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A Novel 3-Dimensional Approach for Cardiac Regeneration

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Abstract

Ischemic heart diseases, such as coronary artery disease and microvascular disease, are cardiovascular pathologies that cause reduced blood supply to the heart muscle. Acute and chronic ischemia cause cardiomyocytes to die, and these cells are not naturally replaced as part of the wound healing process in the heart. To promote neovascularization in the wound bed and in implanted engineered tissues, we have developed a collagen–alginate microspheres scaffold intended for local release of drugs and growth factors in order to recruit host endothelial cells to the area and provide them with geometrical cues to form new vessels. Optimization of alginate microspheres included modulation of nitrogen pressure, alginate and CaCl_2 concentrations, nozzle size, and velocity of extrusion to achieve monodisperse populations of 100 μm diameter microspheres with protein release over 3 days. *In vitro* incorporation of fibroblasts in the bulk collagen demonstrated cellular compatibility with embedded alginate microspheres. An *in vitro* vessel formation assay, performed with human umbilical vein endothelial cells (HUVECs) immobilized in the collagen phase of the collagen–alginate microspheres scaffolds, showed that HUVECs formed networks following the 3-dimensional pattern of the microspheres even in the absence of growth factor. Implantation of acellular collagen–alginate microspheres scaffolds onto healthy rat hearts confirmed the invasion of host cells at one week. Together, these results suggest that the collagen–alginate microspheres scaffold is a viable, tunable therapeutic approach for directing neovascularization in engineered tissues and in the heart after ischemic events.

I. Introduction

Recent advances in pre-clinical cardiac repair after ischemic events have used scaffolds, glues, tissue constructs and patches implanted or injected in the left ventricle [1–3]. However, the poor vascularization of such systems leads to their time-limited efficacy and poses limits in the dimensions of cell-based cardiac grafts. Tissue constructs thicker than 400 μm are known to be scarcely effective because of the lack of nutrients reaching the cells immobilized in the core of the constructs [4].

We hypothesize that enabling the invasion of host endothelial cells into the implants will improve cardiac regeneration by promoting neovascularization, reducing cardiomyocyte death due to chronic ischemia, and improving the efficacy of implanted therapeutics. Toward this aim, we designed an acellular scaffold that couples the regenerative properties of collagen [5,6] with the advantages provided by alginate microspheres dispersed in the polymeric gel. The microspheres are local reservoirs for the release of growth factors (such as vascular endothelial growth factor, VEGF) for the recruitment of host endothelial cells, and also provide geometric cues for new vessel formation in the scaffold.

Alginate and collagen represent two versatile biomaterials that have been extensively used for the production of the implantable scaffolds due to their biocompatibility and biodegradability [7–10]. In particular, alginate is a negatively charged polysaccharide that can rapidly form microspheres in the presence of calcium ions at mild temperature and pH, being particularly suitable as vehicle for the immobilization of cells and protein/drug therapeutics [7–10]. The addition of positively charged molecules, such as VEGF, to alginate before the production of the microspheres results in the formation of ionic interactions between the molecules and the biomaterial, thus leading to improved loading and retention of the therapeutics in the microspheres. Finally, the immobilization of alginate microspheres in 3-dimensional collagen scaffolds allows for a spatially controlled release of therapeutics to ultimately recruit host endothelial cells to the site of implant.

Here we report *in vitro* optimization and characterization of the collagen–alginate microspheres scaffold and preliminary *in vivo* studies where acellular scaffolds were implanted onto the epicardial surface of healthy rat hearts.

II. Materials and Methods

A. Production of alginate microspheres

Alginate microspheres were produced by the extrusion method, spraying alginate solution (Sigma, 71238) in a calcium chloride (Fisher Sci, S25223) gelling bath with the VarJ30 Bead Generator (Nisco, Switzerland). The size and shape of the microspheres were optimized by varying alginate concentration (0.25% w/v – 2% w/v in phosphate buffered saline (PBS)), nitrogen pressure (80 mbar – 200 mbar), nozzle size (0.1 mm or 0.35 mm), velocity of extrusion (6 ml/h – 18 ml/h) and distance to gelling bath. After preparation, the microspheres were washed twice in PBS before being used for experiments.

B. Release of a model protein from alginate microspheres

α -Chymotrypsin (Sigma, C4129) was used as a model protein to study the release kinetics from alginate microspheres. 1 mg/mL chymotrypsin was mixed with 1% or 2% (w/v) alginate solutions before extrusion. Microspheres were produced by extruding alginate-chymotrypsin solutions in 0.15, 0.5 and 1 M CaCl_2 , using nitrogen pressure of 120 mbar and 180 mbar (for 1% and 2% alginate formulations, respectively), velocity of extrusion of 18 ml/h, and nozzle size of 0.35 mm. After preparation, microspheres were washed twice with PBS and then incubated in 1 mL PBS up to 7 days. The release kinetics of α -chymotrypsin were measured with bicinchoninic acid (BCA) assay (Thermo Sci, PI-23227), a colorimetric assay which measures the concentration of protein in the incubation solutions, according to manufacturer instructions. Briefly, at each time stage (1h, 8h, 1, 2, 3 and 7 days), 500 μL of incubation solution was removed and replaced with 500 μL of fresh PBS. The removed sample was frozen (-20°C) until analyzed with the BCA assay. 25 μL of the sample solutions were incubated with 200 μL of BCA Working Reagent for 30 min at 37°C , according to the manufacturer protocol. Sample absorbance was read at 562 nm with a microplate reader (SpectraMax M5) and protein content compared to a standard curve (bovine serum albumin). Unloaded microspheres were produced with the same parameters and used as control.

C. Production of acellular and cellular collagen-alginate microspheres scaffolds

Alginate microspheres were incorporated into collagen scaffolds by mixing on ice 1.25 mg/mL collagen (Advanced BioMatrix, Type I from rat tails, 5056-A) with alginate microspheres at varying densities (10–250 mg/mL). The constructs were then poured in custom-made rectangular PDMS molds (0.5 cm × 2 cm) cast from laser-etched acrylic and placed in a well of a 6-well plate, then left at room temperature for 45 min to gel. For the preparation of mouse embryonic fibroblast (MEF) tissue constructs, MEFs (8×10^6 cells/mL) were mixed on ice with 2.5 mg/mL collagen before adding alginate microspheres, and then the constructs were allowed to gel at room temperature. After 45 min, MEF medium was added to the well and was replaced every other day.

D. In vitro assay for vascular network formation in collagen-alginate microspheres scaffolds

For the evaluation of vascular network formation in the collagen-alginate microspheres scaffold, human umbilical vein endothelial cells (HUVECs, Lonza, C2519A) were treated with Green BODYPI Cell Tracker (Life Technology, C2102) and mixed with collagen and alginate microspheres (cell density of 1×10^6 cells/mL and microspheres density of 50 and 100 mg/mL) on ice. Constructs were poured into the wells of a 96-well plate for culture and imaging. EGM-2 medium (Lonza, CC-3162) was added after 45 minutes to the gelled constructs and changed every other day. Network length and area were analyzed for up to 36 h from fluorescent images taken in triplicate from 8 wells per condition.

E. Acellular scaffold implantation on healthy rat hearts

All animal procedures were conducted in accordance with the US NIH Policy on Humane Care and Use of Laboratory Animals and the Brown University Institutional Animal Care and Use Committee (IACUC protocol #1310000025).

Acellular scaffolds with alginate microspheres (40 mg/mL, 100 μ m diameter) in 1.25 mg/mL collagen were implanted on the anterior free wall of the left ventricle of 8-week old male Sprague-Dawley rats (Charles River Laboratory). Briefly, rats were anesthetized with 2–5% inhaled isoflurane supplemented with oxygen, intubated and ventilated. Body temperature was maintained at 37°C by placing the animal on an electric thermal pad with a rectal probe providing temperature feedback (Harvard Apparatus). After exposure of the heart via thoracotomy, constructs were sutured onto the epicardial surface of the left ventricle from apex to base with 8–0 suture at either end of the construct. The chest was closed aseptically and animals were allowed to recover. Rats received analgesic (buprenorphine 0.01–0.05 mg/kg) twice daily for up to 2 days post-surgery. After 7 days, animals were sacrificed by pentobarbital overdose (180 mg/kg) and hearts were harvested and embedded in frozen blocks in OCT medium. Cross-sectional sections of the hearts (20 μ m thick) were stained with picosirius red with fast green for histological evaluation.

III. Results and Discussion

A. Production of alginate microspheres

Alginate microspheres were produced using the Var J30 Bead Generator, which utilizes coaxial nitrogen flow with aerodynamically assisted jetting. This method reliably produced microspheres with diameters ranging from 50 to 450 μm (Fig. 1). Microsphere diameter and size distribution can be tightly controlled by varying nitrogen pressure, alginate and CaCl_2 concentrations, nozzle size, velocity of extrusion, and distance between the nozzle and the gelling bath. In this study, we aimed at obtaining microspheres with mean diameters of 100 μm to fit into the scale of existing engineered tissue scaffolds and provide physical, geometric cues for the invasion of the host endothelial cells into the collagen–alginate microspheres scaffolds.

Using constant nitrogen pressure (120 mbar), varying alginate concentration (1% or less) resulted in microspheres with diameters in the range of 40 to 80 μm with narrow size distributions (Fig. 1a). Higher alginate concentrations (2% w/v) resulted in multiple peaks with diameters 100 μm (Fig. 1). Because higher alginate concentrations may be required for optimizing drug loading and delivery, 2% w/v alginate was used with increasing nitrogen pressure up to 180 mbar, which produced a mono-dispersed population with a 100 μm diameter (Fig. 1b).

B. Release of a model protein from alginate microspheres

α -Chymotrypsin was chosen as a model protein to study the release kinetics from alginate microspheres because its isoelectric point and molecular weight are similar to a number of growth factors that could be used as therapeutics released locally from the scaffold, including VEGF, bFGF and SHH. Specifically, VEGF, a growth factor inducing angiogenesis and neovascularization [11], has $\text{pI}_{\text{VEGF}}=8.5$ and $\text{MW}_{\text{VEGF}}=21\text{--}24$ kDa while α -chymotrypsin has $\text{pI}_{\alpha\text{-Chym}}=8.75$ and $\text{MW}_{\alpha\text{-Chym}}=25$ kDa. A burst release was observed during the first 24h for microspheres produced with 1% and 2% w/v alginate (Fig. 2). The burst release was followed by a slow but sustained release of the model protein (3%–5%) in the next 48h for the 1% w/v alginate formulation (Fig. 2). Notably, the cumulative protein release was dependent upon CaCl_2 concentration of the gelling bath, with 1 M CaCl_2 resulting in the highest release. Further, higher alginate concentration (2% w/v) enabled higher cumulative release, up to ~95% of loaded protein. We hypothesize that faster gelling of the microspheres resulted in the formation of a thinner external barrier to enhance total protein release and an increased charge density provided by Ca^{2+} ions produced an electrostatic repulsion of the positively charged protein (at neutral pH).

Choice of formulation parameters for optimization of our cardiac scaffold rests upon a few key concepts related to our purpose of inducing vascularization into the scaffold. The invasion of the host endothelial cells into the collagen–alginate microspheres scaffold should be both a localized and rapid process, in order to maintain viability of native or implanted cells within the region. Therefore, a burst release is acceptable, particularly since it will be localized, but a somewhat sustained release is also desirable. Too long of a release window raises concerns about the viability of the released protein itself. Therefore, we consider the

1% w/v alginate microspheres as the optimal formulation to carry on the optimization of the scaffolds, particularly since we have loaded 1 mg/mL protein and require 5–20% release for inducing activity of endothelial cells with angiogenic growth factors. Ongoing studies aim to alter the kinetics and amplitude of protein release from the alginate microspheres in order to minimize the burst release and maximize the slow release up to day 5 by forming chitosan-alginate layers on the external surface of microspheres or by mixing alginate with other synthetic polymers, such as PLA and PGA.

C. Production of cellular collagen–alginate microspheres scaffolds

Fibroblasts immobilized in engineered collagen tissues are known to grow, spread and remodel the polymeric scaffold in few hours [12]. The goal of our experiment, performed with mouse embryonic fibroblasts (MEFs) immobilized in the bulk collagen of our collagen-alginate microspheres scaffolds, was to demonstrate that the presence of the microspheres would not interfere with either the formation of a dense cellular engineered tissue or the growth and remodeling capabilities of the immobilized cells. Alginate microspheres (1% w/v) with an average diameter of 100 μm were dispersed in the collagen scaffolds at low and high densities (Fig. 3) and cellular remodeling of the scaffolds was monitored by bright field imaging up to 48h.

Alginate microspheres were clearly visible in the scaffolds, creating 3-dimensional patterns with microsphere density dependent upon loading concentration (Fig. 3b,c). Physical organization was not controlled. MEFs grew around the microspheres and contraction of the scaffolds with dispersed alginate microspheres (measured as construct diameter) was not different from that of the collagen-only control. Therefore, our collagen–alginate microspheres scaffold is compatible with cells immobilized in the bulk collagen.

D. Vessel network formation in collagen–alginate microspheres scaffolds

To understand how alginate microspheres provide geometric cues to physically direct vessel formation, we immobilized human umbilical vein endothelial cells (HUVECs) in collagen-alginate microspheres scaffolds *in vitro* with varying densities of unloaded microspheres. HUVECs are able to form perfusable networks in the space between densely packed alginate microspheres in microfluidic systems [13], but we wanted to assess how network morphologies were altered in our scaffold where microspheres are more sparse. We observed a similar behavior on the macroscopic scale: after 2 days of culture, HUVECs formed networks around the microspheres, appearing to use the microsphere surfaces to form 3 dimensional networks (Fig. 4). Improved network length and area were observed in the scaffolds where alginate microspheres (100 μm diameter) were dispersed at 50 and 100 mg/mL versus control (Fig. 4e).

E. Acellular scaffold implantation on healthy rat hearts

We successfully implanted acellular collagen–alginate microspheres scaffolds (100 μm diameter, 40 mg/mL) on healthy rat hearts. At 7 days, the scaffold is clearly visible on the epicardium (arrows, Fig. 5a), adhering to host tissue without formation of a fibrous capsule. Cells have infiltrated the scaffold as shown by the presence of fast green-positive regions within the scaffold. Higher magnification shows void regions where microspheres were

likely located (Fig. 5b), however dissolution of alginate during histological processing limits analysis, and development of methods to assess microspheres in implants is ongoing.

Identification of invading cell types is required to fully assess the therapeutic benefits of our collagen–alginate microspheres scaffold for regional neo-vascularization, and we anticipate identifying vascular and inflammatory cells in ongoing analysis.

IV. Conclusions

In the present work, we have developed and optimized the properties of a collagen–alginate microspheres scaffold intended for cardiac regeneration. The results obtained with *in vitro* immobilization of mouse embryonic fibroblasts and human umbilical vein endothelial cells suggest that this scaffold can sustain cell growth in engineered tissue and enable angiogenesis and neovascularization upon implantation. Ongoing studies are evaluating the ability of growth factors (such as VEGF) released from the alginate microspheres to induce the invasion of host endothelial cells into the scaffold in a myocardial infarct model to create a perfused vascular bed and promote contractile function of a damaged heart.

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Figure 1.

Microspheres were produced by varying alginate concentration at 120 mbar pressure (a,b) or by altering nitrogen pressure using 2% w/v alginate (c,d), while keeping constant the nozzle size (0.35 mm) and velocity of extrusion (18 mL/h). Images show a polydisperse population (b) obtained with 2% alginate extruded at 120 mbar (scale bar = 200 μm) and a monodisperse population (d) obtained with alginate 2% extruded with P=180 mbar (scale bar = 100 μm).

Figure 2.

Release kinetics of α -chymotrypsin from 2% (continue lines) and 1% (dotted lines) alginate microspheres show an initial burst and variable cumulative release dependent upon CaCl_2 concentration of the gelling bath.

Figure 3.

MEFs (8×10^6 cells/ml) immobilized in collagen scaffolds with no microspheres (a), 120 mg/mL microspheres (b), and 250 mg/mL microspheres (c) formed uniform tissues. Images acquired after 24h of culture. The arrows show the spatial distribution of the microspheres. Scale bars = 100 μ m.

Figure 4.

HUVECs formed networks in collagen scaffolds with (a,b) no microspheres and (c,d) 100 mg/mL unloaded alginate microspheres. Brightfield (a,c) and fluorescence (b,d) images acquired after 48 h of culture. Scale bars = 200 µm. Network length and area were measured up to 36h (e).

Figure 5.

The implanted acellular collagen-alginate microspheres scaffold was localized to the epicardium and infiltrated by host cells. An apical cross-section (a) identifies the scaffold (red arrows) and higher magnification (b) shows round spaces created by the microspheres (yellow arrows). Picrosirius red stain identifies collagen (primarily of the implanted scaffold) and fast green shows cellular cytoplasm. Scale bar = 2 mm (a).