Biomaterials 125 (2017) 13–22

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Crossing kingdoms: Using decellularized plants as perfusable tissue engineering scaffolds

Joshua R. Gershlak [a], Sarah Hernandez [b], Gianluca Fontana [c], Luke R. Perreault [a], Katrina J. Hansen [a], Sara A. Larson [b], Bernard Y.K. Binder [d], David M. Dolivo [b], Tianhong Yang [e, f], Tanja Dominko [b, g], Marsha W. Rolle [a], Pamela J. Weathers [b], Fabricio Medina-Bolivar [e, f], Carole L. Cramer [e, f], William L. Murphy [c, h, i], Glenn R. Gaudette [a, *]

[a] Biomedical Engineering, Worcester Polytechnic Institute, Worcester, MA, United States
[b] Biology and Biotechnology, Worcester Polytechnic Institute, Worcester, MA, United States
[c] Orthopedics and Rehabilitation, University of Wisconsin School of Medicine and Public Health, Madison, WI, United States
[d] Department of Surgery, University of Wisconsin School of Medicine and Public Health, Madison, WI, United States
[e] Department of Biological Sciences, Arkansas State University, Jonesboro, AR, United States
[f] Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR, United States
[g] Center for Biomedical Sciences and Engineering, University of Nova Gorica, Slovenia
[h] Biomedical Engineering, University of Wisconsin-Madison, Madison, WI, United States
[i] Material Sciences and Engineering, University of Wisconsin-Madison, Madison, WI, United States

ARTICLE INFO

Article history:
Received 4 October 2016
Received in revised form
8 February 2017
Accepted 9 February 2017
Available online 10 February 2017

Keywords:
Regenerative medicine
Tissue engineering
Decellularization
Perfusable scaffold
Plants

ABSTRACT

Despite significant advances in the fabrication of bioengineered scaffolds for tissue engineering, delivery of nutrients in complex engineered human tissues remains a challenge. By taking advantage of the similarities in the vascular structure of plant and animal tissues, we developed decellularized plant tissue as a prevascularized scaffold for tissue engineering applications. Perfusion-based decellularization was modified for different plant species, providing different geometries of scaffolding. After decellularization, plant scaffolds remained patent and able to transport microparticles. Plant scaffolds were recellularized with human endothelial cells that colonized the inner surfaces of plant vasculature. Human mesenchymal stem cells and human pluripotent stem cell derived cardiomyocytes adhered to the outer surfaces of plant scaffolds. Cardiomyocytes demonstrated contractile function and calcium handling capabilities over the course of 21 days. These data demonstrate the potential of decellularized plants as scaffolds for tissue engineering, which could ultimately provide a cost-efficient, "green" technology for regenerating large volume vascularized tissue mass.

1. Introduction

The need for organs and tissues available for transplantation far exceeds their availability. More than 100,000 patients can be found on the donor waiting list at any given time and an average of 22 people die each day while waiting for a donor organ or tissue to become available [1]. Tissue engineering has made significant strides over the past decade through the development of tissue grafts, increasing the potential number of viable solutions for these patients. However, there are still issues impeding their translation into the clinic. One of the major factors currently limiting clinical applicability of tissue engineered solutions is the lack of a functional vascular network [2]. Without a viable vascular network, the 100–200 μm oxygen diffusion limit within tissues cannot be overcome, consequently restricting the size of graft that can be engineered and retain viability. Most current bioengineering techniques are unable to create patent perfusion vessels. Techniques such as the loading of scaffolds with pro-angiogenic factors [3], cellular-guided vascular network formation [4], and microfabricated designs [5] have demonstrated limited success in fully recapitulating native vasculature. Furthermore, microvasculature

* Corresponding author.
E-mail address: gaudette@wpi.edu (G.R. Gaudette).

http://dx.doi.org/10.1016/j.biomaterials.2017.02.011

(<10 μm in diameter) cannot be functionally fabricated with current biofabrication techniques, such as 3-D printing.

Instead of attempting to engineer a vascular network, current focus has shifted towards bio-inspired approaches, further driven by the advent of perfusion-based techniques for decellularization [6]. Decellularization removes cellular material from a tissue or organ leaving behind an acellular scaffold consisting of extracellular matrix (ECM), the composition of which depends on the tissue or organ from which it was derived [7], while preserving an intact vascular network [8]. By removing the cellular material of a donor's tissue, a decellularized graft would be rendered non-immunogenic while retaining gross organ structure [9]. Decellularized tissues and organs can then be recellularized with a patient's own cells to create an autologous graft [8]. Native biochemical composition and hierarchical tissue structure of a potential decellularized graft are derived from the donor of the tissue or organ. This inherently leads to inconsistency among tissues or organs derived from different patients, or decellularized using different methods, due to confounding variables such as age, organismal or tissue pathology, and the specifics of the decellularization protocol [7,10,11]. In particular, protein compositional analysis through mass spectrometry has shown drastic differences in the composition of decellularized tissues between different patients [12,13]. Decellularized mammalian tissues are also in short supply and, even when available, are expensive. Furthermore, a considerable amount of additional research needs to be conducted before entire decellularized organs can be considered as a practical option clinically [14]. Consequently, a more consistent, cost-effective and readily available tissue source for decellularization would yield improved prospects by increasing viable numbers of grafts at a significantly lower cost.

Most current bioengineering approaches are limited by the physical and intellectual isolation of basic research in different organisms to their respective biological kingdoms. This critical challenge can be overcome by exploiting *cross-kingdom* contributions within the same bioengineered platform. Plants and animals exploit fundamentally different approaches to transporting fluids, chemicals, and macromolecules, yet there are surprising similarities in their vascular network structures (Fig. 1). Plant vasculature follows Murray's Law [15], which is the physiological law describing the tapered, branching network design of the human cardiovascular system [16]. Structures within plant tissue [17], like human tissue [18], exhibit varied mechanical properties, enabling varied functions. Plant cell walls are composed of a variety of polysaccharides, the most prominent of which are cellulose, pectin, and hemicellulose [19]. Cellulose, which is the most abundant component of plant cell walls, is a well-studied biomaterial for a variety of clinical applications [20]. Cellulose is biocompatible and has been shown to promote wound healing [21]. Furthermore, cellulosic tissue engineering scaffolds derived from decellularized apple slices have shown the ability for mammalian cell attachment and proliferation [22] and were found to be biocompatible when implanted subcutaneously *in vivo* [23]. Pectin [24] and hemicellulose [25] have also been studied as biomaterials for bone tissue engineering and wound healing, respectively. The innate similarities and apparent biocompatibility of plant ECM spurred us to look *across kingdoms* and investigate whether plants and their innate vasculatures could serve as perfusable scaffolds for engineering human tissue. Decellularization techniques were applied to different plant species and tissues in order to generate acellular, pre-vascularized tissue engineering scaffolds. The abundance and rapid growth of many plant species also provides for a less costly, more plentiful and sustainable scaffold material.

2. Materials and methods

Plant Decellularization: Spinach and parsley were acquired from a local market. *Artemisia annua* leaves (SAM cultivar, specimen voucher MASS 00317314) were harvested from soil-grown plants. Peanut hairy roots were generated through *Agrobacterium rhizogenes*-mediated genetic transformation [26,27]. Decellularization for the different plant types was adapted from whole organ perfusion decellularization techniques [6–8]. Spinach leaves were cannulated through the petiole and parsley stems were cannulated via the basipetal end of the stem segment. Cuticles were removed from the plants via serial treatment with hexanes (98%, Mixed Isomers, Alfa Aesar, Haverhill, MA) and 1x PBS. A 10% sodium dodecyl sulfate (SDS) in deionized water solution was perfused through the cannulas for 5 days, after which they were perfused with a 0.1% Triton-X-100 in a 10% sodium chlorite bleach (Aqua-Tab, Beckart Environmental, Kenosha, WI) in deionized water solution for 48 h. Sterile deionized water was then perfused for an additional 48 h. Perfusion was accomplished from a constant pressure head of 152 mmHg and flow was initiated by gravity. *A. annua* and peanut hairy roots were decellularized using the same technique but instead of cannulation and perfusion, they were soaked in the solutions. After decellularization was complete, tissues were stored in sterile deionized water at 4 °C until needed for up to two weeks.

Histological Analysis: Leaf samples were cut into ~1 cm squares, preferentially cut with the main vascular channel of the leaf down the middle of the square. Roots and stems were cut into approximately ~1 cm length pieces. Tissue samples were fixed overnight in an ATP-1 automatic tissue processor (Triangle Biomedical Sciences, North Carolina) and then embedded in paraffin.

Paraffin blocks were sectioned at 14 μm. Samples that were stained using Sass's Safranin and Fast Green protocol for plant staining were done as previously reported [28]. In short, sections were stained for 1 h in aqueous 1% (w/v) Safranin-O, and then rinsed in deionized water for approximately 5 min, or until all residual dye was removed from the sections. Sections were dehydrated in 70%, then 95% ethanol and dipped for 10 s in Fast Green FCF (0.1% w/v in 95% ethanol). Sections were washed in two changes of 100% ethanol (2 min/step) and cleared in two changes of xylene (2 min/step). Samples were stained for lignin for 10 min in a saturated solution of phloroglucionol in 20% hydrochloride, as previously reported [29]. Samples were visualized through use of a DMLB2 upright microscope (Leica Microsystems, Buffalo Grove, IL).

Scanning Electron Microscope Imaging: The preparation of samples for Scanning Electron Microscopy (SEM) analysis consisted of fixation with 1.5% glutaraldehyde in freshly prepared 0.07 M sodium cacodylate buffer for 2 h. The samples were then rinsed in 0.07 M sodium cacodylate with the addition of 2.5% sucrose and dehydrated by immersion in a graduated series of ethanol in H_2O, serially in the following concentrations: 30, 50, 80, and 95%. Then samples were immersed in hexamethyldisilazane (HDMS) in ethanol serial solutions in the following concentrations: 30, 50, 80, and 95%. The samples were left to dry on the sample holder and then gold sputter coated prior to imaging in SEM.

DNA/Protein Quantification: Both native and decellularized leaves were put in centrifuge tubes in a liquid nitrogen bath and ground with a pestle. Fragments were further processed by pulling through a 25-gauge syringe needle and by sonication with 5 pulses performed 3 times to reduce leaf fragment size. DNA was then measured using a CyQUANT Direct Cell Proliferation Assay (Thermo Fisher, Waltham, MA) and protein was measured using a Coomassie (Bradford) Protein Assay Kit (Thermo Fisher). Concentrations were determined using a Victor3 spectrophotometer (Perkin Elmer, Waltham, MA).

Perfusion Studies: Decellularized spinach leaves were removed