Engineering biodegradable polyester elastomers with antioxidant properties to

attenuate oxidative stress in tissues

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Supplemental Data

S1. Polymer characterization

S1.1 Free carboxylic acid groups

A modified version of a toluidine blue dye binding assay was used to assess the

negative charges from carboxylic acid moieties on the POC and POCA polymers [1].

Polymer discs were incubated with 0.5 mM dye solution at pH 10 for 10 minutes.

Polymers were rinsed with sodium hydroxide solution at pH 9 to remove non-complexed

dye. The dye was desorbed from the film in 50% acetic acid solution and measured at

633 nm using UV-visible spectrophotometer and compared against dye standards to

determine total amount of bound toluidine blue dye.

S1.2 Contact angle

To determine hydrophilicity of POC and POCA, static water-in-air contact angle measurements were taken using a custom-built contact angle goniometer (components from Rame-Hart, Inc., Mountain Lakes, NJ). Polymers were acid leached in DMEM media until pH neutral, thoroughly rinsed with MQ water and lyophilized before contact angle measurements. Contact angles of POC and POCA were not significantly different (p=0.094).

S1.3 Hydrolytic degradation

To assess the hydrolytic degradation rate of POC and POCA, polymer discs were incubated under physiological conditions *in vitro* in PBS at 37°C and mass loss over time was measured. After PBS was removed at specified time intervals, the polymers were lyophilized and weighed (W_t). The mass loss percentage was calculated as $100*(W_o – W_t)/ W_o$, with W_o the initial weight of lyophilized discs. No significant change was observed in free carboxylic acid groups (p=0.1343), while degradation rate was slightly increased upon incorporating ascorbic acid (p=0.001)(**Figure S1**), with polymer discs losing structural integrity after 2 months of incubation in PBS at 37°C.
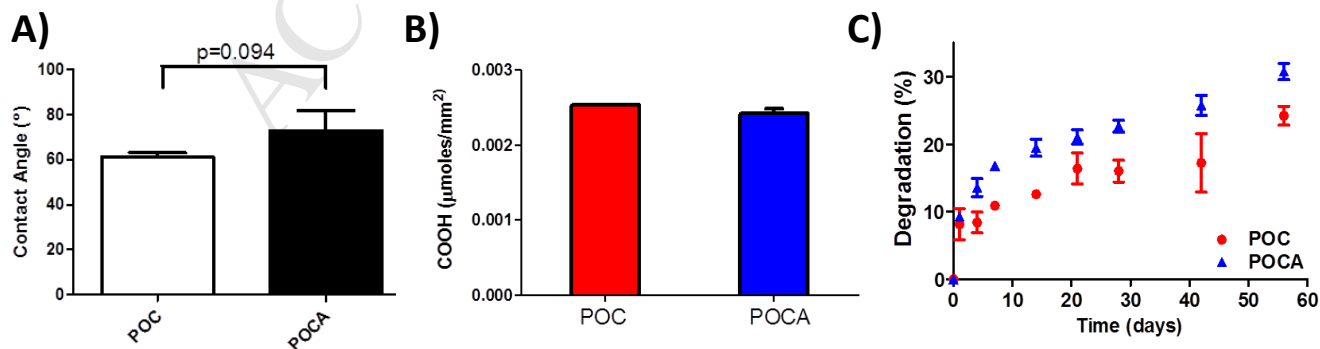
Figure S1. Hydrophilicity (A), free carboxylic acid (COOH) groups (B) and hydrolytic degradation rate (C) were not significantly affected by the incorporation of ascorbic acid in the backbone of citric-acid based polydiolcitrates. Mean±SD, N≥3.

S2. Ascorbic acid release

Ascorbic acid release from polymer discs was measured using the ascorbic acid-characteristic UV peak at 264 nm. Briefly, polymer discs were incubated in PBS at 37°C (50 mg/mL) and at each time point, supernatant was collected and fresh PBS added to samples. UV spectrum was measured using Tecan Safire plate reader and the absorbance value at 264 nm recorded. Ascorbic acid concentrations were determined using a standard curve from known ascorbic acid solutions in PBS.

Ascorbic acid gradually was released from the POCA film, but not from POC, as expected (**Figure S2**). Within a 2-month time frame (until polymer discs lost structural integrity), the total released amount from POCA was 5.31 ± 0.64 µg/mg polymer, which accounts for approximately 5.6% of the total theoretical amount of ascorbic acid present in POCA. The remaining ascorbic acid either comes out as part of small molecular weight co-oligomers with citrate and octanediol (**Figure S3**), or remains in the backbone of the POCA polymer.
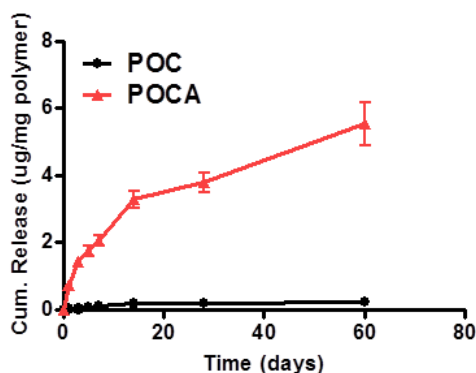
Figure S2. Free ascorbic acid is released from POCA over time. Since no evidence of free ascorbic acid was found in the prepolymer, released ascorbic acid is likely from hydrolytic degradation of polyester bonds. Mean ±SD, N=3.

S3.Characterization of polymer degradation products

To characterize the short-term degradation products from POC and POCA polymers, supernatants were collected after 2 hours of incubation of polymer discs in MQ water at 37°C. Polymer solutions were directly plated without use of a matrix using a matrix-free LDI-MS method. Spectra were collected with a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems). A 355 nm Nd:YAG laser was used as a desorption/ionization source, and all spectra were acquired with 20 kV accelerating voltage using positive reflector mode.

Spectra for both POC and POCA supernatants show peak clusters for small molecular weight co-oligomers of one octanediol with one citrate (C1D1) up to three octanediol and citrate monomers (C3D3). Furthermore, free citric acid was observed in both spectra at 192 m/z, while only POCA spectrum showed a strong peak at 176 m/z indicative of free ascorbic acid (**Figure S3**). The free ascorbic acid-specific peak, combined with measured free ascorbic acid release over time and absence of ascorbic acid-specific peaks in prePOC leads us to the conclusion that ascorbic acid is mainly incorporated in the polymer's backbone and not dispersed in its free form. Moreover, the appearance of free ascorbic acid-specific peaks in releasates after only 2 hours of incubation is due to short-term hydrolytic degradation instead of just leaching of unreacted monomers.
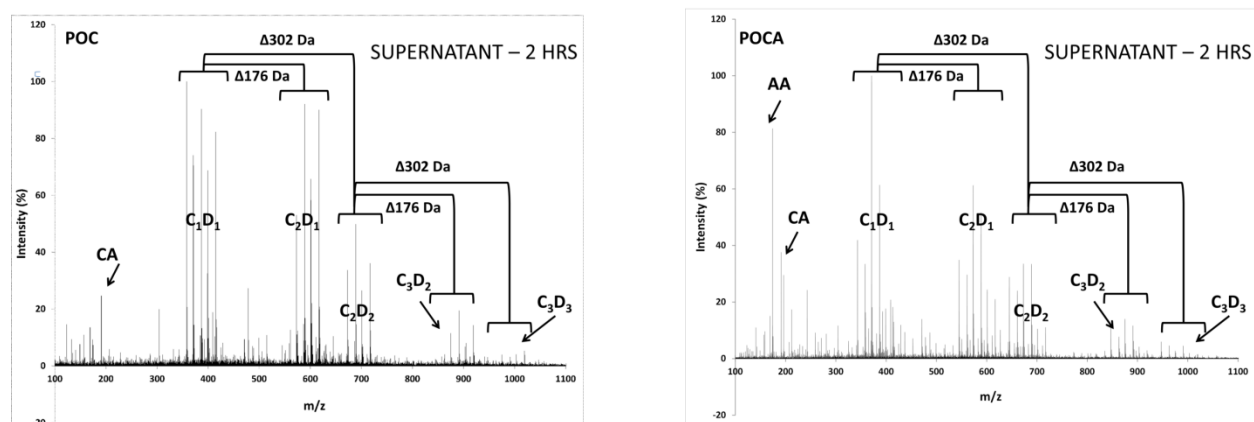
Figure S3. LDI-MS spectra of poly(octanediol-co-citrate) (POC) and poly(octanediol-co-citrate-co-ascorbate) (POCA) after 2 hours of incubation in MQ at 37°C. Both spectra show spaced peak clusters indicative of small co-oligomers of octanediol and citric acid, while only POCA shows a strong peak for ascorbic acid.

S4. Free radical scavenging by monomers

To assess the free radical scavenging capacity of monomers, the scavenging of ABTS and DPPH was monitored. Monomers were added to ABTS working solution (see main article for protocol) at concentrations reflecting their respective contents in POC and POCA multiplied by 50 mg/mL, and solutions were incubated at 37°C. At each time point, absorbance of the solutions was measured at 734 nm for ABTS and 515 for DPPH. All measurements were performed in triplicate. The antiradical activity was measured as % inhibition of free radicals by measuring the decrease in absorbance compared to control solutions. Octanediol does not show any radical scavenging activity, while citric acid scavenges free DPPH radical similarly to POC activity on DPPH (**Figure S4**). Ascorbic acid shows instantaneous scavenging of both ABTS and DPPH radical, indicating that the slower scavenging by POCA polymer is due to slow release of ascorbic acid, e.g. through hydrolytic degradation.
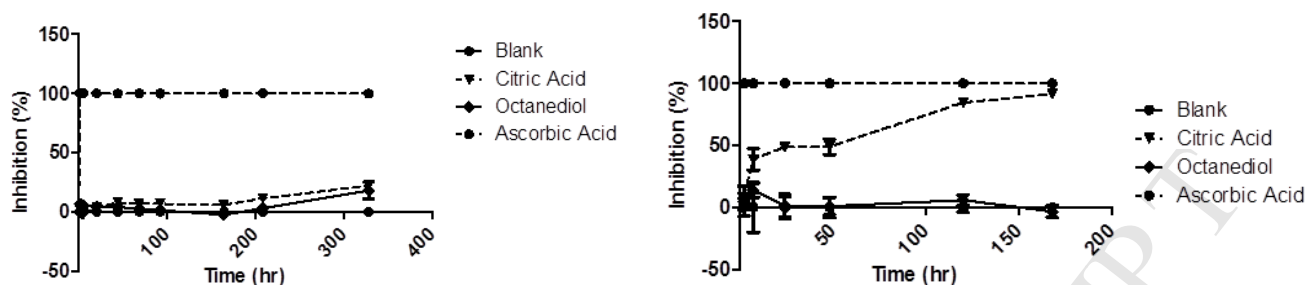
Figure S4. Free radical scavenging of ABTS (left) and DPPH (right) radicals by monomers. N≥3, Mean ± SD.

S5. Antioxidant capacity of polymer degradation products

To assess whether the polymers' short-term degradation products or releasates have AO capacity, polymers were incubated in PBS for several hours, after which the supernatants were collected and tested for AO capacity. To test the free radical scavenging capacity of POC and POCA supernatant, the scavenging of ABTS was monitored. Supernatant solutions were added to ABTS working solution (1:10 ratio) and incubated at 37°C. At each time point, absorbance of the solutions was measured at 734 nm. All measurements were performed in triplicate. The antiradical activity was measured as % inhibition of free radicals by measuring the decrease in absorbance compared to control solutions.

The inhibition of Iron chelation activity was assessed by adding collected supernatants to a solution of 0.4 mM $FeCl_2$ and 1 mM ferrozine indicator ligand in PBS (1:1 ratio). Absorbance was monitored at 534 nm.

The β-carotene-bleaching test was used to assess inhibition of lipid peroxidation by polymer short-term degradation products. Polymer supernatants were incubated with

β-carotene solution (1:10 ratio) and effects on oxidation measured. During that period, the absorbance was monitored at 470 nm, starting immediately after sample preparation.

Interestingly, POCA short-term degradation products exhibit both free radical scavenging and lipid peroxidation inhibition properties, while POC only inhibits lipid peroxidation (**Figure S5**). Since free ascorbic acid was detected in POCA supernatant, the ability to counter ABTS radical was expected. Moreover, these results are indicative that additional free radical scavenging by POCA compared to POC is due primarily to free ascorbic acid released from POCA through hydrolytic degradation. Although citric acid was detected in supernatants, no detectable iron chelation was present. Possibly the citric acid concentrations are too low to lead to any significant competition with ferrozine for iron. This is further indicated by the ability to inhibit beta-carotene bleaching, mainly caused by iron, by POC supernatant.
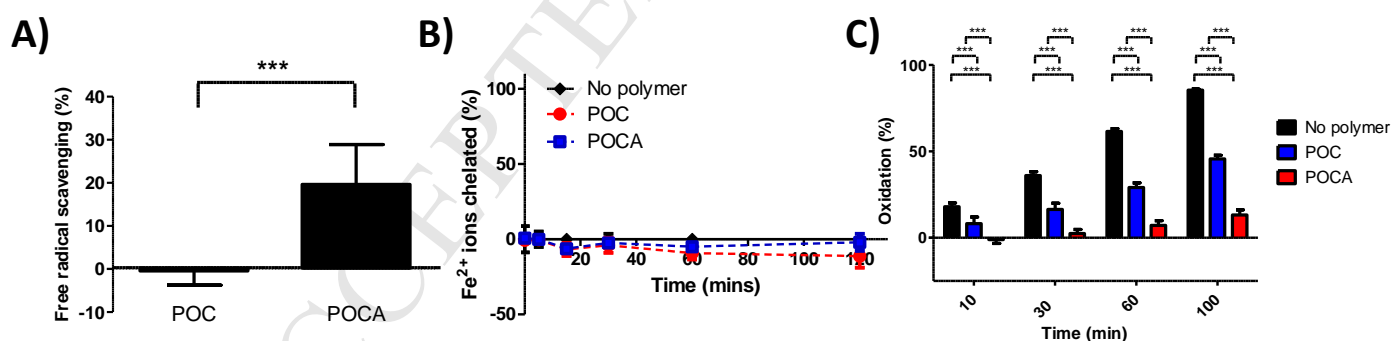

Figure S5. A) POCA short-term degradation products inhibit free radical scavenging of ABTS, while POC does not. B) Degradation products of POC and POCA do not chelate iron. C) POC and POCA degradation products both inhibit lipid peroxidation, with POCA having a stronger effect. N≥3, Mean ± SD. *p<0.05 **p<0.01, ***p<0.001

S6. Long-term antioxidant capacity

To assess long-term retention of intrinsic AO properties, polymers were degraded for 1 month in PBS solution at 37°C, rinsed 3x in MQ water and lyophilized before further use. For protocol, see main article 4.4.1.

POC and POCA, after 1 month of hydrolytic degradation, for the most part show similar radical scavenging and iron chelating results, although there is no significant difference in iron chelation between POC and POCA anymore (**Figure S6**). This may be due to the partial degradation providing increased access for iron to citric acid. Lipid peroxidation inhibition, on the other hand, was decidedly decreased after 1 month of degradation. Although POCA still significantly prevented oxidation at all time points, POC only significantly lowered oxidation at the 30 minute time point. It is currently unclear why lipid peroxidation inhibition decreased upon partial degradation. Nonetheless, both POC and POCA retain most of their AO functionality over time.
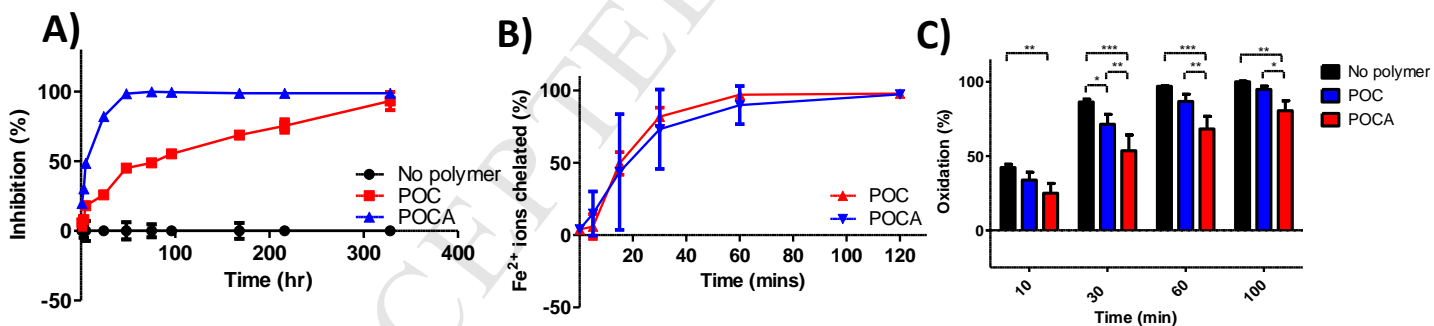

Figure S6. ABTS (A) assay shows free radical inhibition by both POC and POCA even after 1 month of hydrolytic degradation. POC and POCA still both chelate iron ions after 1 month of degradation, but now both polymers have equally strong chelating properties (B). Degraded POC and POCA both inhibit lipid peroxidation, although effects are much less pronounced than with fresh polymers(C). N≥3, Mean ± SD. *p<0.05 **p<0.01, ***p<0.001

S7. Effect of free ascorbic acid on menadione challenge to cells

To measure the effect of free ascorbic acid (AA) on cell viability upon menadione exposure, human umbilical vein endothelial cells (HUVECs) were cultured on tissue culture plastic (TCP) plates. Upon reaching 80% confluence, cells were treated overnight with different concentrations of ascorbic acid. Cells were then exposed to 50 µM of menadione in full growth media. After 1 hour of exposure to menadione, cells were washed with PBS and stained with Calcein-AM and Ethidium homodimer (Life Technologies, Carlsbad, CA) according to manufacturer's instructions to assess viability. Ascorbic concentrations of 250 and 500 µM provided protection from rapid cell death, with 500 µM showing significant numbers of HUVECs still spread and viable after 1 hour (**Figure S7**).
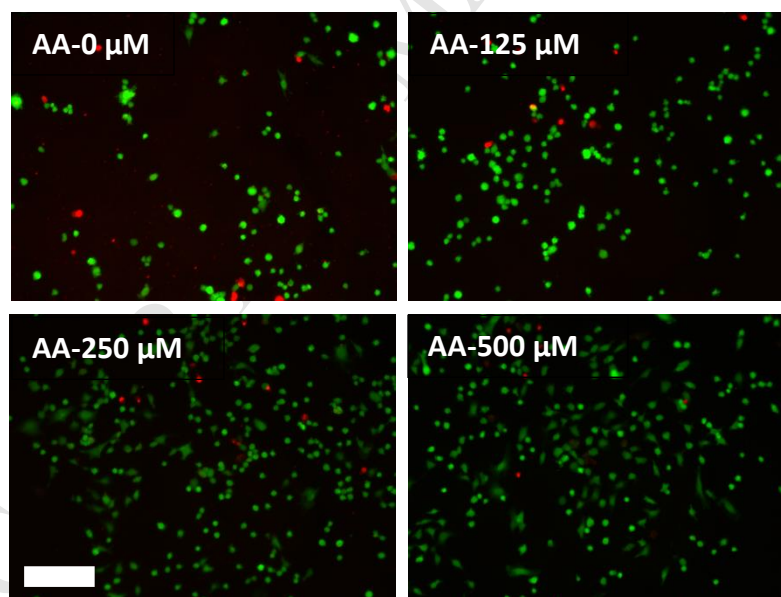

Figure S7. Free ascorbic acid prolongs viability of cultured HUVECs after 1 hour of a 50 µM menadione exposure, which induces rapid cell death due to excessive oxidative stress. Green indicates calcein AM stain for live cells, red indicates ethidium homodimer for dead cells. Bar=200 µm.

S8. Polymer-coated ePTFE for aortic interposition model

S8.1 Graft coating

To fabricate POC-coated vascular grafts, thin-walled ePTFE tubes (Inner diameter: 1.52±0.05 mm, intermodal distance: 25±10 µm, wall thickness:101.6±25.4 µm, Zeus Inc., Orangeburg, SC) were coated with 10% pre-polymer using an infusion-coating method (**Figure S8**). Briefly, grafts were closed at one end and polymer solution was slowly infused though the grafts using a syringe attached to the other side. After ethanol evaporation, this process was repeated once. Coated grafts were post-polymerized at 80 °C for 4 days, ethylene oxide gas sterilized, acid leached in DMEM media, washed 3x with PBS and lyophilized before further use. For visual assessment of polymer coating, grafts were sputter-coated with a 7-nm layer of gold and observed using scanning electron microscopy (SEM 3400N, Electron Probe Instrumentation Center, Northwestern University).
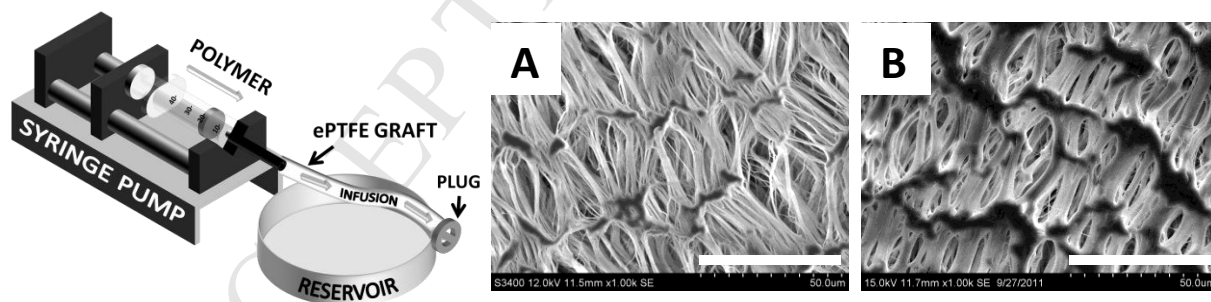

Figure S8. ePTFE grafts were coated with a 10 w/w% polymer solution using an infusion coating method (schematic). SEM images of ePTFE before (A) and after (B) infusion coating. Bar=50 µm.

S8.2 Graft Compliance

The grafts' compliance (C) was measured using a custom-built setup [2] and expressed as percent change per millimeter of mercury $\times 10^{-2}$, determined according to Eq. (S1)

$$C=10^4(D_{sys}-D_{dia})/D_{dia}(P_{sys}-P_{dia}) \tag{S1}$$

where D_{sys} and D_{dia} are systolic and diastolic scaffold diameters (mm), respectively, and (P_{sys} and P_{dia}) are the systolic and diastolic intraluminal fluid pressures (120 and 80 mmHg), respectively.

Upon coating with POC and POCA, the weights of grafts were increased by 25.2±4.8% (**Figure S9A**, p=0.014), and no significant change in compliance was observed (**Figure S9B**), indicating that the current method of infusion-coating the grafts does not alter the stiffness of the grafts, which could negatively impact long-term implantation outcomes.
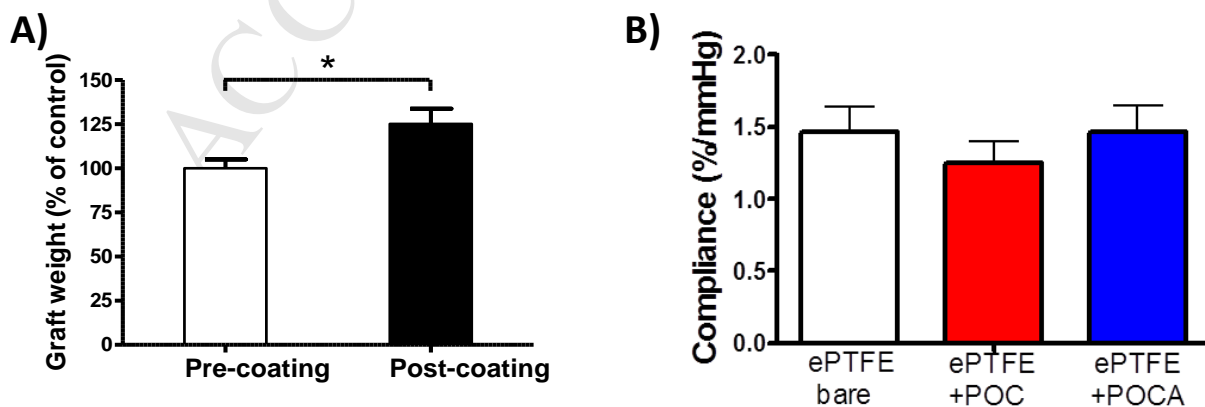
Figure S9. Infusion coating ePTFE grafts with a 10% w/v polymer solution in ethanol (2x) resulted in a weight increase of 25.2±4.8 % (A), P=0.014. Polymer-coated ePTFE grafts did not show any significant change in compliance (B). Mean ± S.D., N.≥4.

S9. Platelet adhesion

Whole blood was collected from adult volunteers into ACD anticoagulant. The blood was centrifuged at 250 g for 15 min to obtain platelet-rich plasma (PRP) supernatant. The PRP preparation and platelet suspension buffer (PSB) used for this experiment were described previously[3]. PRP, diluted 1:10 in PSB, was incubated at 37 °C for 60 min with acid-leached POC- and POCA-coated 48-well plates and gently rinsed with warm PBS. Glass and TCP pre-incubated with DMEM served as positive controls. The number of adherent platelets was determined by detecting the amount of lactate dehydrogenase (LDH) present after cell lysis as previously described [4]. Briefly, adherent platelets were lysed by incubation with 2% Triton-PSB buffer for 30 min at 37 °C. A colorimetric substrate for LDH (Roche Diagnostics Corporation, Indianapolis, IN) was added and incubated for 20 min at 37 °C. The reaction was stopped with the addition of 1N hydrochloric acid. The optical density was measured at 490 nm with a reference wavelength of 650 nm. A calibration curve was generated from a series of serial dilutions of a known platelet concentration and used to determine the number of adhered platelets.

POC and POCA were evaluated for platelet adhesion using platelet-rich plasma diluted in platelet suspension buffer (**Figure S10**). Platelet-rich plasma contains clotting factors present in the plasma as well as proteins such as fibrinogen, which are capable of promoting clot formation. There were numerous adherent platelets on both glass and DMEM-exposed TCP surfaces. In comparison, there was a significant reduction of platelet adhesion on both POC- and POCA-coated TCP ($p < 0.05$). The number of platelets on glass and TCP was $17.4 \times 10^3 \pm 4.1 \times 10^3$ and $16.0 \times 10^3 \pm 3.8 \times 10^3$ per mm^2, respectively. In contrast, the number of adherent platelets on POC– and POCA-coated TCP was only $8.5 \times 10^3 \pm 6.3 \times 10^3$ and $5.5 \times 10^3 \pm 1.9 \times 10^3$ per mm^2. Although there was a difference in adherent platelets between POC and POCA, this did not reach statistical significance (p=0.1063).
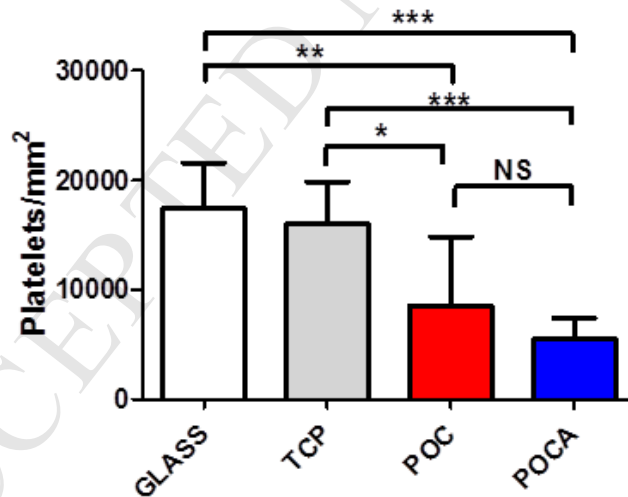

Figure S10 Platelet adhesion is reduced on both POC and POCA. Adhesion quantified by LDH, *$p < 0.05$, N=8, mean ± SD.

S10. *In vivo* neointimal hyperplasia: guinea pig aortic interposition model

POC-ePTFE grafts were fabricated from ePTFE grafts (Inner diameter: 1.52±0.05 mm, intermodal distance: 25±10 µm, wall thickness:101.6±25.4 µm, Zeus Inc., Orangeburg, SC). ePTFE grafts were coated with a 10% w/w polymer in ethanol solution using infusion coating followed by ethanol evaporation. This process was repeated once, after which coated grafts were post-polymerized at 80°C for 4 days. Grafts were then gas-sterilized and acid-leached. Prior to implantation, grafts were washed with 70% ethanol (3X), the ethanol was evaporated and grafts kept in PBS. Grafts were used in a guinea pig aortic interposition model and harvested after 4 weeks, stained with H&E for intimal hyperplasia assessment (for a detailed protocol, see paragraph 2.7.2 of main text). No reduction in neointimal hyperplasia was observed in POC-ePTFE compared to ePTFE grafts (Figure S11). Given that the hydrophilicity of POC and POCA is similar, the observed reduction in neointimal hyperplasia in POCA-ePTFE grafts is likely due to the antioxidant properties of POCA.
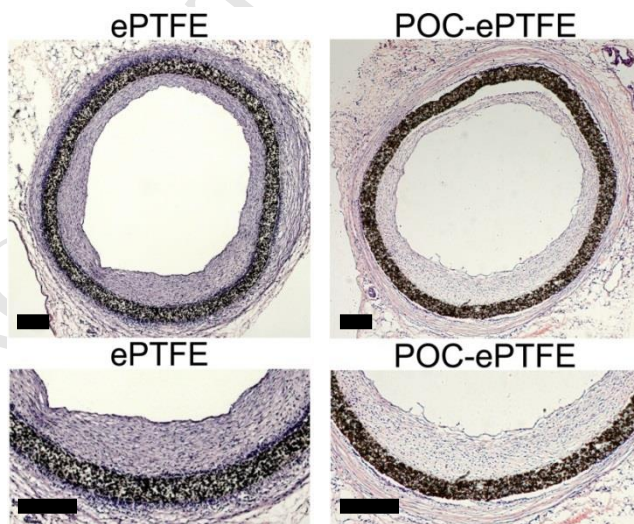

Figure S11. POC-coated ePTFE grafts do not show any reduction in intimal hyperplasia compared to regular ePTFE in a guinea pig aortic graft interposition model after 4 weeks of implantation. Bar=200 µm.

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