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# Well-organized neointima of large-pore poly(L-lactic acid) vascular graft coated with poly(L-lactic-co-ε-caprolactone) prevents calcific deposition compared to small-pore electrospun poly(L-lactic acid) graft in a mouse aortic implantation model

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ABSTRACT

**Objective**: Tissue engineering techniques have emerged that allow bioresorbable grafts to be implanted that restore function and transform into biologically active arteries. However, these implants are susceptible to calcification during the remodeling process. The objective of this study was to evaluate the role of pore size of bioabsorbable grafts in the development of calcification. **Methods**: Two types of grafts were prepared: a large-pore graft constructed of poly(L-lactic acid) (PLA) fibers coated with poly(L-lactide-co-ε-caprolactone) (PLCL) (PLA–PLCL), and a small-pore graft made of electrospun PLA nanofibers (PLA-nano). Twenty-eight PLA–PLCL grafts and twenty-five PLA-nano grafts were implanted as infrarenal aortic interposition conduits in 8-week-old female SCID/Bg mice, and followed for 12 months after implantation. **Results**: Large-pore PLA–PLCL grafts induced a well-organized neointima after 12 months, and Alizarin Red S staining showed neointimal calcification only in the thin neointima of small-pore PLA-nano grafts. At 12 months, macrophage infiltration, evaluated by F4/80 staining, was observed in the thin neointima of the PLA-nano graft, and there were few vascular smooth muscle cells (VSMCs) in this layer. On the other hand, the neointima of the PLA–PLCL graft was composed of abundant VSMCs, and a lower density of macrophages (F4/80 positive cells, PLA–PLCL; 68.1 ± 41.4/mm$^2$ vs PLA-nano; 188.3 ± 41.9/mm$^2$, $p = 0.007$). The VSMCs of PLA–PLCL graft expressed transcription factors of both osteoblasts and osteoclasts. **Conclusion**: These findings demonstrate that in mouse arterial circulation, large-pore PLA–PLCL grafts created a well-organized neointima and prevented calcific deposition compared to small-pore, electrospun PLA-nano grafts.



## 1. Introduction

Atherosclerotic cardiovascular diseases (CVD), including coronary heart disease, carotid artery stenosis, and peripheral arterial disease, is the leading cause of death or impaired quality of life for millions of individuals in the United States [1]. The most successful therapy for CVD is bypass surgery using autologous arteries and veins [2]. Unfortunately, many patients lack suitable donor tissue due to previous surgery or as a result of their underlying vascular disease. Synthetic vascular grafts like expanded polytetrafluoroethylene (Gore-Tex®), polyethylene terephthalate (Dacron®), and polyurethanes are employed in large caliber arteries where flow is high and resistance is low and have a history of long-term success [3]. However, current synthetic small diameter (<6 mm) grafts have not yet shown clinical efficacy due to poor patency as a result of thrombogenesis [4].

Tissue engineered vascular grafts (TEVG) offer the potential of a synthetic conduit that resists thrombogenesis and ultimately transforms into a neovessel capable of growth, remodeling, and repair [5]. The ideal small diameter TEVG for arterial bypass is readily available ("off-the-shelf"), resistant to thrombosis, aneurysmal dilatation and

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ectopic calcification, easily implanted, biocompatible, and capable of transforming into neotissue comparable with that of native artery [6]. However, during the course of neovessel remodeling, these implants are susceptible to calcification, a potentially fatal long-term complication. The effect of scaffold physical structure on the development long-term calcific deposition in the neo-tissue of arterial TEVGs is currently unknown. The objective of this study was to characterize the calcification response between scaffolds fabricated from the same polymer but with different porosities by using a murine aortic implantation model in hopes of guiding future rational TEVG scaffold design.

## 2. Materials and methods

### 2.1. Animals

All animals received humane care in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at Yale University approved the use of animals and all procedures described in this study. 8-week old female SCID/Bg mice were purchased from Jackson Laboratories (ME, USA).

### 2.2. Scaffolds

PLA—PLCL grafts were constructed using a dual cylinder chamber molding system from a nonwoven 100% poly(L-lactic acid) (PLA) fiber mesh (Biomedical Structures, RI, USA) and a 50:50 poly(L-lactic-co-ε-caprolactone) copolymer (PLCL) sealant (Gunze, Kyoto, Japan) as previously described [7]. PLA-nano grafts were composed of PLA nanofibers, which were constructed using electrospinning technology (Gunze). Total porosity, pore size, and fiber size of graft were measured via scanning electron microscopy.

### 2.3. Graft implantation

Twenty-eight PLA—PLCL grafts and twenty-five PLA-nano grafts were implanted as infra-renal aortic interposition conduits using standard microsurgical technique [8]. Animals were followed for 12 months following implantation to evaluate chronic calcification. Post-operatively, no drugs such as anti-platelet or anti-coagulant agents were used.

### 2.4. Histology, immunohistochemistry, and immunofluorescence

Explanted grafts at 4, 8, and 12 months after implantation, along with native abdominal aortas (control) were fixed in 4% paraformaldehyde and embedded in paraffin. 5 μm thick sections were then stained using standardized techniques for Alizarin Red S, Hematoxylin and Eosin (HE), Masson's Trichrome, and Elastica van Gieson (EVG). Quantitative analysis of calcific deposition was evaluated as a percentage of vessel cross-sectional area using Alizarin Red S staining, and measured by Image J software.

Endothelial cells (ECs), macrophages, vascular smooth muscle cells (VSMCs), and transcription factors of osteoblasts and osteoclasts were identified by immunohistochemical staining of paraffin-imbedded explant sections with rabbit anti-CD 31 (1:50, Abcam, MA, USA), rat anti-F4/80 (1:1000, AbD Serotec, Oxford, UK), mouse anti-smooth muscle actin (SMA, 1:500, DAKO, CA, USA), mouse anti-smooth muscle myosin heavy chain (SM-MHC, 1:400, Abcam), mouse anti-runt related transcription factor 2 (Runx2, 1:25, Abcam), and anti-receptor activator of nuclear factor kappa-B ligand (RANKL, 1:100, Abcam). Primary anti-bodies were detected using biotinylated goat anti-rat, -rabbit, and -mouse IgG (1:500, Vector, CA, USA) respectively, followed by binding of streptavidin—horseradish peroxidase and color development with 3,3-diaminobenzidine (Vector).

Immunofluorescent staining for SMA and SM-MHC as markers of SMCs was performed using mouse anti-SMA primary antibody (1:500, DAKO) and rabbit anti-SM-MHC primary antibody (1:1000, Abcam), and Alexa Fluor 647 anti-mouse IgG secondary antibody (1:300, Invitrogen, CA, USA) and Alexa Fluor 488 anti-rabbit IgG secondary antibody (1:300, Invitrogen), respectively. To evaluate expression of transcription factors of osteoblasts and osteoclasts, rabbit Runx2 primary antibody (1:500, Abcam) and

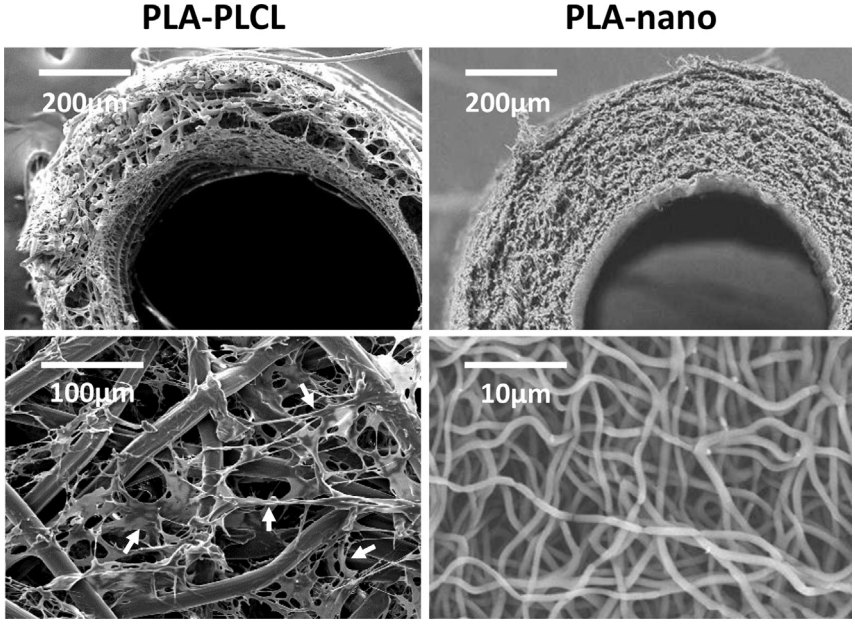


**Fig. 1.** Representative scanning electron microscopy images of implanted grafts. (Left) PLA—PLCL grafts were constructed from nonwoven 100% poly(L-lactic acid) (PLA) fiber mesh and a 50:50 copolymer sealant of poly((L-lactic-co-ε-caprolactone) (PLCL). Total porosity of PLA—PLCL was about 60%, and pore size was about 30 μm. Arrows indicate the PLCL sealant between PLA fibers. (Right) PLA-nano grafts were composed of PLA nanofibers, which were constructed using electrospinning technology. Total porosity of PLA-nano grafts was 70%, and pore size was about 5 μm. Inner luminal diameters of each graft were between 500 and 600 μm.