Fast-degrading elastomer enables rapid remodeling of a cell-free synthetic graft into a neoartery

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Host remodeling is important for the success of medical implants, including vascular substitutes. Synthetic and tissue-engineered grafts have yet to show clinical effectiveness in arteries smaller than 5 mm in diameter. We designed cell-free biodegradable elastomeric grafts that degrade rapidly to yield neoarteries nearly free of foreign materials 3 months after interposition grafting in rat abdominal aorta. This design focuses on enabling rapid host remodeling. Three months after implantation, the neoarteries resembled native arteries in the following aspects: regular, strong and synchronous pulsation; a confluent endothelium and contractile smooth muscle layers; expression of elastin, collagen and glycosaminoglycan; and tough and compliant mechanical properties. Therefore, future studies employing large animal models more representative of human vascular regeneration are warranted before clinical translation. This cell-free approach represents a philosophical shift from the prevailing focus on cells in vascular tissue engineering and may have an impact on regenerative medicine in general.

A key challenge of arterial substitutes is that they need to withstand arterial pressure immediately upon implantation. Thus, the classic approaches to arterial substitutes use strong materials. This is reflected in autografts, synthetic grafts and many tissue-engineered grafts. Tissue-engineered grafts typically show limited host cell infiltration and remodeling even 6–12 months after implantation. In vascular extracellular matrix (ECM), collagen provides strength and remodeling even 6–12 months after implantation. In this work, we describe a fast degrading elastomer, poly(glycerol sebacate) (PGS), because timely degradation is essential for rapid host remodeling and mechanical conditioning is recognized as an important remodeling cue. Elastomers efficiently transduce mechanical stimulation to cells. Second, for graft porosity, we chose highly porous grafts with interconnected pores to enable immediate host cell infiltration. To prevent blood cell loss, we enclosed grafts with a dense, nonwoven sheath. Last, for thromboreistance, we chose heparin coating. The graft is a blood-contacting foreign material prone to clotting, which can occlude grafts and block cell infiltration into the graft wall. Heparin is a well-recognized standard in thromboresistance.

RESULTS

The open porous graft is suturable and resists platelet adhesion

Consistent with the design criteria, our graft consists of a heparin-coated porous tube wrapped with a 15-µm-thin electrospun sheath (Fig. 1b–d). The porous tube is made from PGS fabricated as previously described except a 1-mm mandrel was used. The sheath is a polycaprolactone (PCL) mesh that increases graft strength and prevents bleeding by controlled fibrin formation within the sheath. Composite grafts are stored at ambient temperature. Microcomputed tomography (microCT) morphometric analysis indicates that the inner diameter of the graft is 720 µm, the wall thickness is 290 µm and over 99.99% of the pores are interconnected.

Table 1

High pore interconnectivity is crucial for efficient cell infiltration, the first step in initiating host remodeling. PGS has numerous hydroxyl groups that can form hydrogen bonds with heparin. Thus, heparinization of PGS most likely renders the graft lumen nonthrombogenic and inhibits excessive blood clotting within the graft wall. Heparin coating significantly reduces platelet adhesion on grafts (Fig. 1e–g). Scanning electron microscopy (SEM) reveals that

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hemin coating (2 mg ml\(^{-1}\)) also reduces fibrin formation, and most adhered platelets appear quiescent (Fig. 1e,f). The small pore size of the PCL sheath probably permits fibrin formation within the sheath and prevents leakage. Although very thin, the sheath significantly increases the suture retention force of the graft from 0.11 ± 0.0087 to 0.45 ± 0.031 N and is stronger than the break force of the 9-0 suture (0.26 ± 0.046 N) used for the microsurgical anastomosis (Fig. 1h). Furthermore, the PCL sheath increases elastic tensile modulus from 0.26 ± 0.529 kPa to 3,790 ± 1,450 kPa (P < 0.001).

**Rapid graft remodeling leads to strong and compliant neoartery**

Interposition grafting of ethylene oxide–sterilized grafts was performed in the abdominal aorta of Lewis rats (n = 21) without heparin administration during surgery or systemic heparin treatment after (Fig. 1i). The implantation immediately exposed the grafts directly to 120 mm Hg pressure and an arterial hemodynamic environment. Therefore, in terms of allowing unobstructed blood flow upon implantation, the grafts performed the same function as autografts in bypass surgeries. Grafts reddened with the infiltration of blood cells, but the PCL sheath effectively prevented bleeding. The host rapidly remodeled the grafts performed the same function as autografts in bypass surgery and lower-limb vascular reconstruction\(^{16,17}\). Unimplanted grafts leak due to their high porosity and have no burst pressure to compare. Before implantation, composite grafts were stiffer than native arteries, with no toe region in the stress-strain curve (Fig. 1j). Host remodeling substantially altered the stress-strain curve of grafts with a clear toe region in the neoarteries. Neoarteries appeared softer than the native aorta, although the differences were insignificant. More notably, the compliance of the neoarteries (11% ± 2.2%) is statistically the same as native aorta at 6.7% ± 2.3% in the 80–120 mm Hg range\(^3\). More informative than this single value of compliance is a plot of compliance over the whole pressure range (Fig. 1j). Of note, the neoartery was not only strong but also compliant. The high compliance of neoarteries contrasts with the lower compliance of unimplanted grafts, suggesting extensive graft remodeling.

This fast host remodeling is explained by widespread cell infiltration soon after implantation. The open porous structure of the graft allows extensive cell penetration into the graft wall, and nucleated cells occupied many of the pores within 3 d (Supplementary Fig. 1). Smooth muscle cells extensively infiltrated the graft within 14 d (Fig. 2a). Cells positive for \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA), a protein specific to mural cells including smooth muscle, were widely distributed within the graft wall. Higher magnification illustrated that smooth muscle cells were not organized into circumferential layers at this early stage and were mixed with \(\alpha\)-SMA–negative cells (Fig. 2b,c). Co-staining of endothelial and smooth muscle cells indicated that the smooth muscle layer was separated from the blood by an endothelium
Figure 2  Smooth muscle cell infiltration and organization at 14 d. (a) Smooth muscle cell distribution (α-SMA, green) within the remodeled graft wall. The tissue was split longitudinally, half of which is shown. Native aorta is on the right; its border with the graft is indicated by the dashed line; scale bar, 500 μm. L, lumen. (b) Magnified view of the mid-graft shows distribution of both α-SMA–positive (green) and α-SMA–negative cells. Nuclei counterstained by DAPI (blue); scale bar, 250 μm. (c) Further magnification of the mid-graft to view the complicated smooth muscle cell distribution (α-SMA, green); scale bar, 50 μm. (d) Distribution of endothelial cells (vWF, red) and smooth muscle cells (α-SMA, green) in the graft wall. Immunofluorescent images merged with the bright-field image (darkened to not overwhelm the fluorescent images). Dark spots (*) in the bright-field image might be residual graft material. Scale bar, 100 μm. Bright-field images of original brightness are in Supplementary Figure 2.

(Fig. 2d). By 90 d, the cellularity of the neoartery was slightly higher than but comparable to that of native aortas (2.14 ± 0.34 μg DNA per mg wet weight versus 1.36 ± 0.40 μg DNA per mg wet weight, \( P = 0.06 \)). Bright-field images revealed a band of dark spots mixed with cells located generally closer to the lumen. This acellular matter is probably remnants of graft materials that are visible as dark fibers in bright-field images (Supplementary Fig. 2).

Figure 3  Remodeling of grafts. (a) H&E staining of the grafts during the transition into a neoartery. An area of the neoartery wall containing inflammatory cells is marked by *. The top of the 14-d sample was trimmed to remove the adjoining vein. Image merged from a panel of 100× micrographs; scale bar, 250 μm. (b) Magnified view of the vessel wall shows ECM content and alignment; scale bars, 50 μm. (c) Luminal area of remodeling grafts to assess stenosis and aneurysm formation. There is no statistical difference between the two groups. (d) Changes in number and organization of macrophages (CD68, red). (e) Distribution of M2 macrophages (CD163, red). (f) Number of CD68- and CD163-positive cells quantifies total macrophage number and the proportion of M2 macrophages. (g) Changes of smooth muscle cell (α-SMA, green) organization over time. (h) Distribution of contractile smooth muscle cells (myosin heavy chain, red). All of the immunofluorescent micrographs were counterstained for nuclei by DAPI (blue); scale bars, 50 μm. Data represent means ± s.d. for c and f.
Smooth muscle cells are important for blood vessel function. Immunofluorescent staining revealed the presence of smooth muscle cells within 14 d and a progressively more organized media layer (Fig. 3g,h). Myosin heavy chain is a late-stage differentiation marker for smooth muscle. Strong expression of myosin heavy chain indicated a contractile smooth muscle phenotype in neoarteries (Fig. 3h). Neoarteries stained positive for fibroblast surface protein in the outer layer at 90 d, suggesting the formation of an adventitia-like tissue (Supplementary Fig. 3). The layers were distinct, resembling the trilaminar structure of a muscular artery.

The marked impact of host cells on the graft is further revealed in ECM composition. The neoaorty wall contained substantial amounts of elastin, collagens I and III and glycosaminoglycans (Fig. 4a). The amount of elastin in the neoaorta at 90 d was 77% of that in native aorta, and the amount of total collagen was statistically the same as native aorta (Fig. 4b). These ECM macromolecules aligned circumferentially, mimicking their orientation in native arteries. However, native matrix is more compact and less cellular than in the neoaorta. Nonetheless, high ECM production provides a molecular explanation of the observed match of mechanical properties between the neoaorta and the host aorta.

**Endothelialized neoaorta pulses synchronously with host aorta**

Integration with host tissue is often a major challenge in tissue engineering. Laser Doppler ultrasound imaging of the grafts indicated uninhibited blood flow and strong, synchronous pulsation with host aorta (Fig. 5a and Supplementary Video 1). Regular and clear pulsation of neoarteries indicates thorough integration with host tissue. To the best of our knowledge, this is the first report of any vascular graft that pulses with host arteries. The high patency was corroborated by angiography (Fig. 5b), correlating well with a confluent endothelium that transitions smoothly from neoartery to host aorta. The transition was marked only by the suture (Fig. 5c). von Willebrand factor (vWF) staining revealed a confluent endothelial monolayer covering of the lumen (Fig. 5d,e). A basement membrane separated the endothelial from the smooth muscle layer, as indicated by transmission electron microscopy (Fig. 5f). Basement membrane prevents smooth muscle cell migration to the endothelial layer, thereby preventing intimal hyperplasia21. We found no evidence of intimal hyperplasia in neoarteries. In the four observed cases of graft occlusion (4/21; 19.0%) the cause was acute anastamotic thrombosis in three cases where the rats died. Acute thrombosis was also suspected in one rat that survived an occluded graft, because no graft remodeling was observed in the explant (Supplementary Fig. 4). Acute thrombosis is likely to be due to endothelial injury at the anastomosis in the absence of systemic anticoagulation22. Overall patency as determined by ultrasonography, angiography and necropsy was 80.9% (17 patent grafts/21 total grafts) at time points of up to 90 d. Patency rates were 60% (3/5), 100% (5/5) and 81.8% (9/11) for grafts explanted at days 14, 28 and 90, respectively. This overall graft patency is similar to a previous report of an antithrombogenic vascular graft7.
**TECHNICAL REPORTS**

Graft material is crucial for host remodeling. When we substituted PGS with PCL, with all other graft parameters being identical, graft remodeling was substantially impaired (Supplementary Fig. 5). Only a thin layer of smooth muscle was observed near the lumen. This is consistent with results of other cell-free approaches in arterial tissue engineering where a thin layer of endothelial cells and small amount of smooth muscle were present with graft materials largely intact. Cells within the interstitial space of the graft wall were negative for α-SMA and were potentially inflammatory cells or fibroblasts (Supplementary Fig. 4b). PCL grafts showed poor integration with host tissue even at 90 d, at which a clear boundary was visible and the grafted segment distorted the aorta (Supplementary Fig. 6). Collagen I expression was much higher than collagen III and elastin expression. Collagen I distribution was extensive, spanning the whole graft area from lumen to albumen (outer surface), whereas other ECM proteins were mostly expressed only near the lumen (Supplementary Fig. 6c–f). These results, coupled with the ‘walled-off’ appearance of the H&E stained graft, suggest that collagen I might serve to isolate the PCL from the host.

**DISCUSSION**

Cell-free approaches to tissue engineering are still relatively new and scarce, with a focus on slow-degrading polymers. A 1-mm poly lactide graft was successfully endothelialized in rats, and 10-mm composite poly lactide-poly glycolide grafts showed good patency in canine and porcine models. However, the grafts degraded slowly, limiting cell penetration and causing prolonged presence of foreign materials. Additionally, no compliance data or ECM content were reported. Porcine small intestinal submucosa grafts showed variable patency above 3-mm diameter, but smaller grafts failed by acute thrombosis, despite heparin soaking and systemic heparinization. A 4-mm graft based on small intestinal submucosa was populated by endothelial and smooth muscle cells by 3 months in rabbits. However, no mechanical data or ECM content were reported, and the bradykinin vasoresponse was counterphysiologic. Poly(ester urethane)urea elastomeric grafts were recently shown to be populated by endothelial and smooth muscle cells. Grafts contained both collagen and elastin, but the graft material appeared largely intact at 6 months. A PCL and hydroxyapatite scaffold infused with growth factors regenerated the articular surface of rabbit cartilage, however, resorption of hydroxyapatite is very slow.

Our philosophy departs from the above in that we emphasize rapid graft degradation and host remodeling. To the best of our knowledge, the use of fast-degrading synthetic grafts is a new design perspective to vascular substitutes. This report is a first step that shows promising results, with a nearly complete host remodeling and good integration within 3 months. We believe three features of the reported graft are important: fast degradation, mechanical properties and pore size. We suspect that rapid graft degradation is the most crucial, because it probably induces different inflammatory responses from long-lasting materials. Rapid degradation progressively generates more space for cell infiltration, proliferation and matrix production. Furthermore, fast degradation reduces the duration of host exposure to foreign material. Long-lasting materials cause tissue stiffening from fibrous encapsulation and can activate inflammatory cells to induce neointimal hyperplasia. Matching mechanical properties and optimizing graft pore size may further promote host remodeling and integration. Matching arterial mechanical properties is likely to promote vascular cell differentiation and avoid stress shielding. PGS is the primary component of our grafts, and its modulus is close to that of native aortas (148 ± 55 kPa versus 390 ± 191 kPa) at the strains experienced by new grafts over 80–120 mm Hg. Small pores in our grafts probably pack infiltrating cells close together to promote self-assembly.

As with any new technology, many questions remain to be answered. The applicability of this approach in large animals awaits investigation. Rats have different regeneration potential, and their endothelial cells have a greater capacity to cover the lumen and the grafts than those of humans (especially aged humans). It is crucial to investigate the rate of graft endothelialization and the safety of surgeries with no or low heparin in large animals. The rats we used were healthy, because there are few atherosclerotic animal models with suitable artery sizes. Up to now, only apolipoprotein E-deficient mice are available and widely accepted.

The type and quantity of cells recruited to the graft and their changes over time remain to be studied. The vWF- and α-SMA-negative cells in the neoartery wall at 14 d may be a mixture of progenitor and inflammatory cells. Macrophages are crucial in vascular graft remodeling. A substantial fraction of infiltrating macrophages in our grafts expressed the M2 macrophage marker CD163. A previous study found that mouse vena cava grafts with higher M2 macrophage activation stenosed more. In contrast, none of our grafts showed stenosis, even at 90 d. This could be due to the difference in graft material and design or differences in the animal models. A large number of smooth muscle cells are present in the neoartery wall. Tracing their origin may reveal how to accelerate their recruitment. Progenitor cells in the adventitia of the vena cava and the fascia could facilitate regeneration via paracrine effects or contribute directly to the regeneration of the neoartery. Consequently, parts of grafts contacting the cava and fascia might form a thicker wall more rapidly, accounting for the nonuniform thickness of neoarteries.

In addition to increasing antithrombogenicity, heparin is known to bind, stabilize and potentiate the activity of a wide variety of bioactive molecules. Heparin could localize fibroblast growth factor and vascular endothelial growth factor families of angiogenic factors to grafts, promoting vascular cell infiltration. Heparin may also promote cellular infiltration by binding cell adhesion proteins such as PECAM-1 and L-selectin, adhesive matrix proteins and chemokines. Previous work demonstrated that heparinization improves implant cellularity and angiogenesis. Heparin can also promote remodeling by binding remodeling factors such as tissue plasminogen activator.

PCL improves graft suturability; however, it might not be the optimal shear material. Residues of graft materials observed at 3 months are probably PCL, because solid nonporous PGS degrades within 2 months subcutaneously. The presence of graft material when the neoartery is sufficiently strong and compliant is likely to inhibit host remodeling. Thus, investigation of shear materials with mechanical properties similar to PCL but with a faster degradation is warranted. This research may lead to advanced biomaterials and graft designs that can bring synthetic small arterial grafts closer to clinical translation. Furthermore, the focus on rapid graft remodeling is probably applicable to tissues other than blood vessels, especially when combined with progenitor-cell homing signals.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary information is available in the online version of the paper.*
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AUTHOR CONTRIBUTIONS
W.W. designed experiments, fabricated and implanted the grafts, characterized explants and analyzed data. R.A.A. performed mechanical characterization of grafts and explants, and analyzed data. Y.W. designed experiments and supervised the project. All authors interpreted results and contributed to writing the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Graft fabrication. To fabricate the PGS core, we used a modified salt fusion and leaching method as previously described except that we used a 1-mm mandrel and a 1.25-mm outer mold. To fabricate the PCL sheath we dissolved PCL \((M_w = 80 \text{ kDa}, \text{ Aldrich})\) in 2,2,2-trifluoroethanol (ACROS) at 14% w/v and electrospun the solution onto the rotating PGS-salt template (20 r.p.m.). Immersion in deionized water removed salt from PCL-PGS-salt composites. We lyophilized the grafts (Labconco Freezone 4.5) and stored them in a desiccator at ambient temperature. We used ethylene oxide to sterilize grafts.

Graft characterization. Mechanical testing. To measure elastic modulus and suture retention strength of grafts, we used an electromechanical testing machine (Insight, MTS Systems) with a 5.0-N load cell. We measured tensile strength and elastic modulus as previously described with some modification (detailed in Supplementary Methods). We took ultimate tensile strength as the peak stress of the stress-strain curve. To measure suture retention strength, we placed a 7-0 prolene suture 1 mm from the end of each graft. We fixed the suture around the upper hook and immobilized the grafts in the lower hook. We measured the force needed to pull the suture apart from the graft using a crosshead speed of 2 mm min\(^{-1}\). To evaluate whether suture retention met the microsurgical requirement, we measured the break forces of 9-0 sutures. SEM, microCT and platelet adhesion quantification are detailed in the Supplementary Methods.

Rat studies. Implantation. Rats were cared for in compliance with protocols approved by the Committee on Animal Care of the University of Pittsburgh following NIH guidelines for the care and use of laboratory animals (NIH publication No. 85–23 rev. 1985). We used male Lewis rats (body weight: 200–250 g, Charles River Laboratories, Boston, MA) for experiments. We successfully implanted 27 rats with either PGS-based composite grafts \((n = 21)\) or porous grafts made completely from PCL \((n = 6)\). Surgical survival rate was 87.5% \((21/24)\). Three rats died during surgery due to bleeding from aortic or inferior vena cava injury, and we excluded these from rat counts. We performed interpositional implantation in rat abdominal aorta as follows. A midline abdominal incision exposed the abdominal aorta in rats anesthetized by isoflurane inhalation. To implant the graft, we separated the aorta from the inferior vena cava, cross-clamped the infrarenal abdominal aorta, transected a 4-mm segment, and inserted the composite graft (8–10 mm in length) in the gap. End-to-end anastomosis connected grafts to the native aorta with 9-0 nylon suture. Rats received no postoperative anticoagulation or antiplatelet treatment. We explanted grafts at 14 d \((n = 5)\), 28 d \((n = 5)\) and 90 d \((n = 11)\) after implantation. We killed all rats implanted with PCL-only grafts \((n = 6)\) at 90 days.

X-ray angiography and Doppler ultrasound examination. To detect blood flow in grafts, we used Doppler ultrasound monthly and X-ray angiography immediately before explantation. Details are in the Supplementary Methods.

Biochemical evaluation. To quantify elastin, collagen and DNA content of regenerated and native arteries, we used a Fastin Elastin Assay kit (F2000; Biocolor), Sircol Collagen Assay (MP Biomedicals) and DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer instructions (detailed in the Supplementary Methods).

Mechanical characterization. To prepare aortic segments (1 cm length) for testing, we trimmed off connective tissue and sealed branching vessels with 7-0 suture. We then strained segments longitudinally to 10% and pressurized by infusing with physiologic saline solution using a syringe pump (NE-1000, New Era Pump Systems). To precondition, we cycled aortas between 0 and 130 mm Hg until we acquired reproducible diameter versus pressure curves. We calculated compliance and modulus from the last cycle. A pressure transducer (PX309 Omegadyne), optical micrometer (LS7070, Keyence) and data acquisition system (PowerLab 8/30, AD Instruments) recorded pressure and diameter synchronously. A description of compliance and modulus derivations from diameter versus pressure data is in the Supplementary Methods.

Statistical analyses. A two-tailed Student’s \(t\) test made comparisons between two groups. A one-way analysis of variance followed by Tukey’s Honestly Significant Difference (HSD) post hoc test made comparisons between three or more groups. We confirmed normal distribution of data and homogeneity of variance between groups using IBM SPSS Statistics 19.

Additional methods. A more detailed description of SEM, microCT, platelet adhesion quantification, X-ray angiography and Doppler ultrasound examination, histology, immunofluorescent staining, biochemical evaluation and explant mechanical characterization is available in the Supplementary Methods.