In vivo efficacy of a polymer layered decellularized matrix composite as a cell honing cardiovascular tissue substitute

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1. Introduction

Biologically active materials, which are inert outside the body, but are active upon implantation in the host, have long been considered to be the optimal tissue substitutes in the cardiovascular system. Due to the blood contacting nature of any material implanted in the cardiovascular system, an inert material carries the risk of thrombosis from lack of an endothelial layer, and that of calcification from the material-body immune response it can elicit. On the contrary an active material can hone cells from the circulation, enabling gradual host cell infiltration, tissue deposition and remodeling, and resorption of the initial material from the implant site. The active material needs to be programmed to have adequate mechanical strength at all times, to avoid aneurysmal degeneration of the material, risk of rupture, and death from bleeding.

We recently reported a new hybrid or composite material that can satisfy both properties, i.e. host cell honing and replacement, and also having adequate mechanical strength without thrombosis. This hybrid approach was implemented by electrospinning a layer of polymeric nanofibers that elicit programmed host cellular attraction and honing, onto a decellularized matrix that has a native three-dimensional structure to provide a conducive environment for honed cells to thrive.

This approach builds upon earlier methods for autogenous tissue formation, but addresses their shortcomings in both hemocompatibility and mechanical strength. For example, the method to use biomaterial-host immune response alone to create granulation tissue and create in-situ vascular grafts and valves, was partially successful, but lacked control over the mechanical strength. Tissues formed with this method were either fibrotic and stiff, or weak causing aneurysmal degeneration. Another method was to use synthetic scaffolds that...
are bioreabsorable, with material resorption and tissue replacement occurring over several months [19–21]. This approach has encouraging pre-clinical results, with collagenous host tissue formation onto the implant in 12 months [21], however, even after 24 months, the implanted material was not fully resorbed, eliciting chronic inflammation and calcification [22,23]. Since the infiltrating cells hone into a matrix that is not native and is designed to be dense to maintain adequate mechanical strength, the risk of promoting calcification is higher in this approach.

Our approach is a composite material, with a blood-boring layer of thin biocompatible polymeric nanofibers, electrospun from polycaprolactone-chitosan (PCL:Ch), and a mechanically strong, thick matrix layer of decellularized pericardium that has the three dimensional structure, biological moieties and organization to host the cells that hone into it. The blend of PCL:Ch creates a hydrophobic surface for cellular attachment, without the risk of thrombus formation. In drawing them into electrospun nanofibers, the size of the fibers, their alignment, pore diameter between fibers and their density can be controlled, which are key cues for cellular behavior upon attachment. Decellularized pericardium was chosen, as it is a material that is in use as a cardiovascular tissue scaffolding and has adequate mechanical strength to sustain cyclical loading [24–26]. Detergent based decellularization of this material is possible, while still preserving the native characteristics of the matrix [27].

In this study, we report in-vivo results demonstrating the biocompatibility, hemocompatibility, and remodeling potential of this new material for use as a cardiovascular tissue substitute. Chronic implantation was performed in rodents and sheep, and the material was studied as a scaffold in low- and high-pressure environments.

2. Materials and methods

2.1. Material preparation

The composite material was prepared by electrospinning a blend of PCL:Ch in 12:1 ratio onto the decellularized bovine pericardium. Minor modifications were made to the preparation protocol reported previously [27]. Bovine pericardium (BP) was decellularized with 2% sodium deoxycholate (D6750, Sigma-Aldrich, USA, average MW 1200–5000) for 48 h at 37 °C, in a shaker incubator (Model 420; Orbital shaker, Forma Scientific). Acellularity was confirmed by DNA estimation and histology (hematoxylin and eosin (H&E) and 4,6-diamidino-2-phenylindole (DAPI) staining). A blend of 12% polycaprolactone (PCL, Catalog # 440 744, Sigma Aldrich, USA, mol wt. 70 000–90,000) and 1% chitosan (Ch, Catalog # 417 963, Sigma-Aldrich, USA, mol wt > 1,000,000) was prepared in a solvent, and electrospun onto the decellularized pericardium in the circumferential direction. After 3 h of electrospinning, the polymer-tissue composite was neutralized on 0.5 M NaOH for 10 min, for the free amine of chitosan to interact with the decellularized tissue, and then the material was preserved in 70% ethanol. Decellularization efficacy and structural integrity of the decellularized matrix core were assessed, and polymer-core interface was examined with scanning electron microscopy (SEM) and spectroscopic techniques (infrared and x-ray photon spectroscopy), as reported earlier [13].

Two controls were used – (a) a bovine pericardium that was not treated with any other processes other than decellularization; and (b) an untreated fresh pericardium that was fixed in 0.625% buffered glutaraldehyde solution at a pH of 7.0. All the materials were sterilized, by washing them thrice in sterile, 70% ethanol solution, and then rinsed in sterile saline solution, in a laminar flow hood. The samples were then exposed to ultraviolet light for 30 min, while maintaining sterility, and stored in saline at 37 °C, until the day of implantation.

2.2. In-vivo biocompatibility in a rat subcutaneous model

In-vivo biocompatibility was investigated in a subcutaneous pouch in adult rats. Sprague Dawley rats weighing between 200 and 300 g were purchased from Envigo (Indianapolis, IN), with equal number of male and female rats used for the study (N = 46 total, 23 female, 23 male). Under an approved Institutional Animal Care and Use Committee protocol at Emory University, the animals were placed under deep general anesthesia. The animal was first sedated with 5% isoflurane in 100% oxygen for 5 min, and then maintained at 2% isoflurane in 100% oxygen with a nose cone for rest of the surgical procedure. The rat was placed supine, 1 mL of blood was drawn from the tail vein, and the dorsal side of the rat was shaved to prepare the implantation sites. Carprofen (2.5 mg/kg) and Gentamycin (6 mg/kg, SQ) were administered before the surgical incision was made. A longitudinal incision was made on the dorsal side of the rat, and 10 mm subcutaneous pockets were opened on either side of the incision with blunt dissection, with each pocket distant from one another to isolate their in-vivo remodeling. 10 such pockets were made in each rat, and 2 cm² samples of a chosen material were implanted into each pocket. Pockets were then closed with 4–0 Vicryl (Ethicon Inc.) in an interrupted manner. The rats were weaned off anesthesia and recovered. Buprenorphine (0.02 mg/kg, intramuscular) and Carprofen (2.5 mg/kg, subcutaneous) were administered post-operatively, for analgesia.

Rats were survived to either 1, 4 or 12 weeks after the surgery, with daily observation of wound site for signs of infection.

46 rats were assigned to three groups, with an equal male to female ratio in each group: (Group I) 0.6% glutaraldehyde fixed untreated BP (n = 15), (Group II)- decellularized BP (n = 15) and (Group III)- Bio-Hybrid material consisting of decellularized BP layered with PCL-Ch nanofibers (n = 16). 15 rats each were recruited to groups I and II, and 16 to group III. In each group, 5 rats were terminated at 1 week, 5 at 4 weeks, and 5 at 12 weeks after implantation. At termination, under general anesthesia with isoflurane, a midline incision was performed on the dorsal region, and the subcutaneous pockets were excised to expose the material. A small vascular clip was placed on one corner of the material, to mark the surface facing the skin. The material was carefully excised from surrounding tissue, stored in 10% buffered formalin, and further processed to stain with Hematoxylin and Eosin (H&E), Movat’s Pentachrome and Von Kossa stains to assess cellular infiltration, changes in extracellular matrix and calcification, respectively.

2.3. In-vivo sheep studies

Three 25 kg Suffolk male sheep (Robinson Services Inc, Mocksville, NC) were surgically implanted with the hybrid material using aseptic techniques. Patches of the material were implanted in their carotid artery (high pressure, high flow), left atrium (low pressure, low flow), and main pulmonary artery (low pressure, high flow) that represent different hemodynamic conditions of the cardiovascular system. These procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Emory University.

All animals were pre-treated with Aspirin (81 mg, PO) and Plavix (75 mg, PO), three days prior to surgery. On the day of the procedure, the animals were sedated with Ketamine (4 mg/kg, IV), and Diazepam (0.3 mg/kg, IV), and intubated for mechanical ventilation with 1.5% isoflurane mixed in 100% oxygen. A rumen tube was inserted to empty the stomach and avoid bloating during the procedure. Appropriate medications were given peri-operatively and intra-operatively (flunixin–2 mg/kg, IV; buprenorphine-0.01 mg/kg, IM; bayerl-5 mg/kg, IV; propofol -5 mg/kg, IV; isoflurane-1%-5% in 100% O₂ and heparin- 200 U/kg followed by 50 U/kg maintenance dose) for pain management and maintenance of normal physiological functions. The sheep were cannulated in the femoral artery and vein, to record a baseline angiogram of the carotid artery and the main pulmonary artery. B-mode and color doppler ultrasound of both carotids was performed to evaluate stenosis or thrombosis. A longitudinal incision was performed on the left neck, and the left carotid artery was exposed. The artery was clamped both proximally and distally and a longitudinal incision was made in the vessel to remove a thin width of tissue. A patch of the hybrid material (~20 mm × 5 mm)
was cut and sutured into the open carotid artery incision with 6–0 prolene sutures. Hemostasis was confirmed. The neck was closed in layers with internal muscle layers approximated with 3–0 vicryl and the skin layer with 3–0 PDS sutures. A left thoracotomy was then performed, and access to the heart was achieved through the 4th intercostal space. Epicardiac echocardiography was used to assess baseline cardiac function, and presence of preexisting clots or thrombii. Color Doppler of the pulmonary artery was performed to quantify blood flow rate. The main pulmonary artery was partially clamped using a side biting clamp, and an incision was made in the artery. A 20 mm × 10 mm patch was sutured into the pulmonary artery incision and the clamp was released. Similarly, a hybrid patch was sutured onto the roof of the left atrium, by making an incision into the atrial wall and removing part of it. A post-procedure angiogram was performed to assess flow in the regions where the patches were implanted. Hemostasis was confirmed and the chest was closed in layers. The animals were weaned off anesthesia and once vital signs were stable, were shifted to their cages for recovery. All the animals were followed up to 12 weeks.

At termination, a similar operative procedure was performed. After sedation, ultrasound was repeated to evaluate both the left and right carotids, using color doppler, to examine for flow disturbances near or away from the patch, presence of thrombus and vessel stenosis. Pulmonary artery angiography was also performed to assess the patency. A median sternotomy was then performed, and the heart was accessed by careful dissection of the intrathoracic adhesions. An epicardiac ultrasound was done to assess intracardiac clots, color Doppler in pulmonary artery for flow disturbances, and examination of rest of the cardiac structures was performed. A left atrial angiogram was done at termination by inserting a 5Fr sheath in the LV apex and advancing the tip into the left atrium. The animals were euthanized with 80°C water bath for 20 min in modified citrate buffer (C9999; Sigma) for heat mediated antigen retrieval. After antigen retrieval, nonspecific binding was blocked with overnight incubation with 20% goat serum (S-1000; Vector Laboratories) and 1% bovine serum albumin (BSA; A6003; Sigma) in 1x phosphate-buffered saline (PBS; 806 552; Sigma). Primary antibodies were added next day, that were prepared in optimized concentrations in 1:10 diluted blocking solution (1X PBS: 10% goat serum, 1% BSA and 0.05% Tween 20 (P9416; Sigma)). Tissue slides were incubated overnight at 4°C and thereafter washed with 1x PBS (3x, 5min each). Slides were incubated with secondary antibodies for 2 h at room temperature. For counterstaining of cells, 4',6-diamidino-2-phenylindole (DAPI (62 248, 1 mg/ml; Thermo Scientific: 1X PBS) was applied for 10 min. After drying, the sections were mounted using FluorSave reagent (Sigma, 345 789-20 ML). Negative controls were generated with the same procedure omitting the primary antibody during the first incubation. Tissues for positive controls included right carotid artery from the sheep where the material was not implanted (for alpha SMA and vimentin) and the spleen (for CD163 and iNOS). The following antibody concentrations were used: mouse anti-human CD163 monoclonal EDH8u-1 (Biorad, MCA1853; 1:50), polyclonal rabbit anti-iNOS antibody (Abcam ab3523; 1:50), goat anti-rabbit IgG H&L (Alexa Fluor® 488) (Abcam, ab150077; 1:200), goat anti-mouse IgG H&L Alexa Fluor® 647 (Abcam, ab150115; 1:200), monoclonal rabbit anti-vimentin (D21H3) XP® (Cell Signaling, 5741S; 1:200), monoclonal anti-actin, γ-smooth muscle (Sigma, A2547-100UL; 1:400), goat anti-mouse IgG H&L (Alexa Fluor® 568) (Abcam, ab175473; 1:200).

From the immunohistochemistry images of the rat and sheep explants (stained for different stains: CD163, iNOS, alpha SMA and vimentin), the number of cells/cellular nuclei were quantified using Image J (U. S. National Institutes of Health, Bethesda, Maryland, USA) to: a) compare the differences in the cellular infiltration between the groups (untreated BP, decellularized BP and Bio-Hybrid) at 1, 4 and 12 weeks in the rat explants, and b) compare the cellular infiltration between the Bio-Hybrid and the surrounding native tissue. From the rat tissue sections, 3 samples in each group (untreated BP, decellularized BP and Bio-Hybrid) at each timepoint (1, 4 and 12 weeks) were analyzed. In the sheep explants, 3 sections each of the hybrid material and native surrounding tissue were analyzed for cells. For each sample, three frames were counted for cells, averaged and the means of these cells were compared among the groups. The 20X immunofluorescent color images stained for DAPI were first converted into 8-bit grayscale images to look at the nuclei as white structures and the background was subtracted and threshold adjusted. The images were then processed to count the cellular nuclei and thus the cells.

2.4. Immunohistochemistry assessment of honing cell types

Immunohistochemistry was done on both the rat and the 12 week juvenile sheep explants. In the rats, the explants were stained to identify the inflammatory macrophages phenotype (M1-iNOS and M2-CD163 macrophages), whereas the sheep explants were additionally stained for the presence of smooth muscle actin cells (α-SMA) and cellular damage using Vimentin. 5 μm paraffin sections of the sheep explants were taken and deparaffinized with serial changes in xylene and isopropyl alcohol. The slides were then heated in 96°C water bath for 20 min in modified citrate buffer (C9999; Sigma) for heat mediated antigen retrieval. After antigen retrieval, nonspecific binding was blocked with overnight incubation with 20% goat serum (S-1000; Vector Laboratories) and 1% bovine serum albumin (BSA; A6003; Sigma) in 1x phosphate-buffered saline (PBS; 806 552; Sigma). Primary antibodies were added next day, that were prepared in optimized concentrations in 1:10 diluted blocking solution (1X PBS: 10% goat serum, 1% BSA and 0.05% Tween 20 (P9416; Sigma)). Tissue slides were incubated overnight at 4°C and thereafter washed with 1x PBS (3x, 5min each). Slides were incubated with secondary antibodies for 2 h at room temperature. For counterstaining of cells, 4',6-diamidino-2-phenylindole (DAPI (62 248, 1 mg/ml; Thermo Scientific: 1X PBS) was applied for 10 min. After drying, the sections were mounted using FluorSave reagent (Sigma, 345 789-20 ML). Negative controls were generated with the same procedure omitting the primary antibody during the first incubation. Tissues for positive controls included right carotid artery from the sheep where the material was not implanted (for alpha SMA and vimentin) and the spleen (for CD163 and iNOS). The following antibody concentrations were used: mouse anti-human CD163 monoclonal EDH8u-1 (Biorad, MCA1853; 1:50), polyclonal rabbit anti-iNOS antibody (Abcam ab3523; 1:50), goat anti-rabbit IgG H&L (Alexa Fluor® 488) (Abcam, ab150077; 1:200), goat anti-mouse IgG H&L Alexa Fluor® 647 (Abcam, ab150115; 1:200), monoclonal rabbit anti-vimentin (D21H3) XP® (Cell Signaling, 5741S; 1:200), monoclonal anti-actin,  γ-smooth muscle (Sigma, A2547-100UL; 1:400), goat anti-mouse IgG H&L (Alexa Fluor® 568) (Abcam, ab175473; 1:200).

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Fig. 1. Surgical implantation of the materials in the dorsal subcutaneous region of rats, after which the animals were followed to 1, 4 or 12 weeks and then explanted for histopathological analysis. Bio-Hybrid composite showed increased tissue deposition compared to the untreated and decellularized BP at 12 weeks (image on far right).
2.5. Statistical analysis

Statistical analysis was performed in GraphPad Prism v.9. Data was not tested for normality since the sample size was small, and only three samples were compared in each group. The mean cell count data from the immunohistochemistry images (3 frames/sample) was compared to examine differences between the groups using one-way ANOVA. $p < 0.05$ was considered significant.

Fig. 2. Cellular infiltration in Bio-Hybrid, decellularized BP and glutaraldehyde fixed untreated pericardium explants in a rat subcutaneous implantation model at 1, 4 and 12 weeks in the material-tissue border and the mid-regions. H&E staining of the explants shows higher number of infiltrating cells in the decellularized and Bio-Hybrid samples from 1 to 12 weeks. Mid-region at 12 weeks depicted better repopulation of cells in Bio-Hybrid compared to the other two groups.
3. Results

3.1. Biocompatibility, inflammation, and calcification of the Bio-Hybrid

The hybrid composite material is a combination of decellularized bovine pericardial matrix and an electrospun layer of blended polycaprolactone-chitosan. The polymer-tissue interaction resulting from the formation of newly formed chemical bonds (C=O, C-O, and C-N) was characterized by infrared and x-ray photon spectroscopy. Scanning electron microscopy also demonstrated a white glistening polymer layer at high magnification resulting from the interaction at the interface. These results have been discussed in detail in our previous report [13].

Fig. 1 depicts the dorsal implants in rats, with more tissue deposition by 12 weeks around the hybrid material, and the decellularized BP. Figs. 2–4 depicts the longitudinal remodeling of the hybrid material, decellularized BP and glutaraldehyde fixed untreated BP, in the rat model. H&E staining of the samples at 1, 4 and 12 weeks depicts varying cellularity in the materials, as shown in Fig. 2. At 1-week, cellular accumulation at the interface between the material and the host, was evident in all the materials tested. By 4 weeks, cellular infiltration beyond the interface, into the tissue, was only observed in the hybrid material and decellularized BP, but not in the glutaraldehyde fixed intact BP. This result was consistent at 12 weeks, with extensive cellularity in the hybrid material, some sparse cellularity in the decellularized BP, and no cellularity in the mid region of the glutaraldehyde fixed untreated pericardium.

Fig. 3 depicts extracellular matrix deposition by the infiltrating cells, between different samples. In the hybrid material, collagen that is indicated by yellow staining, is predominant at 1 week. Mucin deposition stained in green and fragmented elastin strands stained in black at 4 weeks. At 12 weeks, further remodeling is evident that resulted in yellow, green, and black staining, indicating presence of native like wavy collagen, mucin and elastin with the possibility that infiltrating cells hone into the native 3D architecture and develop a synthetic phenotype. In the decellularized BP, collagen with some elastin is prominent at 1 week and at 4 weeks, there is evidence of remodeling with much ucgin deposition and some elastin that is retained. At 12 weeks, green staining is prominent, indicating mucin deposition due to the remodeling of the scaffold by the infiltrated cells that deposited new extracellular matrix replacing the collagen. Also, the wavy nature contributed predominantly by the collagen fibers is lost with random orientation of the matrix possibly indicating slight degradation with cellular infiltration. Finally, in the glutaraldehyde fixed intact BP, yellow staining is dominant in all the three time points, which depicts unchanged collagen that is already within the tissue that is cross linked. Cross linking of the pericardium did not allow much cellular infiltration in the, pericardial, cross linked collagen predominant matrix and allow it to remodel over time.

Presence of calcification was assessed with Von kossa staining, the results for which are shown in Fig. 4. In the hybrid material and decellularized BP, there was no calcification seen at any time points, with pink spots that represent cells. In the glutaraldehyde fixed intact BP, there was no calcification at 1 week. But, contrastingly at 4 and 12 weeks the sections stained for brown color, indicating calcification. Supplemental figures 1, 2 and 3 shows the temporal changes in cellularity, remodeling and calcification that was confirmed in 3 rats in each group at each time point. The results described above in all the groups validate the results in multiple animals and varied sex (male:female).

Fig. 5 depicts the inflammatory response and its evolution over 12 weeks after implantation in the sub-cutaneous pocket in rats. At 1 week, iNOS + pro-inflammatory M1macrophages (green) were present at the material-host border region in the three groups (Bio-Hybrid, decellularized BP, and the glut fixed untreated BP). At 4 weeks, iNOS expression...
was evident within the mid region of the Bio-Hybrid and decellularized BP groups and was higher but isolated to the border in the glutaraldehyde fixed intact BP. At 12 weeks, higher levels of iNOS expression was seen in both mid-material and border regions of the Bio-Hybrid, whereas sparse staining was seen in both decellularized BP and glutaraldehyde fixed intact BP. CD163+ stained cells (red), indicative of M2 pro-regenerative macrophages, was sparse in all the materials at 1 week. By 4 weeks CD163+ staining was observed in both Bio-Hybrid and decellularized BP. Staining was seen both within the border and the mid region in the Bio-Hybrid but only at the border of the decellularized BP. At 12 weeks, M2 expression was visualized across the entire thickness in the Bio-Hybrid but dense staining seen in the border of decellularized BP.

The presence of the polymer in the hybrid material samples several weeks after implantation in the host animals, was seen in SEM images of the processed explants. The samples upon dehydration for SEM, depicted a sandwiched layer of decellularized BP overlaid with polymer, along with deposited tissue on both the subcutaneous and muscular sides of the implant. The material-tissue layers were separated by small gaps due to the dehydration step in tissueprocessing, enabling visualization of the polymer on top of the decellularized matrix as white dense electrospun fibers that were distinct from the underlying decellularized tissue, demonstrating slow degradation profile of the polymer as seen in Supplemental figure 4.

3.2. In-vivo juvenile sheep cardiovascular implant studies

None of the sheep implanted with the hybrid material patches elicited any adverse reactions, or thrombotic events, that caused morbidity or mortality. All study animals completed the entire follow-up period of 90 days. Explants from the carotid artery, main pulmonary artery, and left atrium are shown in Fig. 6, Panel I (Bio-Hybrid implant). Tissue dehiscence, aneurysmal degeneration or thrombosis were not observed under direct vision. A glistening layer of neotissue was seen on all the explants shown in Fig. 6, Panel II (Explant after 90 days). Blood flow across the implanted sites was not perturbed in color Doppler at all the three sites of implantation as seen in Fig. 6, Panel III (Flow at patch site). Contrast angiography of carotid, pulmonary artery, and left ventricle angiogram (Fig. 6, Panel IV (Angio at 90 days) also did not show any disturbance in flow or stenosis at the implant site. Data shown in Fig. 6 is representative data for one animal (sheep #7562). Supplemental figures 5 and 6 shows the hemocompatibility for the remaining sheep (animals #7563 and 7564). The site of the implanted material location is indicated in white arrows in Panel IV of the figures. Similar results were seen in the other two animals as well.

Figs. 7–9 depicts the histopathological analysis of the explants from cross sections of the carotid, pulmonary artery and left atrial regions, delineating the explanted Bio-Hybrid samples from the surrounding native tissue. The vertical panels show whole sections, followed by magnified images indicated as black boxes. The horizontal panels are the carotid artery, pulmonary artery and left atrial sections, where the implanted material is indicated within dotted lines and the side facing the blood is labelled accordingly. Fig. 7 shows H&E staining of the carotid, pulmonary artery and left atrial explants, that shows the layered structure of a blood vessel, well organized and oriented cells in the native carotid and pulmonary arteries. In Fig. 7, Panel I and II, both the arteries show good cellular infiltration into the entire thickness of the Bio-Hybrid.
material, that was comparable to the native tissue. This pattern was observed in the left atrial explant (Fig. 7, Panel III) as well with good cellular infiltration into the material. It was difficult to clearly define the borders of the material in the left atrial tissue, compared to the ones in the arteries. Fig. 8 shows pentachrome staining of the explants and depicts remodeling of the explants in the carotid, pulmonary artery and left atrial explants after 90 days. Fig. 8, Panel I, II and III shows the layered structure of the carotid and pulmonary artery as compared to the left atrial tissue. In Fig. 8, all the three explants show a combination of fibrin (pink in color), elastin (black) and mucin (green) in the native tissue. In the carotid explant, staining for collagen represented as yellow in color, is clear. The Bio-Hybrid material showed mucin deposition in all the explants (Panel I, II and III) throughout the sample, evident from the green staining (Fig. 8), with some fibrin seen in the left atrial explant (Fig. 8, Panel III). Von kossa staining did not demonstrate any calcification in any of the explanted carotid, pulmonary and the left atrial samples with absence of any black/brown staining representative of calcium deposition (Fig. 9). Supplemental figures 7 to 11 shows histology after 90 days, from the other two animals, that demonstrated cellular infiltration and matrix remodeling similar to what is described for the animal # 7562, without any calcification in the material.

Fig. 10 depicts the immunohistochemistry of the explants from the three regions. Vimentin positive cells were observed in tissues explanted from the three distinct regions, indicating their viability and the biocompatibility of the material. Among the infiltrating cells, α-smooth muscle actin positive staining was abundant in the carotid and pulmonary artery explants, but not in the left atrial explant. Cellular staining for iNOS, which marks pro-fibrotic M1 macrophages, were high in both the carotid and pulmonary artery explants. However, M2 macrophages (CD163) were also abundant in these tissues, indicating inflammation was paralleled by a reparative response as well. Supplemental figure 13 shows the immunohistochemistry of explants from every animal recruited to this study. Fig. 11 shows the quantification of cells from the rat (Fig. 11a) and sheep (Fig. 11b) explants. From Fig. 11a, there was no difference among the three groups with respect to the number of cells infiltrated into the samples at 1 week (mean cell count: untreated BP = 2213.5 ± 881.7, decellularized BP = 2468.5 ± 637.1 and Bio-Hybrid = 3541.0 ± 1940.3), whereas at 4 weeks, there was a significant increase (p = 0.0027) in the number of cells in the decellularized BP in comparison to the untreated BP (mean cell count: untreated BP = 520.0 ± 677.4 and decellularized BP = 4502.0 ± 1189.3). Also at 4 weeks, there was a significant decrease (p = 0.0401) in the number of cells in the Bio-Hybrid in comparison to the decellularized BP (mean cell count: decellularized BP = 4502.0 ± 1189.3 and Bio-Hybrid = 1577.5 ± 195.8). At 12 weeks, the number of cells in the Bio-Hybrid was significantly higher (p = 0.0334) than the untreated BP but no difference was seen between decellularized BP and the Bio-Hybrid (mean cell count: untreated BP = 294.5 ± 21.92, decellularized BP = 1104 ± 1356.2 and Bio-Hybrid = 3293.5 ± 359.91). Overall, the number of cells decreased in the decellularized BP from 4 to 12 weeks whereas, there was an increase in the Bio-Hybrid. Fig. 11b shows the number of cells in the Bio-Hybrid material in the three locations (carotid, pulmonary and left atrium sites) in comparison to the surrounding native tissue and there was no difference found between the two regions indicating similar extent of cell repopulation in the material.

Table 1 depicts changes in the systemic inflammatory response observed in total and differential blood count, platelets, D-dimer and LDH in the three sheep. The total white blood cell count increased to 8.83 ± 2.69 × 10³/μL (normal range: 4-8 × 10³/μL) by 7 days, which came down to normal range (6.07 ± 0.82) by 60 days. The percentage of neutrophils also increased from 45.00 ± 6.42% to 59.67 ± 6.11% immediately after surgery but normalized after day 1 (46.0 ± 9.86%) and plateaued rest of the study duration until termination. The average lymphocyte count remained normal until day 21, after which it remained slightly elevated until termination. Platelets, D-dimer, and LDH, that are indicators of ongoing thrombocytosis and cellular damage, were
measured. The platelet count before surgery was $327.67 \times 10^3/\mu l$ (Baseline 1) and increased after surgery (Baseline 2 = $1074.33 \pm 340.99 \times 10^3/\mu l$) but was within normal limits ($800–1100 \times 10^3/\mu l$) for the three sheep. LDH increased after surgery and normalized after day 21.

4. Discussion

In this in vivo study, the feasibility of a novel Bio-Hybrid material with a cell-honing, sacrificial polymeric layer overlaid onto a decellularized, three-dimensional native matrix core, as a cardiovascular tissue substitute is demonstrated. In both the sub-cutaneous rat implant model, and in implants performed in the cardiovascular system in sheep, by 3 months, the polymeric layer had attracted and honed host cells into the material. The cells honing into the implant were not localized to the polymer layer, but also infiltrated the underlying matrix core. Such cellular infiltration was consistently highest in the Bio-Hybrid approach when compared to other scaffolds such as glutaraldehyde fixed bovine pericardium, or decellularized pericardium lacking a cell-honing polymeric layer. The Bio-Hybrid material was also hemocompatible, without any thrombii forming on the implant, with signs of endothelialization by 3 months. The adhesion of the polymer to the underlying decellularized pericardium by chemical interaction in the Bio-Hybrid composite (previously reported by us) resulted in cell infiltration without non-delamination or peeling. Calcification was not evident by this timepoint, in comparison to the other material types. Overall, data from this study provides
encouraging evidence in support of this new strategy to develop mechanically robust, and potentially host integrating scaffolds for use in the cardiovascular system.

The composite layering of the thin polymeric nanofibrous layer onto the three-dimensional, native matrix scaffold serves dual purposes. In a prior study, we demonstrated that the polymeric layer adds mechanical strength to the decellularized matrix scaffold, and provides additional extensibility [13]. Such improved mechanics are desired, as bare decellularized scaffolds used in the cardiovascular system have failed from aneurysmal degeneration [28,29]. Such degeneration or mechanical failure were not seen in any of the animals in this study, despite using the material in high pressure conditions such as the carotid artery. The second purpose of the polymer is to be a barrier to thrombosis, but also an attractant for host cells to infiltrate the tissue. Thrombosis was not observed in any of the animals at the time of scaffold explantation, which we attribute to the hydrophobicity of polycaprolactone (PCL) and the smoothening of the decellularized matrix with the PCL that did not trigger platelet adhesion [30]. For cellular attachment and infiltration, the hydrophobicity of the PCL does not help. Thus, adding 90% deacetylated chitosan to the material induced some hydrophilicity into the PCL fibers, without causing thrombosis, but enabling cellular attraction, attachment and proliferation [27]. Our data is also supported by other groups that have successfully used polycaprolactone-chitosan small-diameter vascular grafts in sheep, without thrombosis [31].

Cellular infiltration, immune response, and subsequent remodeling of the scaffold material without thrombosis and calcification determines the long-term viability of any biomaterial in-vivo. Macrophages are one of the most studied cellular phenotypes to understand the material’s remodeling ability and macrophage polarization in the chronic inflammatory response commands the fate of the biomaterial based on its phenotype: expression of M1 dominates pro-inflammatory response and M2 expression promotes the pro-regenerative response [8]. These macrophages are known as highly plastic cells that adapt their function depending on the biomaterial microenvironment including structure, composition, pore size and tissue-specific signals secreted by cells that determine differential activation of these cells [32]. In the rat

![Fig. 7. Cellular infiltration in sheep explants from carotid artery, main pulmonary artery and left atrium after 90 days. H&E staining of all the explants showing uniform cellular infiltration in all the locations.](image)
Fig. 8. Matrix remodeling of the sheep explants from carotid artery, main pulmonary artery and left atrium after 90 days. Pentachrome staining of the explants showing predominant deposition of glycosaminoglycans in carotid and pulmonary positions and with less extent in the left atrial explant.

Fig. 9. Calcification of the sheep explants from carotid artery, main pulmonary artery and left atrium after 90 days. Von kossa staining of the explants from all the three locations showing no calcification.
sub-cutaneous implants of this study, acute inflammation resulted in the presence of M1 macrophage positive cells after 1 week, at the material-tissue interface in all the three materials (Bio-Hybrid and control materials). Following the onset of chronic inflammatory response, M1 macrophage expression increased qualitatively and overall quantitative increase in the number of cells after 4 weeks, in the mid-regions of decellularized pericardium and Bio-Hybrid. Since the glutaraldehyde fixed untreated BP was cross linked, it did not allow the inflammatory cells to infiltrate into the mid-regions. Such an observation has been seen in some studies where the cross linking limits the cellular infiltration [33, 34]. The continual presence of chronic inflammation was confirmed with M1 macrophages (iNOS + ve) in the mid-region of Bio-Hybrid and decellularized BP at 12 weeks in the rat, with significantly increased number of cells in the Bio-Hybrid in comparison to the untreated BP, as well as sheep cardiovascular explants with different flow conditions.

Such staining was not seen in glutaraldehyde fixed pericardium, most likely due to its irreversible nature of aldehyde fixation which prevents recipient cellular repopulation, pro-regenerative ECM signaling, and associated remodeling that has been observed in other studies that used glutaraldehyde treated tissues [34].

The cues in the biological ECM based tissue substrates enables resolution of the chronic inflammation and adaptation in the host over time with the expression of M2 macrophages that has been seen in other biomaterials [8,35,36]. In this study, though iNOS + positive cells were high, indicating infiltration of M1 macrophages, as part of the host immune response to the implanted scaffold, heavy staining for CD163 also depicts that the macrophages were shifting towards a more reparative M2 macrophage phenotype, which may favor material regeneration. Such a shift from M1 to M2 polarity indicating a pro-regenerative effect was not reported in other biomaterials used in cardiovascular

Fig. 10. Immunohistochemistry analysis of the Bio-Hybrid sheep explants (carotid, pulmonary, left atrial explants) after 90 days from one animal. Panel A: Alpha smooth muscle actin (Alpha SMA) staining of explants and right carotid artery (positive control) showing positive staining of SMA positive cells in the control, carotid and pulmonary samples while the left atrial samples show minimal staining, Panel B: Inducible nitric oxide synthase (iNOS) staining of explants and spleen tissue (control) showing most positive staining for M1 macrophages in the carotid samples followed by pulmonary and atrial explants, Panel C: CD163 staining of M2 macrophages of explants and few cells in the atrial explants, Panel D: Vimentin staining of explants and control (right carotid artery) showing vimentin positive cells in all the samples. Positive control samples are from the same animal.

Fig. 11. Quantification of cellular infiltration in the rat and sheep explants. A) Quantification of number of cells per frame in the untreated BP, decellularized BP and the Bio-Hybrid explants at 1,4 and 12 weeks showing no difference at 1 week, increase in the decellularized BP at 4 weeks and increase in the Bio-Hybrid at 12 weeks in comparison to the untreated BP, and B) Quantification of number of cells/frame in the Bio-Hybrid (material) in comparison to the surrounding native tissue showing no difference between the two regions.
Immunological response to Bio-Hybrid in sheep for 90 days.

| WBC | Neutrophils | Monocytes | Basophils | Eosinophils | Lymphocytes |
|-----|-------------|-----------|-----------|-------------|-------------|
| 4.10 | 1.27 | 0.57 | 0.00 | 0.00 | 2.67 |
| 0.63 | 0.40 | 0.88 | 0.00 | 0.00 | 6.42 |
| 4.90 | 10.59 | 2.67 | 0.00 | 0.00 | 5.93 |
| (8.30) | (5.93) | (6.42) | (0.00) | (0.00) | (5.93) |

Table 1

Immunological response to Bio-Hybrid in sheep for 90 days.

Data is represented as Average ± SE. B1: before surgery, B2: immediately after surgery, D1: Post-operative day 1, D7: Post-operative day 7, 14 and 21, M1 – 1 month post-operative.

| Parameter | B1 | D1 | D7 | D14 | D21 |
|-----------|----|----|----|-----|-----|
| WBC | 4.10 ± 0.63 | 4.90 ± 0.35 | 5.93 ± 1.60 | 6.27 ± 0.58 | 6.83 ± 0.28 |
| RBC | 8.30 ± 0.23 | 8.43 ± 0.77 | 8.20 ± 0.51 | 8.97 ± 0.58 | 9.27 ± 0.82 |
| HCT | 22.00 ± 1.76 | 23.67 ± 3.38 | 20.99 ± 0.99 | 28.30 ± 0.66 | 26.72 ± 0.64 |
| Platelets count | 327.67 ± 11.90 | 304.33 ± 39.99 | 319.87 ± 39.99 | 356.33 ± 39.99 | 327.67 ± 11.90 |
| Neutrophils | 4.50 ± 0.62 | 4.67 ± 0.12 | 5.97 ± 0.29 | 6.00 ± 0.96 | 6.27 ± 0.58 |
| Monocytes | 2.67 ± 0.33 | 2.00 ± 0.57 | 2.00 ± 0.57 | 1.00 ± 0.57 | 2.00 ± 0.57 |
| Eosinophils | 0.87 ± 0.82 | 0.87 ± 0.82 | 0.87 ± 0.82 | 0.87 ± 0.82 | 0.87 ± 0.82 |
| Basophils | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |

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reconstruction [37–39]. We believe that the M2 macrophage expression in decellularized BP and Bio-Hybrid is due to the biological cues from the natural extracellular matrix and the optimal nano topography of the polymer that provides favorable pore diameter to not only allow cell infiltration but promote M2 macrophage proliferation. At the same time, this mixed M1-M2 response seen in the Bio-Hybrid at the end of 12 weeks in both rats and sheep, makes it inconclusive to determine if the immune response is steering towards fibrosis or regeneration/remodeling at 12 weeks and warrants further long-term studies to understand the material remodeling and resolution of inflammatory response.

Extracellular matrix remodeling is expected after cellular infiltration is observed in in-situ degradable biomaterial remodeling. The infiltrated immune cells, matrix degradation proteins like metalloproteinases, and the resident cells infiltrating from the adjacent tissue contribute to the matrix turnover and neo matrix formation [8]. In this study, the starter matrix of the Bio-Hybrid and the control materials evaluated histopathologically stained for collagen and glycosaminoglycans as indicated in the 1 week explants and as explained in our previous study [13]. The in-vivo studies showed glycosaminoglycan deposition, preservation of native collagen matrix, and cellular infiltration in the Bio-Hybrid implants, whereas the glutaraldehyde fixed untreated pericardium did not enable similar remodeling at 4 and 12 weeks. The infiltrated immune macrophage cells and non-immune cells like fibroblasts are known to deposit neo-extracellular matrix proteins, which has been observed in this study and by others as well [21,40]. With the progression of the immune response, the Bio-Hybrid still showed some collagen and preservation of the wavy structure, whereas the decellularized BP showed loss of collagen wavy structure, and glycosaminoglycans deposition typically indicative of a robust immune response elicited by the decellularized BP seen throughout the thickness. This probably indicates some degradation of the decellularized BP (seen only in histology). There exists less antigenic proteins in the Bio-Hybrid compared to the decellularized BP due to the polymer layering and much slower level of degradation is predicted with adaptive immune response that is matched by extracellular matrix deposition. Native ECM proteins and proteoglycans provide secondary structural support [41] in addition to providing important intracellular signaling cues for regeneration/repair such as proliferation, differentiation, mitogenesis, and chemotaxis [8,42]. The presence of such proteins observed in the Bio-Hybrid material would potentially enable sustained synthesis and organization of the existing and new ECM, to promote cellular proliferation and tissue homeostasis. However, the long-term deposition of matrix proteins such as GAGs, elastin, and collagen, as well as the balance of these proteins needs to be studied in relation to the long-term remodeling of the material. After explantation, tissue retraction and fibrosis were not observed which is likely due to the active matrix remodeling that took place, creating a suitable microenvironment for the cells to proliferate. This was evident by the presence of extracellular proteins, vimentin, and alpha SMA positive interstitial cell phenotypes without tissue thickening.

Other aspects of the biomaterial viability and function are non-thrombogenicity and calcification [8]. Calcification is seen in natural ECM based materials due to factors like lipid content, mechanical stress due to weak matrix, immune response, and lack of anti-calcific proteins. The process of polymer overlay on the decellularized matrix in the Bio-Hybrid reduces the mechanical stress and exposure of antigenic proteins. Storage of pericardium in 70% ethanol additionally contributed to the absence of calcification that has been seen before [43]. The other reason for the absence of glutaraldehyde that has shown calcification in cross linked tissues, however, the degree of crosslinking plays an important role in the degree of calcium deposition [44,45]. Calcification was absent from the Bio-Hybrid samples at all time points in rats and in the juvenile sheep model, whereas some degree of calcification was observed in glutaraldehyde fixed pericardium, similar to a previous report [44]. Long-term anti-calcification potential of Bio-Hybrid needs to be further studied in a juvenile sheep model in comparison to the commercially available materials used.
The preliminary feasibility of the Bio-Hybrid approach in honing host cells and enabling its remodeling is encouraging. This approach provides an opportunity to tune the polymeric layer over a strong decellularized core to elicit the best response from the host and maybe tuned to suit the host's physiological state, age, immunity, and other confounding factors. Thus, the proposed material could be used for multiple applications such as an intracardiac patch to correct septal defects, large diameter vascular grafts, patches for vessel augmentation/repair or as biomaterial to cover the septal occluders and potentially as valve leaflets as well. The material properties of Bio-Hybrid enable us to potentially use it in high pressure regions such as for carotid artery repair as well. The clinical relevance of this material as a patch or shaped into different implants, and their functional efficacy requires long term studies in animals, which is our next step.

5. Conclusion

The proposed Bio-Hybrid material, by layering polymeric nanofibers onto a decellularized native matrix core, is non-thrombogenic, enables honing of host cells into it, and remolds with the host over time, providing a novel active remodeling substitute for use in the cardiovascular system.

Financial support

This work was supported by grants from the American Heart Association (19POST34380522), and Carlyle Fraser Heart Center at Emory University Hospital Midtown (4886580000).

Relationship with industry

M.P. reviewed, extensively revised and approved the animal studies and the explant analysis. J.M. wrote the manuscript, and formed the Credit author statement approved this work.

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