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Scaffold-free vascular tissue engineering using bioprinting

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ABSTRACT

Current limitations of exogenous scaffolds or extracellular matrix based materials have underlined the need for alternative tissue-engineering solutions. Scaffolds may elicit adverse host responses and interfere with direct cell–cell interaction, as well as assembly and alignment of cell-produced ECM. Thus, fabrication techniques for production of scaffold-free engineered tissue constructs have recently emerged. Here we report on a fully biological self-assembly approach, which we implement through a rapid prototyping bioprinting method for scaffold-free small diameter vascular reconstruction. Various vascular cell types, including smooth muscle cells and fibroblasts, were aggregated into discrete units, either multicellular spheroids or cylinders of controllable diameter (300–500 μm). These were printed layer-by-layer concomitantly with agarose rods, used here as a molding template. The post-printing fusion of the discrete units resulted in single- and double-layered small diameter vascular tubes (OD ranging from 0.9 to 2.5 mm). A unique aspect of the method is the ability to engineer vessels of distinct shapes and hierarchical trees that combine tubes of distinct diameters. The technique is quick and easily scalable.

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1. Introduction

The general model of most tissue-engineering strategies rests on the use of exogenous biocompatible scaffolds in which cells can be seeded and matured *in vitro* or *in vivo*, to grow the tissue of interest. Scaffolds have been subject to prolific research and development over the last thirty years and, in general, offer the advantage of good biocompatibility, cell attachment and proliferation, while providing the biological, chemical, and mechanical clues to guide the eventual cell differentiation and assembly into a 3D tissue construct. Scaffold-based tissue engineering has led to significant results in the reconstruction of various tissues and organs and, in some cases, has been further translated to clinical practice [1–6].

Biomaterials-based solutions, though promising, still face general as well as specific challenges. Scaffold choice, immunogenicity, degradation rate, toxicity of degradation products, host inflammatory responses, fibrous tissue formation due to scaffold degradation, mechanical mismatch with the surrounding tissue are key issues, that may affect the long term behavior of the engineered tissue construct, and directly interfere with its primary biological function [7]. An example is myocardial tissue that presents high cell density necessary to assure synchronous beating through gap junctions that tightly interconnect neighboring cells. The use of scaffolds in cardiac tissue-engineering has been associated with reduced cell-to-cell connection, as well as incorrect deposition and alignment of extracellular matrix (ECM; i.e. collagen and elastin), affecting scaffold biodegradation and the force-generating ability of myocardial constructs [8,9]. ECM-related factors are particularly critical in vascular tissue engineering. Largely for this reason the promise of a scaffold-engineered small-diameter blood vessel substitute with mechanical strength comparable to native vessels for adult arterial revascularization, often described as the "holy grail" of tissue-engineering, remains unrealized. Besides the recurrent difficulty to produce elastic fibers *in vitro* [10], the use of scaffolds presents additional problems. The inherent weakness of the gels may hinder the final strength of the tissue-engineered vessel [11]. The presence of residual polymer fragments can disrupt the normal organization of the vascular wall [12,13] and even influence smooth muscle cell (SMC) phenotype [14]. Therefore it is not surprising that the first clinical applications of tissue-engineered vascular grafts have either targeted low-pressure applications [4] or relied on an entirely scaffold-free method termed sheet-based tissue-engineering [15–18] (currently under study also for myocardial reconstruction [19]).

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A variety of techniques have been developed to engineer tissues without any scaffold, composed only of cells and the matrix they secrete. Such techniques are being applied even in areas where scaffolds have had early success such as the engineering of skin, bone or cartilage [20–22]. Despite these developments, scaffold-free tissue-engineering has yet to provide a reliable method to produce custom-shaped tissues in a reproducible, high throughput and easily scalable fashion while keeping precise control of pattern formation, particularly in case of multiple cell types.

To address some of the present challenges, we have recently introduced a rapid prototyping technology based on three-dimensional, automated, computer-aided deposition of "bioink particles" (multicellular spheroids) into a "biopaper" (biocompatible gel; e.g. collagen) by a bioprinter [23,24]. Three dimensional tissue structures such as myocardial patches were formed through the post-printing fusion of the bioink particles similar to self-assembly phenomena in early morphogenesis [25]. Delivery of bioink particles with this technology was rapid, accurate and assured maximal cell density, while showing minimal cell damage that is often associated with other solid freeform fabrication-based deposition methods [26–30] focused mostly on printing cells in combination with hydrogels. The success of the reported technology depended strongly on the collagen biopaper [23,31]. Collagen gelation time was critical for the smooth deposition of the multicellular spheroids. Collagen concentration had to be finely tuned to assure the fusion of the spheroids during the postprinting phase [31]. Layer-by-layer construction lacked precision beyond a few layers due to progressive distortion of the construct, caused by the uneven gelation of successive collagen sheets [23]. Biopaper removal after postprinting fusion was technically challenging. As some of the supporting collagen was incorporated within the construct during the fusion of the spheroids, this method was indeed not entirely scaffold free.

In the present study, we describe and employ a fully biological scaffold-free tissue engineering technology and apply it to fabricate small-diameter multi-layered tubular vascular grafts that are readily perfusable for further maturation. We show that the approach circumvents a number of shortcomings associated with scaffolds and achieves the goal of being rapid, reproducible, and easily scalable by means of rapid prototyping.

2. Materials and methods

2.1. Cell culture

Chinese Hamster Ovary (CHO) cells transfected with *N*-cadherin were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS; Atlanta Biologicals, Lawrenceville, GA), antibiotics (100 U/mL penicillin streptomycin and 25 μg/mL gentamicin) and 400 μg/mL geneticin. Besides gentamicin (American Pharmaceutical Partners, IL) all antibiotics were purchased from Invitrogen. Human umbilical vein smooth muscle cells (HUVSMCs) and Human skin fibroblasts (HSFs) were purchased from the American Type Culture Collection (CRL-2481 and CRL-2522 respectively; ATCC, Manassas, VA). HUVSMCs were grown in DMEM with Ham's F12 (Invitrogen) in ratio 3:1, 10% FBS, antibiotics (100 U/mL penicillin–streptomycin and 25 μg/mL gentamicin), 20 μg/mL Endothelial Cell Growth Supplement (ECGS; Upstate, Lake Placid, NY), Sodium Pyruvate (NaPy; Invitrogen) 0.1 M. Human skin fibroblasts (HSFs) were grown in DMEM with Ham's F12 in ratio 3:1, 20% FBS, antibiotics (100 U/mL penicillin/streptomycin and 25 μg/mL gentamicin), glutamine 2 mM, NaPy 0.1 M. Freshly isolated porcine aortic smooth muscle cells (PASMCs) were grown in low glucose DMEM with 10% FBS (Hyclone Laboratories, UT), 10% porcine serum (Invitrogen), L-ascorbic acid, copper sulfate, HEPES, L-proline, L-alanine, L-glycine, and Penicillin G (all aforementioned supplements were purchased from Sigma, St. Louis, MO). All cell lines were cultured on 0.5% gelatin (porcine skin gelatin; Sigma) coated dishes (Techno Plastic Products, St. Louis, MO) and were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Preparation of multicellular spheroids and cylinders and agarose rods

Cell cultures were washed twice with phosphate buffered saline solution (PBS, Invitrogen) and treated for 10 min with 0.1% Trypsin (Invitrogen) and centrifuged at 1500 RPM for 5 min. Cells were resuspended in 4 mL of cell-type specific medium and incubated in 10-mL tissue culture flasks (Bellco Glass, Vineland, NJ) at 37 °C with 5% CO_2 on gyratory shaker (New Brunswick Scientific, Edison, NJ) for one hour, for adhesion recovery and centrifuged at 3500 RPM. The resulting pellets were transferred into capillary micropipettes of 300 μm (Sutter Instrument, CA) or 500 μm (Drummond Scientific Company, Broomall, PA) diameters and incubated at 37 °C with 5% CO_2 for 15 min. For spherical bioink, extruded cylinders were cut into equal fragments that were let to round up overnight on a gyratory shaker. Depending on the diameter of the micropipettes, this procedure provided regular spheroids of defined size and cell number (Fig. 1). For cylindrical bioink, cylinders were mechanically extruded into specifically prepared non-adhesive Teflon or agarose molds using the bioprinter (Fig. 2A and B). After overnight maturation in the mold, cellular cylinders were cohesive enough to be deposited.

To prepare agarose rods, liquid agarose (temperature >40 °C) was loaded into micropipettes (300 or 500 μm ID). Loaded micropipettes were immersed into cold PBS (4 °C). As agarose did not adhere to the micropipette, upon gelation, continuous rods could easily be extruded by the bioprinter using another printing head (Fig. 5C; cf. with Fig. 2A).

2.3. Imaging and visualization

The morphology of multicellular spheroids was analyzed by FESEM (Field Emission Scanning Electron Microscopy). Spheroids were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate buffered saline (PBS) for 90 min, on a low speed shaker. Subsequently, samples were rinsed 3 times for 10 min in PBS. Dehydration was performed by an increasing concentration series of ethanol as follows: 10%, 25%, 50%, 75%, 95%, for 30 min each and finally in 100% ethanol overnight. After critical point drying (in Tousumis Samdri-PVT-3B; Tousimis, Rockville, MD), aggregates were spread on carbon adhesive tabs mounted on stub and sputter coated with platinum to a nominal thickness of 2 nm. Aggregate surface was examined using a Hitachi S4700 cold-cathode field-emission scanning electron microscope at an accelerating voltage of 5 kV.

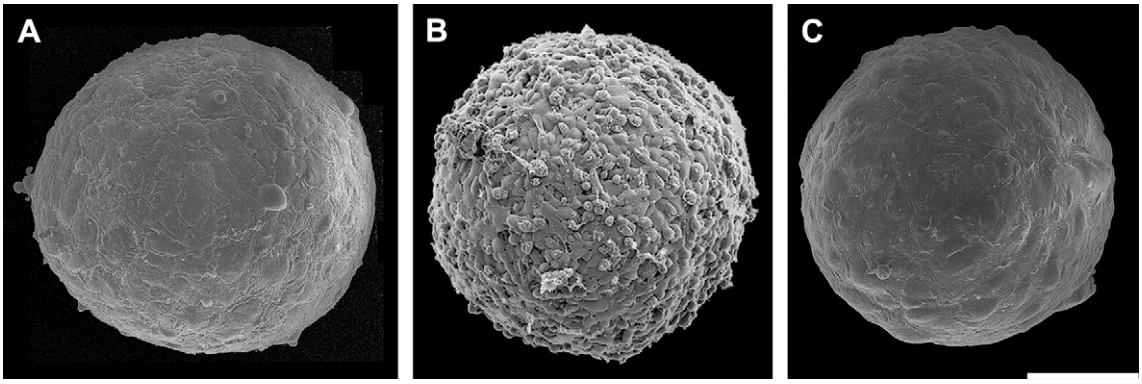

Fig. 1. Scanning electron micrographs of 300 μm diameter multicellular spheroids of HUSMCs (A), CHO cells (B) and HFBs (C) employed in the present study. HUSMC and HSF spheroids display similar morphology with smooth and uniform surface and contain respectively about 8000 and 15,000 cells. In contrast, CHO spheroids assume a berry-like shape suggesting that surface cells adhere more weakly to inner cell layers. Scale bar: 100 μm.