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A polymer–extracellular matrix composite with improved thromboresistance and recellularization properties

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ABSTRACT

Organ engineering using decellularized scaffolds is a potential long-term solution to donor organ shortage. However, this technology is severely limited by small vessel thrombosis due to incompletely recellularized vessels, resulting in exposure of extracellular matrix (ECM) components to platelets and clotting factors in flowing blood. To address this limitation, we designed a polymer–ECM composite and demonstrated its potential to reduce thrombosis and facilitate re-endothelialization in a vascular graft model. Rat aortas were decellularized using a sequential combination of weak detergents followed by a nuclease treatment that resulted in 96.5 ± 1.3% DNA removal, while ECM components and mechanical properties were well maintained. A biodegradable and biocompatible elastomer poly(1,8 octanediol citrate) (POC, 1 wt.%) was infused throughout the ECM at mild conditions (37 °C and 45 °C) and was functionalized with heparin using carbodiimide chemistry. The polymer–ECM composite significantly reduced platelet adhesion (67.4 ± 8.2% and 82.7 ± 9.6% reduction relative to untreated ECM using one of two processing temperatures, 37 °C or 45 °C, respectively); inhibited whole blood clotting (85.9 ± 4.3% and 87.0 ± 11.9% reduction relative to untreated ECM at 37 °C or 45 °C processing temperature, respectively); and supported endothelial cell—and to a lesser extent smooth muscle cell—adhesion *in vitro*. Taken together, this novel POC composite may provide a solution for thrombosis of small vessel conduits commonly seen in decellularized scaffolds used in tissue engineering applications.

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1. Introduction

Organ transplantation is the only definitive treatment for end-stage organ failure. The shortage of functional donor organs, the need for immunosuppression, and the development of allo-antibodies remain major hurdles limiting extension of organ transplantation to all patients in need [1]. The concept of whole-organ tissue engineering has emerged as a promising, long-term, alternative approach for organ replacement [2]. Decellularization, the process of removing allogeneic or xenogeneic cells from donor organs also removes cell-based antigenic ligands that may cause immune rejection, leaving behind the three-dimensional extracellular matrix (ECM) as an intact scaffold as a template to develop whole organs from donor scaffolds and recipient cells [3,4].

One major challenge in whole-organ tissue engineering is thrombus formation within the microvasculature of organoids developed from decellularized scaffolds, often due to incomplete coverage of thrombogenic ECM proteins by endothelial cells [5]. To address this immediate hurdle in organ engineering, we developed a strategy to immobilize the anticoagulant heparin onto ECM surfaces that are exposed to blood in a method that may be applied to any ECM-based tissue engineering system. Specifically, we incorporated a biodegradable polyester elastomer, poly(1,8 octanediol citrate) (POC) to link heparin to ECM, building upon a novel strategy earlier reported by our group to biofunctionalize expanded-polytetrafluoroethylene (ePTFE) vascular grafts with endothelial cells or immobilized heparin [6,7]. We also report a critical

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modification of the post-polymerization manufacturing protocol performed at physiologic temperatures to retain matrix structure as the natural protein composition of ECM leads to a more tenuous and temperature-dependent stability compared to synthetic materials such as ePTFE. POC exhibits excellent biocompatibility, supports endothelialization, and provides carboxyl and hydroxyl groups for chemical modification with bioactive macromolecules [8]. Heparin remained active for at least 4 weeks when linked to POC coated ePTFE vascular grafts via carbodiimide chemistry [6,7]. We hypothesize that a similar strategy can be applied to link heparin to an ECM-based tissue-engineering scaffold using physiologic-range temperatures to improve hemocompatibility of exposed ECM for potential use in tissue engineering and regenerative medicine applications.

We developed a strategy to decellularize rodent aortas as a model ECM scaffold to evaluate the efficacy of immobilizing heparin onto ECM matrices hybridized with POC at 37 °C or 45 °C. We show that this strategy decreases platelet binding and clot formation, and allows for recellularization with both endothelial and smooth muscle cells. As the ECM is both easily infused with POC and readily processed under physiologic conditions that support cellular repopulation, this method can be applied to other decellularized organ systems, broadening the ultimate application of this strategy.

2. Materials and methods

2.1. Materials

Triton X-100, sodium dodecyl sulfate (SDS, 20% stock solution) and absolute ethanol were obtained from VWR International (Radnor, PA). Deoxyribonuclease I from bovine pancreas (DNase I, ⩾400 U/mg), Proteinase K from Tritirachium album (⩾30 U/mg), Hoechst 33258 solution, urea, Trizma® hydrochloride (Tris–HCl, 99%), citric acid (99%), 1,8-octanediol (98%), L-cysteine (⩾98%), 2-(N-morpholino)ethanesulfonic acid (MES, ⩾99%), N-(3 dimethylaminopropyl)-N-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS, 98%), 1,6-diaminohexane, Toluidine Blue O, sodium chloride, potassium chloride, magnesium chloride, calcium chloride, sodium phosphate monobasic, dextrose, apyrase, and bovine serum albumin (BSA) were obtained from Sigma–Aldrich (St. Lois, MO). Heparin sodium (200 U/mg) was obtained from Celsus Laboratories (Cincinnati, OH). Quant-iT™ PicoGreen® dsDNA Assay Kit and Alexa Fluor 488 Phalloidin were obtained from Life Technologies (Carlsbad, CA). Complete mini protease inhibitors and LDH cytotoxicity detection kit were obtained from Roche Molecular Diagnostics (Pleasanton, California). BCA protein assay kits were obtained from Thermo Scientific (Rockford, IL), and Rat Fibroblast Growth Factor (FGF) ELISA kit was obtained from Bmassay (Beijing, China).

2.2. Preparation of decellularized aortas

Animal care was performed in accordance with the NIH Guide for Care and Use of Laboratory Animals, and experiments using animals were approved by the Animal Care and Use Committee of Northwestern University (NU-IACUC), Chicago, IL. Male Sprague Dawley rats (200–250 g) were used for aorta recovery. Briefly, donor rats were anesthetized, abdomen shaved, and operative field disinfected using standard surgical technique. The abdomen was entered through a midline incision. The abdominal aorta was exposed after removing internal organs, and a 3–4 cm segment of the aorta was removed. All donor rats were euthanized by exsanguination at the end of the procedure and 5–10 ml of blood was collected into ACD anticoagulant test tubes (BD Biosciences, Franklin Lakes, NJ) for platelet adhesion and whole-blood clotting assays described below. Recovered aortas were stored at −20 °C until they were decellularized.

Rat aortas (3–4 cm long in length) were washed with deionized (DI) water and then decellularized by submersion in 1% Triton X for 48 h followed by 1.5% SDS for 48 h. During the treatment, aortas were immersed in each solution with continuous stirring at room temperature. Decellularized aortas were incubated with 100 U/ml DNase I solution at 37 °C for 4 h to remove residual DNA and subsequently washed and stored in DI water at 4 °C.

2.3. Characterization of decellularized aortas

2.3.1. Imaging

Decellularized aorta and native aortas were embedded in paraffin, and sectioned into 5 μm thick slices using a microtome. Hematoxylin and eosin (H&E) staining, Masson's trichrome staining and Weigert's elastin staining were performed by the Northwestern University Mouse Histology Core. Histological sections were imaged by transmitted light microscopy with a 10× objective (Carl Zeiss AG, Germany). Aortas were further imaged with scanning electron microscopy (SEM). Briefly, all samples were fixed with 2.5% glutaraldehyde for 1 h and dehydrated in ethanol prior to coating with osmium tetroxide. SEM images were taken on a Hitachi 3500 N at the Northwestern University EPIC facility. Lastly, native and decellularized aortas were embedded in Optimal Cutting Temperature compound (OCT), frozen and sectioned (10 μm thickness), and stained with Hoechst-33258 (5 μg/ml). Florescent microscopy (Nikon TE2000U, Japan) was used to image the sections under an ultra violet (UV) filter.

2.3.2. DNA analysis

After treatment with detergents, decellularized aortas were incubated with DNase I solution (0, 100 and 400 U/ml) for 4 h at 37 °C. Native aortas were used as controls. Native or decellularized aortas were then lyophilized, weighed to obtain dry mass, and digested overnight at 60 °C with Proteinase K (15 U/ml in Tris–HCl buffer). DNA content in the digested solutions was quantified with a PicoGreen dsDNA assay kit (Life Technologies, Carlsbad, CA) as per the manufacturer's instruction.

2.3.3. Protein analysis

Native and decellularized aortas were lyophilized, weighed and carefully grinded into small pieces. Fresh extraction buffer (2 M urea, 50 mM Tris–HCl, 5 mg/ml heparin sodium, and complete mini protease inhibitors 1 tablet/10 ml) were added to the minced aortas and incubated on a rocker at 4 °C overnight. Samples were then centrifuged at 12,000g for 30 min at 4 °C to obtain protein extracts. The extraction and centrifugation process was repeated, and the solution containing recovered protein was added to the first time extracts. The total extract solution was then dialyzed in DI water with 3500 MWCO, and analyzed with a micro BCA protein assay and rat FGF ELISA kit for total protein content and growth factor retention, respectively.

2.3.4. Mechanical testing

Tensile tests were performed on aortas before and after decellularization ($n = 3$) using Instron 5544 Materials Testing Machines (Instron, Norwood, MA). Each aorta sample (~4 cm length, 1.2 mm diameter) was gripped on either side of the proximal and distal ends (5 mm gripping length from each end), leaving a 30 mm gauge length for testing, which was calibrated for each sample prior to each testing. All samples were hydrated during testing to maintain physiologic conditions and a stroke rate of 1 mm/min across 0–10% strain. A stress–strain curve was plotted for each sample and Young's modulus (E) was calculated. All data

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