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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 infra-renal aortic interposition conduits in 8-week-old female SCID/Bg mice, and followed for 12 months after implantation.

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Conflicts of Interest

CB and TS receive grant support from Gunze Ltd.

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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; $68.1 \pm 41.4/\text{mm}^2$ vs PLA-nano; $188.3 \pm 41.9/\text{mm}^2$, $p = 0.007$). 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.

Keywords

Atherosclerotic cardiovascular diseases; Calcification; Tissue engineering; Bioabsorbable grafts; pore size; electrospinning technique

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 (<6mm) 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 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% para-formaldehyde and embedded in paraffin. 5 μ m 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-embedded 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 rabbit anti-RANKL primary antibody (1:100, Abcam), and Alexa Fluor 488 anti-rabbit IgG secondary antibody (1:300, Invitrogen) were used for immunofluorescent staining, respectively.

2.5. RNA extraction and reverse transcription quantitative polymerase chain reaction

Explanted grafts at 12 months after implantation and native abdominal aortas were frozen in optimal cutting temperature (OCT) compound (Sakura Finetek, CA, USA), and sectioned into twenty 30 μm sections using a Leica CM 1950 cryostat (Leica Biosystems, Wetzlar, Germany). Excess OCT compound was removed by centrifugation in PBS. Total RNA was extracted and purified using the RNeasy mini kit (Qiagen, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed using High Capacity RNA-to-cDNA Kit (Applied Biosystems, CA, USA). All reagents and instrumentation for gene expression analysis were obtained from Applied Biosystems. Quantitative polymerase chain reaction (qPCR) was performed with a Step One Plus Real-Time PCR System using the TaqMan Universal PCR Master Mix Kit. Reference numbers for primers are: eNOS (Mm00435217_m1), Itgam (Mm0043455_m1), Runx2 (Mm00501580_m1), bone morphogenetic protein 2 (Bmp2, Mm01340178_m1), RANKL (Mm00441906_m1), and HPRT (Mm00446968_m1). The results were analyzed using the comparative threshold cycle method and normalized to endogenous reference gene HPRT, and reported results as relative values ($2^{-\Delta\text{CT}}$) to the mean gene expression of control native aorta.

2.6. Statistical analysis

Results are expressed as mean $\pm$ standard deviation. The number of experiments is shown in each case. Data of continuous variables between PLA-PLCL group and PLA-nano group were evaluated by student's *t*-test. Comparisons between multiple groups were done using one-way ANOVA followed by Tukey HSD. Spearman analysis was used for correlation analysis. A probability value of less than 0.05 was considered significant. All statistical analysis was performed using SPSS software (ver.20) (IBM, NY, USA).

3. Results

3.1. Graft construction

Total porosity of PLA-PLCL was about 60%, and average pore size was about 30 μm [7]. Diameter of PLA fiber of PLA-PLCL graft was about 20 μm . Total porosity of PLA-nano was about 70%, and average pore size was about 0.7 μm . Diameter of PLA-nano fiber was about 0.7 μm . Each scaffold was 3.0 mm in length and inner luminal diameters were between 500 and 600 μm (Fig. 1).

3.2. Animal survival

Twenty-eight PLA-PLCL grafts and twenty-five PLA-nano grafts were implanted as infra-renal interposition aortic conduits. Two animals in each group died during the peri-operative recovery period. PLA-PLCL grafts had dilated 4 months after implantation to have a luminal diameter of about 1.0 mm, which is almost twice as large as the implanted scaffold,

however, these graft diameters stabilized at 1.0 mm for the remainder of the observation period. As a result of aneurysmal dilatation and subsequent graft rupture, twelve mice of the PLA-PLCL group died [9]. There was no aneurysmal formation and graft rupture in PLA-nano group, although 2 mice of the PLA-nano group died of undetermined causes during the course of observation period. Autopsy within 24 hours after death did not identify any graft related complications such as rupture or occlusion.

3.3. Calcified depositions in neointimal layer and graft layer

Alizarin Red S staining of PLA-nano explants at 12 months demonstrated calcified depositions in the neointimal layer, which was defined as the layer between the endothelium and the graft material. Conversely, in the PLA-PLCL group, few grafts developed neointimal calcifications, although similar amounts of calcified depositions were present in the graft layer of both groups (Fig. 2A). Calcification area in all layers of PLA-nano was higher than that of PLA-PLCL (PLA-nano; 30.9 ± 29.1 vs PLA-PLCL; 3.0 ± 2.9 , $p = 0.04$) (Fig. 2B). The neointima of the PLA-PLCL group was thicker than that of the PLA-nano group (PLA-PLCL; $60.7 \pm 24.7 \mu\text{m}$ vs PLA-nano; $22.5 \pm 10.5 \mu\text{m}$, $p = 0.01$) (Fig. 2C), and there was negative correlation between calcification area (%) and intimal thickness ($y = -0.4965x + 37.597$, $r^2 = 0.716$, $p = 0.001$) (Fig. 2D).

3.4. PLA-PLCL supports well-organized neointimal formation with collagen and elastin

HE staining demonstrated complete degradation of PLCL sealant at 12 months following implantation, but some PLA fibers remained. There was dense cellular infiltration within PLA-PLCL grafts at the 12 month time point, and neotissue formation of PLA-PLCL graft resembled native aorta (Fig. 3). Extracellular matrix components including collagen and elastin were evaluated by Masson's Trichrome and EVG staining, and showed a positive deposition of both in the neointima of the PLA-PLCL group (Fig. 3). At 12 months, the PLA-nano group demonstrated abundant remaining fiber and a lack of cellular infiltration, yet there was a thin intimal layer on the luminal surface that was composed of collagen and elastin (Fig. 3).

3.5. Endothelial cell coverage on luminal surface and macrophage infiltration into neotissue

Immunohistochemical staining for CD31 showed endothelial cell coverage of the luminal surface of both PLA-PLCL and PLA-nano at 12 months after implantation (Fig. 4A). Furthermore, there was no significant difference in gene expression of eNOS between two the groups (PLA-PLCL: 0.17 ± 0.06 vs PLA-nano: 0.20 ± 0.06 , $p = 0.99$), although the levels of these were lower than that of native aorta (Fig. 4C).

F4/80 staining for macrophages showed that inflammation in the neotissue was present, however, there appeared to be regional differences between the localization of macrophages in the two groups (Fig. 4A). In the intimal layer, the density of macrophages in PLA-nano was higher than that of PLA-PLCL (F4/80 positive cells, PLA-PLCL; $68.1 \pm 41.4/\text{mm}^2$ vs PLA-nano; $188.3 \pm 41.9/\text{mm}^2$, $p = 0.007$) (Fig. 4B). In the scaffold layer, a higher density of macrophages was present around remaining PLA fibers in the PLA-PLCL group compared to the PLA-nano group (positive cells/ mm^2 , PLA-PLCL; 909.9 ± 143.4 vs PLA-nano; 105.6

± 90.0 , $p < 0.001$) (Fig. 4B). Relative gene expression of *Itgam*, a macrophage marker, tended to be higher in the PLA-PLCL group than in the PLA-nano group, reflecting increased cellular infiltration into the more porous PLA-PLCL scaffold, but there was no significant overall difference between the two groups (PLA-PLCL: 11.9 ± 4.0 vs PLA-nano: 6.8 ± 2.3 , $p = 0.061$) (Fig. 4C).

3.6. Vascular smooth muscle cell proliferation in neointima of implanted grafts

Immunohistochemical staining for SMA and SM-MHC showed abundant VSMCs in the neointima of the PLA-PLCL group at 12 months after implantation (Fig. 5). On the other hand, there were few SMA or SM-MHC positive cells in the thin neointimal layer of the PLA-nano group (Fig. 5).

3.7. Expression of transcription factors for osteogenesis and osteoclastogenesis in vascular smooth muscle cells

Osteogenic progenitors are thought to arise from transdifferentiation of mature VSMCs, and *Runx2* and *BMP2* are transcription factors of this lineage [10]. *RANKL* is one of the key transcription factors associated with osteoclast differentiation [11].

Runx2 and *RANKL* were increased in the neointima of PLA-PLCL (Fig. 6A), and smooth muscle cells, defined by SMA positive cells, expressed both of these factors 12 months after implantation (Fig. 6B). Furthermore, RT-qPCR revealed that gene expression of *Runx2* and *RANKL* in PLA-PLCL were higher than those in PLA-nano (*Runx2*, PLA-PLCL: 15.8 ± 3.33 vs PLA-nano: 1.44 ± 0.71 , $P < 0.001$; *RANKL*, PLA-PLCL: 10.9 ± 1.86 vs PLA-nano: 0.60 ± 0.43 , $P < 0.001$) (Fig. 6C), although, no difference was observed between groups in gene expression of *BMP2* 12 months after implantation (PLA-PLCL: 1.13 ± 0.24 vs PLA-nano: 0.82 ± 0.23 , $P = 0.11$) (Fig. 6C).

4. Discussion

Calcific degeneration remains one of the major obstacles facing the translation of TEVGs for arterial repair [12]. In this study, we demonstrated little evidence of calcification in the neointimal layer of large-pore PLA-PLCL grafts. In contrast, severe calcification occurred in the thin neointimal layer of the small-pore electrospun PLA-nano graft. Poly(l-lactic acid) (PLA) and poly(ϵ -caprolactone) (PCL) are commonly used biodegradable materials for constructing arterial scaffolds due to their history of successful clinical usage [13]. Combining these materials with additional synthetic polymers to create copolymers such as poly(l-lactic-co- ϵ -caprolactone) (PLCL) allows for rational tuning of mechanical properties and degradation rates through precise control of polymer composition ratios and molecular weights. We created the seamless PLA-PLCL conduit by sealing PLA mesh with a PLCL solution. Since the relative mass of PLCL sealant was much lower than that of the PLA mesh in the PLA-PCLA graft (Fig. 1), we argue that the PLA is the dominating polymer in this scaffold. Furthermore, our previous studies have shown that the PLCL coating completely degrades 4 months after implantation in a mouse model identical to that presented in this study [9].

In the present study, neotissue formation in the PLA-nano group was inhibited, we observed abundant remaining scaffold fibers, and inflammation in the thin neointima of the PLA-nano group was sustained 12 months following implantation. Nano-fibers created by an electrospinning technique are thought to be a desirable material for fabricating arterial conduits, because they yield scaffolds of high porosity, large surface area, and very small fiber sizes, which are characteristics comparable to fibrils of extracellular matrix components in human tissues [14]. Additionally, they have been shown to improve endothelialization [15] and achieve clinically sufficient burst pressures [16]. Nano-fiber based scaffolds using biodegradable polymers have become a commonly proposed technique for constructing tissue engineered arterial grafts, and have demonstrated favorable surgical and mechanical properties with a high patency rate in arterial implantation models [17–19]. Primary characteristics of graft remodeling, such as luminal endothelialization, transmural cellular ingrowth, and neocapillary formation within the scaffold, dynamically progress according to the degradation rate of the scaffold [20]. Wu et. al. demonstrated that a novel, fast degrading elastomer developed well-organized neotissue in a rapid remodeling process without any calcification in a rat aortic implantation model [21]. In contrast, the high density (small pore size) of the nanofiber fabric used in the current study appears to have a long period of polymer degradation without transmural cellular migration into the scaffold. We suggest that the combined effect of slow degradation and low porosity elicited a prolonged foreign body reaction and neotissue remodeling resulting in calcified depositions. A review of existing literature supports our findings, as many groups using electrospun TEVGs with fiber diameters of less than 1 μm demonstrated little or no cellular infiltration [22]. Our previous work indicates that cellular infiltration is critical in generating viable vascular neotissue [23]. Furthermore, macrophage related inflammation is understood to be a key contributor to osteogenesis in early stages of atherosclerotic intimal calcification [24], although, we did not collect data analyzing macrophage phenotype as it relates to calcification in the present study.

The thin intima of PLA-nano grafts had components of extra cellular matrix including collagen and elastin, but few VSMCs were observed when compared to that of PLA-PLCL grafts. While VSMC proliferation can lead to neointimal hyperplasia, our results indicate that there was a negative correlation between intimal thickness and calcification area in the graft. Since normal VSMCs have a potential to prevent calcium deposition, some degree of neointimal VSMC proliferation may be required to prevent calcification.

On the other hand, several studies have demonstrated that VSMCs can undergo osteogenic differentiation and calcification [25, 26], although the precise mechanisms underlying osteogenic differentiation of vascular cells during atherosclerosis remain undefined. In the present study, neointimal VSMCs of PLA-PLCL expressed both Runx2 and RANKL, consistent with contractile markers specific to smooth muscle. During the process of vascular calcification, VSMCs can lose their lineage markers such as SMA and SM-MHC, and gain osteogenic makers. Runx2 is thought to be an early marker of VSMC transdifferentiation, is implicated in osteogenic differentiation and calcification of VSMCs, and the upregulation of Runx2 is associated with arterial calcification [25, 27]. RANKL is a member of the tumor necrosis factor superfamily, which is the key regulator for osteoclast formation. Under atherosclerotic conditions, mineral resorption by osteoclasts is delicately

balanced to maintain osteogenesis with mineral deposition by osteoblasts [28]. One study demonstrated that expression of RANKL in VSMCs was enhanced by Runx2 via a direct binding to the RANKL promoter [29]. One possible reason that such little calcific deposition was observed in the neointima of our PLA-PLCL grafts even after 12 months is that the upregulation of both osteogenesis and osteoclastogenesis eliminated calcium deposition.

Although well-organized neointima was demonstrated in large pore PLA-PLCL graft, aneurysmal rupture was observed in 46% of implanted those grafts. To solve this issue, TEVG design may require two approaches, including stronger reinforcement, such as combinational use of electrospinning, and improvement of cellular growth and extra cellular matrix deposition into the scaffold [9]. We utilized SCID/Bg mice according to our previous experience demonstrating lower rates of TEVG thrombosis and stenosis when implanted in this strain, and this specific model might have affected the results of the current study. Based on this limitation, we have since developed an aortic implantation model for TEVG in the wild type C57BL/6 mouse by using anti-platelet and anti-coagulant drugs to reduce acute thrombosis and stenosis following TEVG implantation. PLCL or its degradation products might affect cell migration, proliferation, or differentiation in the remodeling neotissue, and could lead to development of long-term calcific deposition. Finally, we recognize that the results of the present study do not clearly support an optimal scaffold material or material structure for creation of arterial TEVG; we have identified two ends of a design spectrum within which the ideal arterial TEVG scaffold is situated. Further research is now required to isolate parameters in addition to porosity, such as degradation kinetics, polymer type, and mechanical profile, which in combination will yield an optimized arterial scaffold.

In conclusion, the present study demonstrated that large-pore PLA grafts with a PLCL coating (PLA-PLCL) created a well-organized neointima and prevented calcified deposition in contrast to small-pore PLA-nano grafts. Particularly, prolonged macrophage infiltration in the neointima of PLA-nano scaffolds may cause calcification, and regeneration of VSMCs may prevent calcification by sustaining a balance of osteogenesis and osteoclastogenesis in the neointima of tissue engineered arterial grafts during the remodeling process.

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References

1. Lim LS, Haq N, Mahmood S, Hoeksema L. Atherosclerotic cardiovascular disease screening in adults: American College Of Preventive Medicine position statement on preventive practice. *Am J Prev Med.* 2011; 40:381, e1–10. [PubMed: 21335273]
2. Suma H. Arterial grafts in coronary bypass surgery. *Ann Thorac Cardiovasc Surg.* 1999; 5:141–5. [PubMed: 10413758]
3. Ravi S, Chaikof EL. Biomaterials for vascular tissue engineering. *Regen Med.* 2010; 5:107–20. [PubMed: 20017698]

4. Kannan RY, Salacinski HJ, Butler PE, Hamilton G, Seifalian AM. Current status of prosthetic bypass grafts: a review. *J Biomed Mater Res B Appl Biomater*. 2005; 74:570–81. [PubMed: 15889440]
5. Vacanti JP, Langer R. Tissue engineering: the design and fabrication of living replacement devices for surgical reconstruction and transplantation. *Lancet*. 1999; 354(Suppl 1):S132–4. [PubMed: 10437854]
6. Tara S, Rocco KA, Hibino N, et al. Vessel bioengineering. *Circ J*. 2013; 78:12–9. [PubMed: 24334558]
7. Roh JD, Nelson GN, Brennan MP, et al. Small-diameter biodegradable scaffolds for functional vascular tissue engineering in the mouse model. *Biomaterials*. 2008; 29:1454–63. [PubMed: 18164056]
8. Mirensky TL, Hibino N, Sawh-Martinez RF, et al. Tissue-engineered vascular grafts: does cell seeding matter? *J Pediatr Surg*. 2010; 45:1299–305. [PubMed: 20620335]
9. Tara S, Kurobe H, Maxfield MW, et al. Evaluation of remodeling process in small-diameter cell-free tissue-engineered arterial graft. *J Vasc Surg*. 2014 in press.
10. Thompson B, Towler DA. Arterial calcification and bone physiology: role of the bone-vascular axis. *Nat Rev Endocrinol*. 2012; 8:529–43. [PubMed: 22473330]
11. Wu M, Rementer C, Giachelli CM. Vascular calcification: an update on mechanisms and challenges in treatment. *Calcif Tissue Int*. 2013; 93:365–73. [PubMed: 23456027]
12. de Valence S, Tille JC, Mugnai D, et al. Long term performance of polycaprolactone vascular grafts in a rat abdominal aorta replacement model. *Biomaterials*. 2012; 33:38–47. [PubMed: 21940044]
13. Athanasiou KA, Niederauer GG, Agrawal CM. Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid/polyglycolic acid copolymers. *Biomaterials*. 1996; 17:93–102. [PubMed: 8624401]
14. Li WJ, Laurencin CT, Cateson EJ, Tuan RS, Ko FK. Electrospun nanofibrous structure: a novel scaffold for tissue engineering. *J Biomed Mater Res*. 2002; 60:613–21. [PubMed: 11948520]
15. Wu H, Fan J, Chu CC, Wu J. Electrospinning of small diameter 3-D nanofibrous tubular scaffolds with controllable nanofiber orientations for vascular grafts. *J Mater Sci Mater Med*. 2010; 21:3207–15. [PubMed: 20890639]
16. Drilling S, Gaumer J, Lannutti J. Fabrication of burst pressure competent vascular grafts via electrospinning: effects of microstructure. *J Biomed Mater Res A*. 2009; 88:923–34. [PubMed: 18384169]
17. Pektok E, Nottelet B, Tille JC, et al. Degradation and healing characteristics of small-diameter poly(epsilon-caprolactone) vascular grafts in the rat systemic arterial circulation. *Circulation*. 2008; 118:2563–70. [PubMed: 19029464]
18. Kuwabara F, Narita Y, Yamawaki-Ogata A, et al. Long-term results of tissue-engineered small-caliber vascular grafts in a rat carotid arterial replacement model. *J Artif Organs*. 2012; 15:399–405. [PubMed: 22806242]
19. Mrowczynski W, Mugnai D, de Valence S, et al. Porcine carotid artery replacement with biodegradable electrospun poly-e-caprolactone vascular prosthesis. *J Vasc Surg*. 2014; 59:210–9. [PubMed: 23707057]
20. Sung HJ, Meredith C, Johnson C, Galis ZS. The effect of scaffold degradation rate on three-dimensional cell growth and angiogenesis. *Biomaterials*. 2004; 25:5735–42. [PubMed: 15147819]
21. Wu W, Allen RA, Wang Y. Fast-degrading elastomer enables rapid remodeling of a cell-free synthetic graft into a neoartery. *Nature medicine*. 2012; 18:1148–53.
22. Rocco KA, Maxfield MW, Best C, Dean EW, Breuer CK. In Vivo Applications of Electrospun Tissue-Engineered Vascular Grafts: A Review. *Tissue Eng Part B Rev*. 2014 in press.
23. Hibino N, Yi T, Duncan DR, et al. A critical role for macrophages in neovessel formation and the development of stenosis in tissue-engineered vascular grafts. *FASEB J*. 2011; 25:4253–63. [PubMed: 21865316]
24. Aikawa E, Nahrendorf M, Figueiredo JL, et al. Osteogenesis associates with inflammation in early-stage atherosclerosis evaluated by molecular imaging in vivo. *Circulation*. 2007; 116:2841–50. [PubMed: 18040026]

25. Speer MY, Yang HY, Brabb T, et al. Smooth muscle cells give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries. *Circ Res.* 2009; 104:733–41. [PubMed: 19197075]
26. Sun Y, Byon CH, Yuan K, et al. Smooth muscle cell-specific runx2 deficiency inhibits vascular calcification. *Circ Res.* 2012; 111:543–52. [PubMed: 22773442]
27. Steitz SA, Speer MY, Curinga G, et al. Smooth muscle cell phenotypic transition associated with calcification: upregulation of Cbfa1 and downregulation of smooth muscle lineage markers. *Circ Res.* 2001; 89:1147–54. [PubMed: 11739279]
28. Doherty TM, Uzui H, Fitzpatrick LA, et al. Rationale for the role of osteoclast-like cells in arterial calcification. *FASEB J.* 2002; 16:577–82. [PubMed: 11919160]
29. Byon CH, Sun Y, Chen J, et al. Runx2-upregulated receptor activator of nuclear factor kappaB ligand in calcifying smooth muscle cells promotes migration and osteoclastic differentiation of macrophages. *Arterioscler Thromb Vasc Biol.* 2011; 31:1387–96. [PubMed: 21454810]

Highlights

- Large pore PLA-PLCL scaffold induces well-organized neoartery after 1 year.
- Small pore PLA nanofiber scaffold results in poor neotissue formation.
- PLA nanofibers induce chronic inflammation and significant calcification.
- PLA-PLCL neo-artery prevents macrophage infiltration and ectopic calcification.
- PLA-PLCL VSMCs expressed transcription factors of both osteoblasts and osteoclasts.

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 .

Fig. 2.

Evaluation of calcified deposition and neointimal thickness. **(A)** Alizarin red S staining showed that neointimal calcified depositions were noted only in the PLA-nano grafts, although, positive staining in the graft layer of both groups was observed 12 months after implantation. **(B, C)** Calcification area and intimal thickness were measured using Image J software (data presented as mean $\pm$ standard deviation), and evaluated by student's *t* test. Calcification area of the PLA-nano grafts were higher than that of PLA-PLCL grafts, and the neointima of PLA-PLCL grafts was thicker than that of PLA-nano grafts. **(D)** Spearman analysis was used to evaluate correlation between calcification area and intimal thickness, and there was negative correlation between these parameters.

Fig. 3.

Scaffold cellular infiltration and extracellular matrix deposition at 12 months after implantation. Hematoxylin and Eosin (HE) staining demonstrated dense cellular infiltration into PLA-PLCL graft and complete degradation of PLCL sealant. Masson's Trichrome and Elastica van Gieson (EVG) staining showed deposition of collagen and elastin in both the thick neointima of PLA-PLCL grafts and the thin neointima of PLA-nano grafts, respectively.

Fig. 4. Endothelialization and macrophage infiltration of the grafts at 12 months after implantation. (A) Representative histological image of (left) endothelial cell staining (CD 31) and (right) macrophage staining (F4/80). CD31 staining showed endothelial cell coverage of luminal surface in both groups, and F4/80 staining demonstrated a difference in macrophage localization between both groups. (B) The number of F4/80 positive cells (/mm²) was counted for the quantitative analysis of macrophage infiltration into scaffold using immunohistochemical staining images (mean $\pm$ standard deviation), and evaluated by student's *t* test. In the intimal layer, the density of macrophages in PLA-nano grafts was higher than that of PLA-PLCL grafts, although, in the scaffold layer, more macrophages

existed around the remaining PLA fibers in PLA-PLCL grafts compared to PLA-nano grafts. (C) Gene expression was analyzed by RT-qPCR using the $\Delta\Delta$ CT method. Data are expressed as fold change over native aorta expression (mean $\pm$ standard deviation), and evaluated by one-way ANOVA followed by Tukey HSD. There was no significant difference of gene expression of eNOS or Itgam between two groups.

Fig. 5. Vascular smooth muscle cell proliferation in neointima of PLA-PLCL graft at 12 months after implantation. Immunohistochemical staining with low and high power magnifications of smooth muscle actin (SMA) and of smooth muscle myosin heavy chain (SM-MHC) demonstrated abundant VSMCs in neointima of PLA-PLCL grafts, although, few cells positive for these markers were found in the thin neointimal layer of the PLA-nano grafts.

Fig. 6.

Expression of transcription factors of osteoblasts (Runx2) and osteoclasts (RANKL) in smooth muscle cells within neointima at the 12 month time point. **(A)** Immunohistochemical staining revealed that both Runx2 and RANKL were increased in the neointima of PLA-PLCL grafts. **(B)** Smooth muscle cells, defined by SMA positive cells, expressed both of these factors. **(C)** Gene expression was analyzed by RT-qPCR using the CT method. Data are expressed as fold change over native aorta expression (mean $\pm$ standard deviation), and evaluated by one-way ANOVA followed by Tukey HSD. Runx2 and RANKL in PLA-

PLCL grafts were higher than those in PLA-nano grafts, although, no significant difference was found in expression of BMP2.