Published in final edited form as:

Biomaterials. 2017 November ; 144: 166–175. doi:10.1016/j.biomaterials.2017.08.014.

Vascular Scaffolds with Enhanced Antioxidant Activity Inhibit Graft Calcification

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Abstract

There is a need for off-the-shelf, small-diameter vascular grafts that are safe and exhibit high long-term patency. Decellularized tissues can potentially be used as vascular grafts; however, thrombogenic and unpredictable remodeling properties such as intimal hyperplasia and calcification are concerns that hinder their clinical use. The objective of this study was to investigate the long-term function and remodeling of extracellular matrix (ECM)-based vascular grafts composited with antioxidant poly(1, 8-octamethylenecitrate-co-cysteine) (POCC) with or without immobilized heparin. Rat aortas were decellularized to create the following vascular grafts: 1) ECM hybridized with POCC (Poly-ECM), 2) Poly-ECM subsequently functionalized with heparin (Poly-ECM-Hep), and 3) non-modified vascular ECM. Grafts were evaluated as interposition grafts in the abdominal aorta of adult rats at three months. All grafts displayed antioxidant activity, were patent, and exhibited minimal intramural cell infiltration with varying degrees of calcification. Areas of calcification co-localized with osteochondrogenic differentiation of vascular smooth muscle cells, lipid peroxidation, oxidized DNA damage, and cell apoptosis, suggesting an important role for oxidative stress in the calcification of grafts. The extent of calcification within grafts was inversely proportional to their antioxidant activity: Poly-ECM-

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Hep>ECM>Poly-ECM. The incorporation of antioxidants into vascular grafts may be a viable strategy to inhibit degenerative changes.

Graphical abstract

Keywords

Vascular grafts; Extracellular matrix; Calcification; Oxidative stress; Antioxidant

1. Introduction

There is an urgent need to develop clinically acceptable, readily available, small diameter (<6 mm) vascular grafts for patients with severe vascular disease that do not have suitable veins or arteries for autologous grafts or are not appropriate candidates for placement of intravascular stents. Synthetic vascular grafts fabricated from expanded polytetrafluoroethylene (ePTFE) and polyester (Dacron) are not commonly used to reconstruct small blood vessels due to complications such as thrombosis, stenosis and infection.[1, 2] Moreover, synthetic grafts cannot be biologically remodeled in the body or grow with the patient, which is a significant barrier to their use in pediatric cardiac and vascular surgeries.[3] Biological vascular grafts such as allografts (e.g. CryoVein, human saphenous and femoral veins; CryoLife) and xenografts (e.g. Artegraft, bovine carotid arteries; Artegraft Inc) are on-demand, off-the-shelf options; however, clinical trials have not convincingly demonstrated any advantage in patient outcome compared to synthetic grafts.[4]

Bioengineered, acellular vascular grafts fabricated from biodegradable synthetic polymers such as polycaprolactone,[5] decellularized vascular extracellular matrix (ECM),[6, 7] or synthetic polymer/ECM composites[8, 9] have been evaluated as alternatives to vein and radial artery grafts, which are the current gold standard conduits for lower extremity and cardiac revascularization. Rigorous long-term safety and efficacy assessment *in vivo* must be completed to evaluate time-dependent durability of bioengineered vascular grafts because *in vitro* studies alone or short-term *in vivo* experiments do not capture time-dependent degeneration of the implanted material. Despite this need, very few studies have critically assessed long-term (defined as 3 months for small animals,[5] 12 months for large animals[10] and humans[11]) safety and efficacy of bioengineered vascular grafts *in vivo*. Although common evaluative end-points predominantly focus on graft stenosis due to neointimal hyperplasia or intravascular thrombosis, intra-graft calcification is often

overlooked despite its importance in vascular tissues such as biological heart valves,[12, 13] smooth muscle biology,[14] and arterial mineralization in patients with vascular disease, diabetes, and chronic kidney failure (patients likely to be recipients of vascular grafts).[15] Therefore, intra-graft calcification is a pathology that can lead to vascular graft failure in the long-term.

The detailed mechanisms by which calcifications form within vascular grafts are not known. Nevertheless, several studies highlight the critical role of oxidative stress in the pathology of most forms of arterial mineralization.[16, 17] In this study, we hypothesized that improving antioxidant properties of vascular grafts leads to favorable graft remodeling, by reducing tissue and cellular damages (e.g. inflammation, apoptosis, and calcification) caused by oxidative stress. We previously reported the synthesis of antioxidant, citric based polymers[18, 19] and the preparation of antioxidant, citric acid-based polymer-ECM composite vascular grafts whereby the polymer coating was functionalized with heparin to enhance graft thromboresistance.[8, 9] When compared to vascular ECM grafts, the polymer-ECM composite grafts exhibited reduced neointimal hyperplasia after one month of implantation in the abdominal aorta of Sprague Dawley rats. [9]. Herein we report a correlation between the extent of antioxidant activity, oxidative tissue damage, and calcification in vascular grafts. We suggest that manipulating the antioxidant properties of the graft may be a novel strategy to reduce intra-graft calcification.

2. Materials and Methods

2.1. Source of vascular ECM

Male Sprague Dawley rats weighing 200–250 g (Charles River Laboratories, Chicago, IL) were used as tissue (aorta) donors or recipients of vascular grafts. All animal care was performed in accordance with the NIH Guide for Care and Use of Laboratory Animals, and all experiments using animals were approved by the Animal Care and Use Committee of Northwestern University (Chicago, IL). The descending thoracic and abdominal segments of the aorta (3–4 cm in length) were recovered from each donor animal by the Microsurgical Core at Northwestern University (Chicago, IL) as previously described.[8]

2.2. Preparation of vascular grafts

Three types of ECM-based bioengineered vascular grafts were prepared. Decellularization of rat abdominal aortas was performed with Triton-X, sodium dodecyl sulfate, and Deoxyribonuclease I as described previously by us,[8] and used as control group (ECM). Poly (1,8-octamethylene citrate)-co-cysteine (POC-Cys) was synthesized with 1,8 octanediol, citric acid and L-cysteine with molar ratio 1:1:0.2, as described previously,[20] and hybridized onto decellularized aorta ECM at 45°C followed by 2-β mercaptoethanol treatment as experimental group 1 (Poly-ECM).[9] Lastly, the polymer-ECM composite was reacted with N-[β-maleimidopropionic acid] hydrazide (BMPH)-heparin via click chemistry to immobilize heparin onto the Poly-ECM as experimental group 2 (Poly-ECM-Hep).[9]

2.3. Antioxidant activity measurements

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, Sigma-Aldrich, St Louis, MO) based free-radical scavenging assay was performed to evaluate the antioxidant activity of each graft.[19] Briefly, all three types of grafts (n=3 per group) were weighed and incubated in a solution of ABTS (7 mM) and sodium persulfate (2.45 mM) at 37 °C. At each time point (days 0, 3, 6 and 9), ABTS solution was sampled, diluted with MQ water 1:10 and the absorbance measured at 734 nm. The antioxidant activity was expressed as percent free-radical scavenging by measuring the decrease in absorbance compared to control ABTS solutions (0% free-radical scavenging), and normalized to the weight of each graft.

2.4. Binding of Calcium to the grafts

All three types of vascular grafts were incubated in simulated body fluid (SBF)[21] at 37 °C under aseptic conditions, with fresh SBF changed weekly. At 1, 2 and 3 months after incubation, an n=3 samples from each group was collected, rinsed with PBS and weighed. The samples were then incubated in 0.1 M hydrochloric acid overnight, and assayed for calcium concentration using a calcium colorimetric assay kit (Sigma-Aldrich, St Lois, MO). The results were expressed as calcium content normalized to the mass of each graft.

2.5. Implantation and Non-invasive imaging of vascular grafts

A rat abdominal aorta interposition model previously described by us[9] was used to evaluate all three types vascular grafts (1 cm in length, n=4 for each group), using a sutured end to end anastomosis. All vascular grafts were sterilized via ethanol and rehydrated with sterile PBS prior to implantation. All implantation surgeries were conducted at the Northwestern University Microsurgical Core (Chicago, IL). Biweekly vascular ultrasound imaging was performed in accordance with guidelines for noninvasive vascular laboratory testing[22] at the Center for Comparative Medicine (CCM), Northwestern University (Chicago, IL) using an M7/M7T Diagnostic Ultrasound System with an L14-6S probe (Mindray Bio-Medical Electronics, Shenzhen, China). Briefly, animals were anesthetized via isoflurane inhalation (1%–5%) using VetEquip inhalation anesthesia system, and the abdomen around the surgical area was shaved and ultrasound gel was applied. Duplex ultrasound with color Doppler was first performed using the probe to locate the abdominal aorta and the inferior vena cava. B-mode ultrasound was then used to locate the proximal and distal end-to-end anastomosis along the abdominal segment of the vascular graft. Images were acquired with both Doppler and B-mode. Additionally, spectral Doppler waveform analysis was performed at the site of implanted vascular grafts to quantify the velocity of blood flow within the graft.

2.6. Tissue collection

Three months after implantation, the animals were anesthetized for terminal blood collection and tissue recovery. Blood was collected from the inferior vena cava of each animal. A sample of anticoagulant whole blood and a sample of separated serum (0.5 ml each) from each animal was sent to the Clinical Pathology lab at Charles River RADS (Wilmington, MA) for a complete blood count (CBC) and a complete chemistry panel analysis, respectively. The implanted grafts, including the end-to-end anastomotic sites and adjacent

native aortic tissue, were recovered and rinsed in PBS to remove excessive blood and fixed in 4% formaldehyde. Each graft was sectioned into proximal, middle and distal segments (2–3 mm). A segment (2 mm) of native artery proximal to the implanted graft from each animal was also recovered and used as a normal tissue control.

2.7. Histology and histomorphometric evaluation

Each segment (graft and native artery) was processed for histology by embedding in paraffin and sectioning into 5 μ m thick sections. Sections were stained with H&E, von Kossa, Alizarin red, Toluidine blue, Safranin O, or Masson's trichrome using standard histological methods. Stained tissues were imaged by bright field microscopy with a 4 $\times$, 10 $\times$ or 20 $\times$ objective (Nikon TE2000U, Melville, NY). Quantitative analysis to measure the inner circumference of vascular grafts was carried out using ImageJ (National Institute of Health, Bethesda, MD) on digitized images of H&E stained tissue. Data are expressed as the percentage of dilation relative to the initial graft inner circumference calculated previously[8]. Semi-quantitative and quantitative evaluation of the extent of calcification within tissues was performed on digitized images of von Kossa-stained tissues using two different methods: (1) A pathology-based scoring method (Score 0: no calcification; Score 1: calcification involving <50% of graft thickness or <50% of its total surface area; Score 2: calcification involving >50% of graft thickness or >50% of its total surface area; Score 3: calcification involving entire graft thickness or entire surface area) described previously,[23] and (2) Digital measurement using ImageJ software of the graft area that stained positive for the von Kossa stain normalized by the total area of vascular graft. Each graft was analyzed at three different segments (proximal, middle and distal) along the graft length, and compared across the three groups of grafts (ECM, Poly-ECM and Poly-ECM-Hep).

2.8. Immunohistochemical evaluation

Proximal, middle and distal segments of each engineered graft or sections of native arteries as a control were probed for endothelial nitric oxide synthase (eNOS), alpha smooth muscle actin (α -SMA), bone sialoprotein (BSP), osteopontin (OPN), type II collagen (COL II), CD 68, tumor necrosis factor alpha (TNF- α), T-Cell Marker, 8-Oxo-2'-deoxyguanosine (8-OXO-dG), 4-Hydroxynonenal (4-HNE), Matrix metalloproteinase 9 (MMP 9) and Matrix metalloproteinase 13 (MMP 13). Supplemental Table 1 includes antibodies, manufacturer information and dilutions for reagents. All sections were incubated with primary antibodies in a humidity chamber overnight at 4 $^{\circ}$ C, and were subsequently probed with a fluorescently conjugated secondary antibody for 2h at room temperature. Apoptosis was evaluated using a fluorescence-based terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay using an *in situ* cell death detection kit (Sigma-Aldrich, St Louis, MO) following the manufacturer's instructions. All fluorescence stained images were acquired with 20 $\times$ objective by fluorescence microscopy (Nikon TE2000U, Melville, NY). Quantitative analysis for neointima area and endothelial coverage was determined based on the area that stained positive for α -SMA and eNOS, as previously described.[9] Briefly, the area of neointima and the length of endothelial lining was measured and normalized to the inner circumference of the vascular graft using Image J.

2.9. Statistics

Due to the non-normal distribution of the calcification data set, calcification data were analyzed for statistical significance by Kruskal-Wallis test by ranks and plotted with the box and whisker diagram. All data other than calcification analysis were assumed to be normally distributed, and analyzed for statistical significance by ANOVA with a Tukey–Kramer post-test. All analyses were performed with SigmaStat (San Jose, CA). For all comparisons, $p < 0.05$ was considered statistically significant.

3. Results

3.1. POC-cys and heparin addition influence graft antioxidant activity and calcium binding

The free-radical scavenging (antioxidant) activity of bioengineered vascular grafts (ECM, Poly-ECM, and Poly-ECM-Hep) was evaluated *in vitro*. Unmodified ECM control grafts used to establish baseline graft properties exhibited a degree of inherent antioxidant activity throughout the 9-day duration of the assay; however, ECM grafts that were hybridized with POC-cys (Poly-ECM) exhibited higher antioxidant activity ($67.1 \pm 5.9\%$ Poly-ECM vs $54.0 \pm 0.6\%$ ECM on day 9, $p=0.0188$). In contrast, addition of heparin to POC-cys-modified grafts (Poly-ECM-Hep) exhibited a significant decrease in antioxidant activity ($33.7 \pm 0.9\%$ Poly-ECM-Hep on day 9) relative to both ECM ($p<0.01$) and Poly-ECM ($p<0.01$) grafts (Figure. 1A). When measured for up to 15 days, the percent free radical scavenging plateaued and remained stable for all groups after day 9. Calcium binding to vascular grafts gradually increase for all graft preparations incubated in simulated body fluid [68] at 37°C over the course of three months. The increase in binding is more significant with polymer coating (Poly-ECM) and heparin conjugation (Poly-ECM-Hep) than ECM grafts alone (ECM) (Figure. 1B).

3.2. Bioengineered grafts remain patent with evidence of intra-graft mineralization and dilation by ultrasound imaging

Duplex ultrasonography with color Doppler and basic mode (B-mode) revealed that all three graft preparations (ECM, Poly-ECM, Poly-ECM-Hep) remained patent throughout the three-month study. Mineralization within the walls of Poly-ECM-Hep grafts were first observed at two months, as depicted by increased echogenicity of the vascular wall on serial B-mode ultrasound imaging of the middle region of the implant. The area and intensity of mineralization progressed over time and was present in each segment (proximal, middle and distal) at three months (Figure 2, A). By three months, all grafts from ECM and Poly-ECM-Hep groups became calcified to a degree that can be observed by B-mode ultrasound. However, only half (2 out of 4) of the animals from the Poly-ECM group became mineralized.

Although the lumen of the bioengineered vascular grafts remained patent throughout the study, spectral Doppler waveform analysis revealed significant reduction in pulsatile blood flow peak velocity for all groups over time from two weeks to two months (ECM: $45.7 \pm 10.3\%$ reduction, $p=0.0019$; Poly-ECM: $37.4 \pm 7.0\%$ reduction, $p=0.0409$; Poly-ECM-Hep: $20.9 \pm 19.4\%$ reduction, $p=0.0277$), though the blood flow velocity stabilized thereafter (Figure 2, B–D). The statistically significant decrease in peak velocity from 2

weeks to 2 months indicates progressive graft dilation, which was also confirmed by digital analysis of histologic images of grafts at three months (Supplementary Figure S1). All grafts preparations were found to be dilated by 20–40% beyond the graft inner circumference prior to implantation. Nonetheless, the degree of graft dilation was not significantly different ($p>0.05$ via ANOVA) among the three preparations of vascular grafts (Supplementary Figure S1, C).

3.3. Bioengineered grafts remain patent with evidence of osteochondral differentiation and mineralization

All grafts preparations showed few signs of cell invasion into the graft walls at three months after implantation (Figure 3). All graft types remained patent without any occlusion at the anastomoses or along the entire graft length. Despite the lack of cellular infiltration within the wall of the graft, a neointima was noted to form on the luminal side of all vascular grafts, with signs of mineralization within the neointima noted on H&E staining. Mineralization was confirmed to be comprised predominantly of calcium, indicated by von Kossa (black) and Alizarin red staining (Figure 3A). The calcified regions were primary within the neointima; however, some mineralization was also observed within graft walls. Mineralization was in the form of small individual calcium deposits (Supplementary Figure S2A) or large crystals between the elastin fibers (Supplementary Figure S2B) and was present at the interface between the neointima and the graft wall (Supplementary Figure S2C). Importantly, none of the native arterial segments proximal or distal to the bioengineered vascular grafts showed any signs of calcification or other abnormality. The calcified regions also stained positive for glycosaminoglycans (GAG) with Safranin O (orange) and Toluidine Blue O (purple) stains, suggesting the presence of chondrocytes that secrete chondroitin sulfate (Figure 3A). The ECM composition within the calcified neointima was rich in collagen (blue-green color on Masson's trichrome stain, Figure 3A) and the cell morphology appeared to resemble chondrocytes, indicating calcification through the process of chondroid metaplasia.

All vascular grafts from animals implanted with ECM (4 out of 4) and Poly-ECM-Hep (4 out of 4) developed signs of calcification at various regions (proximal, middle region and/or distal end). However, only half of the vascular grafts from the Poly-ECM group (2 out of 4) developed any signs of calcification (Figure 3A). The degree of calcification in each graft was evaluated using a semi-quantitative pathology-based scoring system (Supplementary Figure S3)[23] and a quantitative measurement of calcified area relative to total graft area by digital image analysis.[24] Both methods confirmed that Poly-ECM exhibited the least amount of calcification (Figure 3B and 3C) throughout the entire length of the graft.

The presence of endothelial cells (ECs) on the luminal aspect of each graft was revealed by eNOS staining. ECs were found both in the calcified regions and non-calcified regions (Figure 4A). Approximately 70–80% of the lumen was confluent with ECs at three months (ECM: $74.6 \pm 14.7\%$; Poly-ECM: $73.9 \pm 11.0\%$; Poly-ECM-Hep: $80.0 \pm 8.3\%$), with no significant difference between the graft preparations or along anatomical length of the graft (Figure 4B). Neointima tissue formed within the inner wall of the bioengineered vascular graft and expressed alpha-smooth muscle actin (α -SMA), a vascular smooth muscle cell

(VSMC) marker, in non-calcified regions (Figure 4A). Importantly, at three months neointimal cells lose VSMC markers and express collagen II and osteoblast markers bone sialoprotein and osteopontin in calcified regions (Figure 4A), suggesting that some cells begin to develop an osteochondrogenic phenotype. Calcified regions also stained positive for matrix metalloproteinase 13 (MMP 13, Figure 4A), a collagenase involved in the restructuring of collagen matrix, specifically collagen II, during bone mineralization.[25] In regions without calcification, cells on the graft do not express bone sialoprotein or osteopontin. However, a small amount of collagen II was found in non-calcified regions, suggesting some chondrogenic differentiation (Figure 4A).

The area of neointima, regardless of chondroid formation or calcification, was quantified and normalized to the inner circumference of each graft (Figure 4C). For all groups, a similar amount of regenerated neointima tissue formed throughout the 1 cm graft length at the proximal (normalized neointima area to graft inner circumference, ECM: $73.4 \pm 31.1 \mu\text{m}$; Poly-ECM: $90.9 \pm 19.4 \mu\text{m}$; Poly-ECM-Hep: $86.0 \pm 24.0 \mu\text{m}$), middle (ECM: $67.0 \pm 26.2 \mu\text{m}$; Poly-ECM: $72.1 \pm 16.7 \mu\text{m}$; Poly-ECM-Hep: $66.4 \pm 30.9 \mu\text{m}$) and distal (ECM: $103.6 \pm 16.3 \mu\text{m}$; Poly-ECM-Hep: $70.1 \pm 8.5 \mu\text{m}$; Poly-ECM-Hep: $96.0 \pm 26.5 \mu\text{m}$) regions at three months after implantation. No significant difference in the normalized area of neointima was found at the proximal end or the middle region of the three bioengineered vascular graft preparations (ECM, Poly-ECM, and Poly-ECM-Hep). However, at the distal end, a lower amount of neointima tissue was observed in the Poly-ECM group ($70.1 \pm 8.5 \mu\text{m}$) compared to the ECM group ($103.6 \pm 16.3 \mu\text{m}$, $p < 0.05$) and the Poly-ECM-Hep group ($96.0 \pm 26.5 \mu\text{m}$, $p < 0.05$).

3.4. Oxidative stress is strongly associated with neointima calcification

Complete blood cell counts (Supplementary Table S2) and blood chemistry panels (Supplementary Table S3) show that all measured indices are within the normal ranges, indicating absence of systemic infection, chronic kidney diseases or liver dysfunction. All tissues (native arteries, neointima in the calcified and non-calcified regions, and the adventitia) stained negative for inflammatory makers, such as CD 68, TNF- α and T cell markers (Supplementary Table S1, Supplementary Figure S4), indicating minimal to no inflammation at the conclusion of the three-month study.

Interestingly, apoptosis and oxidative stress appeared to strongly associate with neointima calcification, regardless of the group of the graft (Figure 5). In the calcified regions of all groups, cell apoptosis, identified by TUNEL staining, was observed in most of the cells not only within the calcified neointima, but also on the outer aspect of the graft wall. In contrast, few to no sign of apoptosis was observed in native arteries or in non-calcified regions. Biomarkers of oxidative stress, including 8-hydroxy-2' - deoxyguanosine (8-OHdG) for DNA oxidation and 4-Hydroxynonenal (4-HNE) for lipid peroxidation, were observed in the calcified neointima, but not in native arteries. Some cells in the non-calcified neointima also stained positive for 4-HNE, possibly indicating that they are at the early stages of osteochondrogenic differentiation (Figure 5). Together, these observations point to oxidative stress as a possible mediator/initiator of bioengineered tissue calcification. The sources of oxidative stress, however, have yet to be identified.

4. Discussion

The need for a readily available small-diameter vascular graft with clinically acceptable patency rates remains, and significant research and development efforts continue to pursue this goal.[26, 27] Since acute thrombosis and progressive development of intimal hyperplasia are primary causes of low patency rates for prosthetic grafts, much effort has been put into improving hemocompatibility and compliance of vascular grafts. In our previous work, we developed several strategies to immobilize heparin onto decellularized ECM-based vascular grafts to reduce thrombosis, while preserving mechanocompatibility that led to a corresponding reduction in intimal hyperplasia.[8, 9, 28] However, the current study identifies significant mechanical (dilation) and biochemical (calcification) changes over a long-term durability analysis of bioengineered acellular vascular grafts. These long-term changes need to be clearly identified, carefully monitored, and thoroughly understood for clinical translation to be successful.

POCC biodegrades via hydrolysis, and lasted for more than four months when incubated in PBS at 37 °C *in vitro*, based on the blue auto-fluorescence of POCC. [8] However, in the current study, this autofluorescence was no longer detectable three months after implantation, suggesting that POCC degrades faster *in vivo* than *in vitro*. In addition, we previously observed that Poly-ECM-Hep had the least amount of neointima tissue one month after implantation, possibly due to the known effect of heparin in suppressing smooth muscle cell proliferation.[29] However, it is likely that by three months, bound heparin is also degraded from the Poly-ECM-Hep scaffold by cellular[30] and/or enzymatic[31] processes *in vivo*, and therefore no longer inhibits smooth muscle cell proliferation.

Calcification continues to be a major problem associated with cardiovascular biological implants, predominantly bioprosthetic heart valves.[5, 23, 32, 33] The characteristics of calcification in vascular graft neointima, as observed in this study, closely resemble those observed in vascular calcification, a pathological condition associated with aging, atherosclerosis, chronic kidney disease and diabetes.[34] Vascular smooth muscle cells lose their original phenotype and undergo osteochondrogenic transdifferentiation, developing ectopic mineralization during the process of vascular calcification.[35–39] However, vascular calcification is a systemic process in cardiovascular-related diseases, whereas the neointimal calcification observed in our study is restricted only to vascular grafts and not native tissue. We initially hypothesized that strong binding of calcium to vascular grafts may result in high local calcium content within the graft, and may contribute to the development of calcification in the neointima. After incubation of bioengineered vascular grafts (ECM, Poly-ECM and Poly-ECM-Hep) in simulated body fluid, a significantly higher Ca^{2+} concentration was observed in Poly-ECM-Hep, possibly due to the rich $-\text{COOH}$ from poly(diols) and heparin that promptly chelate calcium.[69, 70] However, this is inconsistent with our observation *in vivo*, where more calcification was observed in the ECM group (4 out of 4 animals developed calcification) relative to the Poly-ECM group (2 out of 4 animals developed calcification). This result suggests that additional mechanisms, other than passive calcium deposition, contribute to the calcification of bioengineered vascular grafts. It is likely that calcification within neointima occurs first through chondrogenic differentiation, where VSMCs differentiate into chondrocytes, followed by

osteogenic differentiation, where chondrocytes deposit mineral and differentiate into osteoblasts, in a manner that is similar to progressive vascular calcification.[40] Through our *in vivo* and *in vitro* studies, we identified similar factors to those described in systemic vascular calcification that contribute to vascular graft neointima calcification, as explained below.

Degradation of elastin [41, 42] and collagen [43] by MMPs is strongly correlated with vascular calcification. The activation of MMPs leads to vascular calcification, [44, 45] while the inhibition of MMP activity attenuates vascular calcification. [46, 47] Therefore, inhibition of ECM degradation via MMP inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs), may also inhibit calcification in vascular grafts. Interestingly, vascular grafts developed from materials other than ECM, such as poly ϵ -caprolactone (PCL)[5] and ePTFE[23], also develop calcification over time, possibly due to ECM proteins produced by infiltrating host cells. Although calcium [48] and phosphate[49] are both known mediators of vascular smooth muscle cell calcification, our study found no sign of hypercalcemia or hyperphosphatemia in blood (Supplementary Table 1).

Change in oxidative stress levels appear to be at least one of the key triggers of the phenotypical change toward the osteochondral lineage at areas of calcification. Oxidative stress induced by agents such as advanced glycation end products (AGEs)[50, 51] and oxidized low density lipoproteins (ox-LDLs)[52–54] is known to induce vascular calcification through modulation of the osteogenic transcription factor Runx2.[16] The current study demonstrates that signs of oxidative stress, including oxidized DNA damage, lipid peroxidation and apoptosis (Figure 5) co-exist with neointimal calcification. It is possible that AGEs and ox-LDLs present in the blood stream exhibit high affinity to vascular graft and neointima tissue, where a wholly intact endothelium has yet to form to protect their direct interactions. The percentage of EC coverage at three months increased by approximately 30% over time relative to the first month after implantation (ECM: $46.1 \pm 17.8\%$; Poly-ECM: $54.0 \pm 20.0\%$; Poly-ECM-Hep: $57.3 \pm 15.7\%$).[9] In contrast, all native arteries proximal or distal to bioengineered vascular grafts were completely covered with ECs on the luminal surface, and no calcification was observed in the native arteries where an intact endothelium was present.

Moreover, we show that the trends in the extent of calcification *in vivo* among the graft preparations aligns with the *in vitro* redox status of bioengineered vascular grafts (Figure 1), where less calcification was observed in the Poly-ECM group (higher antioxidant activity) than the ECM group, and more calcification was observed in the Poly-ECM-Hep grafts (lowest antioxidant activity). The capability of various forms of poly(diols-citrate), including POC,[18] POC-ascorbate,[19] and poly(polyethylene glycol citrate-co-N isopropyl acrylamide)[55] to attenuate oxidative stress has been previously reported. The inclusion of citric acid and thiol groups in POC-cys likely had an additive effect on the scavenging of free radicals.[56] However, the observed decrease in the antioxidant activity, relative to both Poly-ECM and ECM alone, was unexpected as there is no published evidence to suggest that heparin may function as a pro-oxidant. It is possible that the antioxidant thiols originally present in the cysteine residues of the ECM[57] and POC-Cys are rendered inactive upon conjugation with heparin-maleimide. Therefore, a potential strategy to develop vascular

grafts that are resistant to calcification is to fabricate prosthetic vascular grafts from materials that are either strongly antioxidant or resistant to binding of oxidative molecules (e.g. AGEs and ox-LDL). Since calcification in vascular grafts share many of the same characteristics as vascular calcification, the mechanistic understanding and therapeutic development may also be transferable.

The incorporation of an antioxidant polymer into bioengineered vascular grafts to reduce oxidative stress and related calcification, as demonstrated in this study, may also be applied to other biomaterial devices and implants, such as bioprosthetic cardiac valves, where calcification is the most common cause of clinical failure.[58] However, one limitation in the current study is the relatively small number of animals used (n=4) and the fact that calcification was not completely eliminated in the Poly-ECM group (2 out of 4 animals developed calcification at three months). Future work will focus on incorporating polymers with stronger antioxidant properties (e.g. poly[1,8-octanediol-co-citrate-co-ascorbate][19]) into bioengineered vascular grafts, evaluating their effect in calcification and graft fate in a larger number of animals for a longer period of time (6, 12 and 18 months), and further investigating the molecular mechanisms involved in calcification and graft remodeling. Nevertheless, this study highlights the importance of long-term evaluation of bioengineered vascular prostheses *in vivo*, and provides mechanistic insight and a potential solution to the long-term complications of bioengineered vascular ECM conduits.

In addition to the problem of calcification, dilation of vascular grafts could lead to severe anastomotic failure, aneurysms, ruptures and hemorrhage, as has been observed in autologous venous grafts,[59, 60] Dacron grafts,[61, 62] and decellularized xenografts.[63, 64] Strategies to stabilize ECM-based vascular grafts to prevent dilation include chemical crosslinking using carbodiimide chemistry to reduce ECM degradation[63] and perivascular polymer coating with electrospun PCL to prevent expansion.[65] However, stabilization of the ECM scaffold with either chemical crosslinking or polymer coating could also lead to increased stiffness and compliance mismatch between native arteries and vascular grafts and result in a severe inflammatory response and increased neointimal hyperplasia.[9] Therefore, balancing matrix degradation and minimizing new tissue formation remains an important challenge in vascular tissue engineering to minimize graft failure in the clinical setting. The lack of cellular remodeling observed in our study may be explained by the fact that after decellularization, the ECM scaffold is composed of collagens and elastin fibers that are highly compacted together when compared to native arteries with cells.[8] The tight matrix organization and low porosity may limit cell infiltration, resulting in decreased tissue remodeling within the arterial ECM-based scaffold, even with cells pre-seeded and cultured *in vitro*, as observed by us[8] and by others.[66, 67] Partial enzymatic degradation of the ECM scaffold to increase porosity could potentially improve repopulation by cells through transmural migration *in vivo*; however, graft mechanical properties may be compromised. Therefore, strategies that aim to increase scaffold porosity to allow cell infiltration without compromising structural integrity and mechanical strength are warranted. In addition, modification of the arterial ECM scaffold with growth factors that recruit host vascular cells may improve vascular tissue remodeling.[66, 68] Future work will focus on enhancing transmural cell migration and tissue remodeling, which in turn may minimize graft dilation.

5. Conclusions

In conclusion, we report several significant changes in bioengineered acellular vascular grafts over a long-term implantation study, including graft dilation, lack of remodeling and calcification. We found that calcification and oxidative damage play major roles in the long-term maintenance of vascular ECM conduits. Addition of antioxidants may be a viable strategy to limit degenerative changes and improve long-term durability. We provide valuable insight on the importance of critical, long-term assessment of small diameter vascular substitutes, made from any material, and demonstrate the necessity of developing therapeutic strategies to overcome these complications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research reported in this publication was supported by the National Institute of Biomedical Imaging and Bioengineering and the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health under award numbers 5R01EB017129 to GA and K08DK101757 to JAW. This work is also supported by the McCormick Foundation via Northwestern Memorial Hospital, the Dixon Translational Research Grants Innovation Award from the Northwestern Memorial Foundation, American Heart Association (AHA) Midwest Affiliate Postdoctoral Fellowship (14POST20160091) to BJ, and the Chicago Biomedical Consortium (CBC) Postdoctoral Award (PDR-008) to BJ. The authors would also like to thank the Northwestern University Microsurgical Core (Chicago, IL) for performing animal surgeries, and Dr. Tracy Gluckman for training and technical assistance in ultrasound imaging. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Figure 1.

In vitro characterization of bioengineered vascular grafts for their antioxidant properties (A) and calcium binding capability (B). ABTS free radical scavenging assay for vascular grafts shows higher antioxidant activity with polymer coating (ECM-Polymer) and lower antioxidant activity with heparin conjugation (ECM-Polymer-Hep), compared to ECM graft alone (ECM) over 9 days (A). Calcium binding to vascular grafts increases over three months for all groups, and the increase in binding is more significant with polymer coating (ECM-Polymer) and heparin conjugation (ECM-Polymer-Hep) than ECM graft alone (ECM) (B). * indicates $p < 0.05$ per one-way ANOVA ($n=3$).

Figure. 2.

Non-invasive vascular ultrasonography of Poly-ECM-Hep vascular grafts implanted in a rat abdominal aorta interposition model. B-mode ultrasound (**A**) at one, two and three months after surgery shows the morphology of the vascular graft (red arrow) and the compressible inferior vena cava (blue arrow), with signs of calcification in the graft (yellow arrow) appearing at various time points after surgery. Duplex ultrasound with color Doppler shows blood flow in the Poly-ECM-Hep vascular graft (red) and the inferior vena cava (blue) (**B**), and the blood flow velocity measured in the center of the implanted Poly-ECM-Hep vascular graft (**C**). Comparison between blood flow velocity within the graft over time in rats treated with ECM, Poly-ECM and Poly-ECM-Hep grafts during the three-month study (**D**).

Figure. 3.

Histological evaluation of bioengineered vascular grafts and native arteries recovered at three months after implantation. Native arteries and vascular grafts are stained with H&E, von Kossa, Alizarin red, Toluidine blue, Safranin O and Masson's trichrome (A). Scale bar=100 μ m. The degree of calcification was assessed via semi-quantitative scoring (B) and quantitative measurement of digital images (C) for ECM, Poly-ECM and Poly-ECM-Hep vascular grafts at the proximal, middle and distal regions. * indicates $p < 0.05$ per Kruskal-Wallis test by ranks ($n=4$).

Figure 4.

Immunofluorescence evaluation of normal arteries and bioengineered acellular vascular grafts at regions of vascular calcification at three months after implantation. Native arteries, the non-calcified and the calcified regions of vascular grafts are stained with eNOS/ α -SMA, BSP/ α -SMA, OPN, COL II, and MMP 13 (A). Yellow dash lines outline the bioengineered acellular vascular graft wall, L indicates the lumen side, and scale bar=100 μ m. The percent of endothelial cell coverage relative to the graft inner circumference (B) and the area of neointima tissue relative to the graft inner circumference (C) were measured on digitized images for ECM, Poly-ECM and Poly-ECM-Hep vascular grafts at the proximal, middle and distal regions. * indicates $p < 0.05$ per one-way ANOVA ($n=4$).

Figure. 5. Evaluation of apoptosis (TUNEL staining, red), oxidized DNA damage (8-OHdG, green) and lipid peroxidation (4-HNE) among native artery, vascular graft non-calcified region and vascular graft calcified regions in all groups. Yellow dash lines outline the bioengineered acellular vascular grafts, L indicates the lumen side, and scale bar=100 μ m.