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Microfabricated blood vessels undergo neoangiogenesis

Kyle A. DiVito [a, 1], Michael A. Daniele [b, c, *, 1], Steven A. Roberts [a], Frances S. Ligler [c], André A. Adams [a, *]

[a] Center for Bio/Molecular Science & Engineering, U.S. Naval Research Laboratory, 4555 Overlook Ave. SW, Washington D.C., 20375, United States
[b] Department of Electrical & Computer Engineering, North Carolina State University, 890 Oval Dr., Raleigh, NC 27695, United States
[c] Joint Department of Biomedical Engineering, North Carolina State University and University of North Carolina-Chapel Hill, 911 Oval Dr., Raleigh, NC 27695, United States

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ABSTRACT

The greatest ambition and promise of tissue engineering is to manufacture human organs. Before "made-to-measure" tissues can become a reality [1–3], however, three-dimensional tissues must be reconstructed and characterized. The current inability to manufacture operational vasculature has limited the growth of engineered tissues. Here, free-standing, small diameter blood vessels with organized cell layers that recapitulate normal biological functionality are fabricated using microfluidic technology. Over time in culture, the endothelial cells form a monolayer on the luminal wall and remodel the scaffold with human extracellular matrix proteins. After integration into three-dimensional gels containing fibroblasts, the microvessels sprout and generate extended hollow branches that anastomose with neighboring capillaries to form a network. Both the microfabricated vessels and the extended sprouts support perfusion of fluids and particles. The ability to create cellularized microvessels that can be designed with a diameter of choice, produced by the meter, and undergo angiogenesis and anastomoses will be an extremely valuable tool for vascularization of engineered tissues. To summarize, ultraviolet (UV) photocrosslinkable poly(ethylene glycol) and gelatin methacrylate polymers used in combination with sheath-flow microfluidics allow for the fabrication of small diameter blood vessels which undergo neoangiogenesis as well as other developmental processes associated with normal human blood vessel maturation. Once mature, these vessels can be embedded; perfused; cryogenically stored and respond to stimuli such as chemokines and shear stresses to mimic native human blood vessels. The applications range from tissue-on-chip systems for drug screening, characterization of normal and pathologic processes, and creation and characterization of engineered tissues for organ repair.

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1. Introduction

Ideally, engineered human blood vessels should possess tunable dimensions and be free standing so that they can be easily handled and integrated into three-dimensional tissues, as well as be connected to perfusion sources. The constructs should also be amenable to storage for later use and self-sustaining in terms of repair and angiogenesis [1–9].

Earlier attempts to produce vasculature for tissue models have used de-cellularized cadaver materials as scaffolds [10], bio-printing of large blood vessels [11], random outgrowth from endothelial cell spheroid cultures or patterned microchannels [12,13]. These approaches have been partially successful, yet fail to provide both the range of microvessel diameters found in normal vasculature and the desired control over microvessel placement within a regenerating tissue [14–18]. A key challenge addressed here is the creation of human vasculature that has not only controllable size and cellular architecture but which can produce new angiogenic growth for long term tissue sustainability, a feature that many other technologies, including 3D printing, have yet to fully realize.

Human vasculature forms through a series of developmental processes. Endothelial sprouting from a main vessel (angiogenesis) launches a network of smaller tubular branches each capable of further sprouting to support blood flow (tubulogenesis). Critical junctions (anastomoses) between arterial and venule capillary beds

* Corresponding authors.
E-mail addresses: mdaniel6@ncsu.edu (M.A. Daniele), andre.adams@nrl.navy.mil (A.A. Adams).
[1] These authors contributed equally to this work.

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establish a network of arteries/veins, arterioles/venules and capillaries [19]. These three combined processes are essential for tissue maintenance, yet have not been fully replicated in synthetic systems. To date, these processes have largely been accomplished using endothelial spheroid cultures embedded in matrices or pre-patterned microchannels and have not yet been observed from free-standing porous synthetic blood vessels.

The objective of this study is to develop an efficient method of constructing human blood vessels that are both representative and complimentary to microscale human blood vessels. Preferably, these fabricated vessels would then support the formation of a primitive network of blood vessels via the natural processes of angiogenesis, tubulogenesis and anastomosis (Fig. 1), and we sought to characterize the developmental events that occur using our technology. The experimental design begins by creating HEMVs with a desired diameter via hydrodynamic focusing. When seeded into a three-dimensional matrix, the human endothelial microvessels (HEMV) initiate neovascularization which proceeds from angiogenesis through anastomosis. As HEMV technology creates hollow tubular structures with cellular components in place, the ability to immediately perfuse tissue eliminates significant delays associated with tubular development from endothelial networks derived from spheroid cultures. Furthermore, as the HEMV is constructed using a micro-porous [20], biodegradable material, removal of the scaffold is not required. These advantages, together with the possibility for long term storage of HEMV, make the HEMV technology highly useful for *in vitro* tissue engineering and tissue-on-chip analytical systems.

2. Materials and methods

2.1. Fabrication of microvessels

Microvessels were produced using the microfluidic fabrication method previously described [20–22] with minor modifications. Briefly, poly(ethylene glycol) (PEG) tetrathiol and poly (ethylene glycol) acrylate were purchased from JenKem USA (Allen, TX) and resuspended at a 1:1 M ratio in a 1X phosphate buffered saline solution containing 0.4% Irgacure2959 Sigma Aldrich (St. Louis, MO). Gelatin-methacrylamide (GelMA) has been widely identified as a successful substrate for cell attachment in a multitude of bioengineering applications [23,24]. Synthesis of GelMA, using porcine-derived gelatin Sigma Aldrich (St. Louis, MO) was performed as previously described [20] and lyophilized gelatin-methacrylamide was included in the previously mixed PEG polymer also at a 1:1 ratio to provide support for cellular attachment. The final concentration of the mixed polymers is 6% w/w (3% poly(ethylene glycol) and 3% gelatin-methacrylamide). Purified protein components include a mixture of fibronectin (50 μg/mL) from Corning (Bedford, MA), rat-tail collagen-1 (75 μg/mL) and hyaluronic acid (18 μg/mL), both from Sigma Aldrich (St Louis, MO), were added to supplement the poly(ethylene glycol)/gelatin-methacrylamide mixture and further support cellular attachment. The outer sheath fluid is 6% PEG, resuspended in phosphate buffered saline. Cells comprising the lumen are resuspended in a 1% non-photo-crosslinkable gelatin solution; while cells placed in the polymer wall are directly added to the polymer solution. Flow rates for were as follows. Outer sheath 90 μl/min; polymer comprising the microvessel wall 29 μl/min and inner lumen flow is 15 μl/min.

2.2. Microvessel cryopreservation

Day 8 HEMVs were examined for the ability to recover from cryopreservation in three replicate experiments. Briefly, HEMV were removed from the incubator and immediately transferred to cryovials containing endothelial freezing medium (80% EGM-2, 10% fetal bovine serum, 10% dimethyl sulfoxide). Cryovials were then placed into a freezing chamber and transferred to −80 °C overnight.

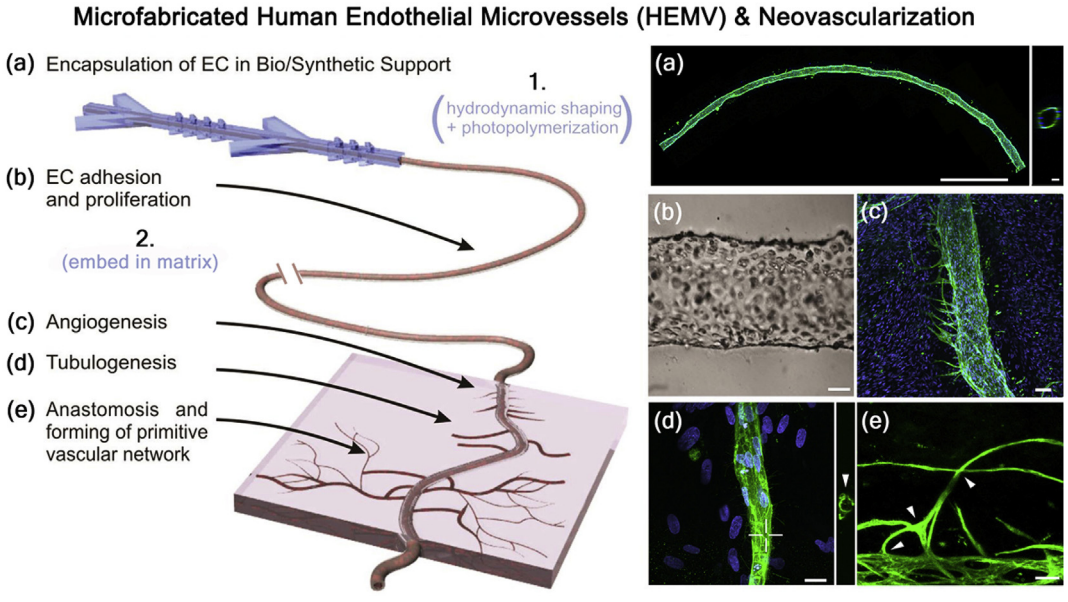

Fig. 1. Neovascularization strategy and implementation. 1. Human endothelial cells (EC) are encapsulated in a bio-macromolecular tubule using a hydrodynamic shaping device. The resultant human endothelial microvessel (HEMV) matures with a coherent endothelial cell lumen. Temporal events a-e (left) correspond to experimental micrographs (right). (a) Representative composite image of ten adjacent fields (10X) taken along the length of a single HEMV immunostained with anti-CD31 (green) demonstrates the ability to produce long continuous HEMVs. Scale, 500 μm; inset 50 μm (b) A confluent monolayer of endothelial cells form along the luminal face of the microvessel. 2. Once embedded in a three-dimensional matrix, the HEMV develop a primitive microvasculature network through traditional vascularization processes, (c) HEMV angiogenesis, the sprouting of endothelial growths from the original HEMV into an extracellular matrix containing dermal fibroblasts (DAPI-stained nuclei, blue), (d) HEMV tubulogenesis (arrowhead), the hollowing of sprouts to support fluid transport, (e) HEMV anastomosis, a developmental process whereby neighboring sprouts form connections (arrowheads) establishing a closed-loop system for circulation. Scale, (b + d) 25 μm; (c + e) 50 μm.