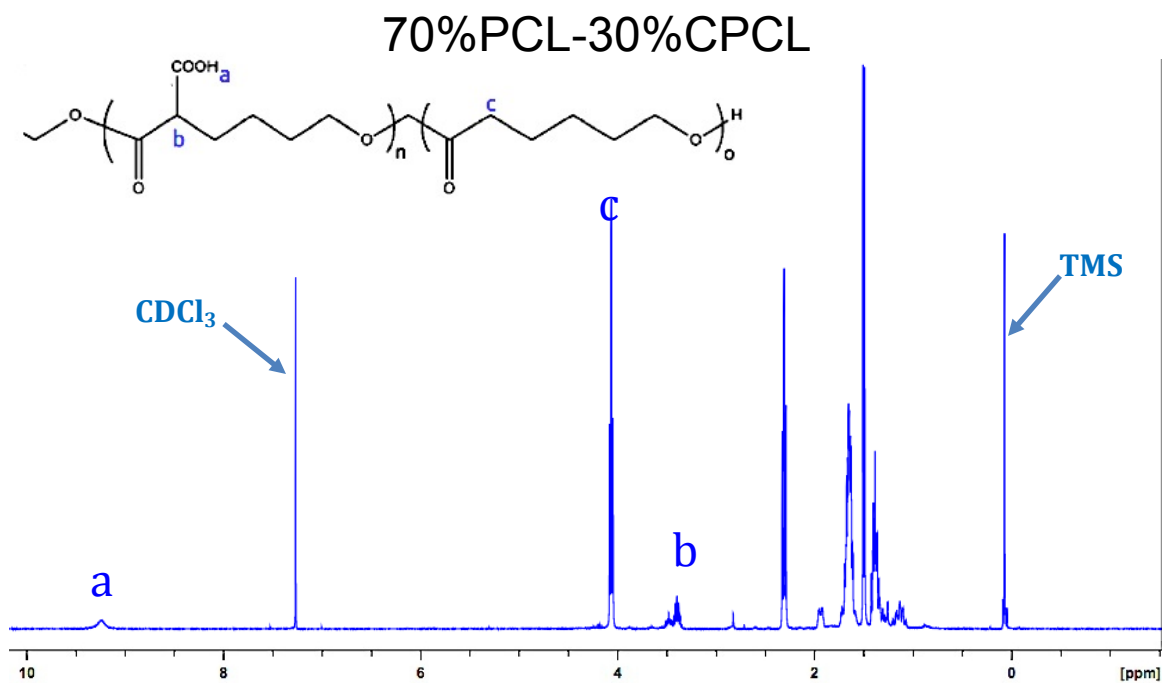
Fig. S1. ¹H-NMR of 70% PCL- 30%CPCL.
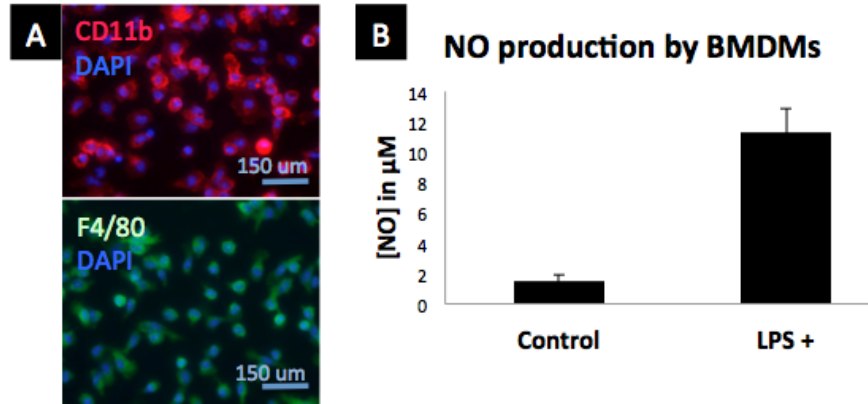
Fig. S2. Characterization of bone marrow-derived macrophages (BMDMs). (A) Proper differentiation to macrophages was confirmed through staining for macrophage markers CD11b and F4/80 prior to seeding on test scaffolds. (B) BMDMs when stimulated with lipopolysaccharide (LPS) overproduced nitric oxide (NO), a characteristic of macrophage response to endotoxins.

References:

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