Tissue Strands as "Bioink" for Scale-up Organ Printing

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Abstract— **Organ printing, takes tissue spheroids as building blocks together with additive manufacturing technique to engineer tissue or organ replacement parts. Although a wide array of cell aggregation techniques has been investigated, and gained noticeable success, the application of tissue spheroids for scale-up tissue fabrication is still worth investigation. In this paper, we introduce a new micro-fabrication technique to create tissue strands at the scale of 500-700μm as a "bioink" for future robotic tissue printing. Printable alginate micro-conduits are used as semi-permeable capsules for tissue strand fabrication. Mouse insulinoma beta TC3 cell tissue strands were formed upon 4 days post fabrication with reasonable mechanical strength, high cell viability close to 90%, and tissue specific markers expression. Fusion was readily observed between strands when placing them together as early as 24h. Also, tissue strands were deposited with human umbilical vein smooth muscle cells (HUVSMCs) vascular conduits together to fabricated miniature pancreatic tissue analog. Our study provided a novel technique using tissue strands as "bioink" for scale-up bioprinting of tissues or organs.**

I. Introduction

Tissue engineering has been focusing on the fabrication of vascularized 3D tissue for decades. Most recently, 3D bioprinting, especially organ printing, has shown great potential to realize automated robotic fabrication of 3D vascularized tissues and organs that are readily available for in vitro studies and/or in vivo transplantation [1]. Tissue spheroids, which are spherical shaped cell aggregates have been studied and shown their potential as the building blocks for organ printing. Without involvement of any hydrogel, tissue spheroids can easily mimic the embryonic development process by self-assembly into larger tissues [2].

Because of their ideal morphological and biological properties, tissue spheroids are great candidate for additive biofabrication of tissues. Visconti and his colleagues used fibroblast and smooth muscle cell derived tissue spheroids to build branched vascular structure [3], showing their exceptional ability of self-assembly and fast maturation. In addition, this technique has been used for β-cells where spheroids ranging from 200-400 μm in diameter produced more insulin than did a monolayer cell culture [4]. Faulkner Jones *et.al* have developed a system to fabricate embryonic stem cell spheroids, which showed high viability as well as maintained pluripotency [5]. Recent studies have developed several approaches for scalable robotic biofabrication of tissue spheroids. A modified hanging-drop system was developed by two companies, which allows high reproducibility, and easiness for robotic integration [6]. Digital microfluidic system has also been used for generating tissue spheroids in large-scale [7]. Most recently, hydrogel micropatterning has also been developed and modified by several groups for 3D formation of micro-scale tissue spheroids. Although, tissue spheroid-based aggregate techniques are promising for advancing tissue engineering, their labor-intensive fabrication in limited scale makes their applicability for large-scale tissue/organ fabrication difficult. Besides fabrication process, printing tissue spheroids sequentially by ensuring contact between each adjacent spheroid is another hurdle, given the extremely critical handling and sterilization conditions [8]. Without ensuring contact, spheroids cannot fuse to each other, easily leaving gaps and openings in the tissue as discussed in our recent review paper [1]. In addition, hydrogels are required as a transferring medium to deposit spheroids. Furthermore, technologies should be developed to prevent spheroid fusion before printing to eliminate nozzle clogging.

In this present work, we present a novel method of bioink generation, which enable scale-up fabrication of vascularized tissues. Tissue strands were fabricated within semi-permeable capsule system directly fabricated by coaxial nozzle bioprinting [9]. Cell viability test revealed minimal cell damage upon fabrication. Tissue strands fusion started as soon as 24 hours post-printing, and nearly completed on day 7. Immunofluorescence staining showed overall tissue specific markers insulin and C-peptide expression on matured tissue strands. Hybrid fabrication of cellular conduits with tissue strands were successfully conducted showing their potential for vascularized tissue fabrication. Our study provides a new method for cell aggregation in tissue strand form, which might serve as feeding material for bioprinting, and may realize 3D organ printing.

II. Materials and Methods

A. Cells and Materials Preparation

Sodium alginate solution was used in this study as biomaterial for vascular conduits fabrication as well as tubular

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capsule fabrication. Sterilized sodium alginate powder was dissolved in deionized water to get 4% (w/v) solutions. Similarly, the cross-linking solution was prepared by dissolving calcium chloride in ultra-purified water (Life Technologies, Carlsbad, CA) at 4% (w/v).

Human umbilical vein smooth muscle cells (HUVSMC) were purchased from Life technology. Upon revitalization, cells were cultured at 37 °C in 5% CO_2 in Medium 231 supplemented with smooth muscle cell growth supplement, 10μg/μl penicillin, 10μg/ml streptomycin, and 2.5μg/μl Fungizone. For vascular conduit fabrication, HUVSMCs were harvested and introduced into alginate solution and then homogenized by Votex mixer (Fisher Scientific, Waltham, MA, USA). HUVSMC-containing alginate solution was used as "bioink" for direct vascular conduit bioprinting with coaxial nozzle system. Mouse insulinoma beta TC3 cells were cultured in DMEM, supplemented with 10% fetal bovine serum, 10μg/ml streptomycin, 1% non-essential amino acid, 1mM sodium pyruvate and 2mM glutamine, in 37 °C in 5% CO_2. All culture media and supplements were purchased from Life Technologies (Carlsbad, CA, USA).

B. Fabrication of Tubular and Cell-laden Conduits

We have previously developed a novel bioprinting approach that enables printing vascular network directly through a coaxial nozzle system. Briefly, 4% sodium alginate tubular capsules were printed by a co-axial nozzle system (22G inner nozzle and 14G outer nozzle), serving as a semi-permeable capsule for tissue strand fabrication, allowing media diffusion. Similarly, 4% (w/v) sodium alginate solution with HUVSMCs at a density of 10×10^6 was used for vascular conduits fabrication [9]. Using the coaxial nozzle, HUVSMCs encapsulated within alginate were printed in tubular shape by maintaining constant controllable flow of crosslinker (4% (w/v) calcium chloride) through the core section of the coaxial nozzle enabling instantaneous gelation and leaving an unblocked vascular conduit. These vascular conduits were further cultured in vitro, and used as macro-vasculature for hybrid tissue fabrication, which is discussed later.

C. Tissue Strands Fabrication and Characterization

Mouse insulinoma bTC3 cells were harvested and centrifuged at 3,500 rpm. The resulting pellet was incubated at 37ºC with 5% CO_2 for overnight in DMEM-based media with 2% fetal bovine serum, supplemented with 10μg/μl penicillin, 10μg/ml streptomycin, and 2.5μg/μl Fungizone (Life Technologies, Carlsbad, CA, USA), in order to have sufficient coherency and mechanical integrity during further processing. Cell pellet was then injected into tubular conduits by a custom syringe unit (Hamilton Company, Reno, NV, USA). The encapsulated structure was incubated for at least 4 days and then dissolved by 1% sodium citrate solution for 10 minutes, leaving pure tissue strands behind. The dimension of tissue strands was measured by *Image J* (National Institutes of Health, Bethesda, MD, USA) analysis on microscopic images over two weeks. Figure 1 demonstrates the procedure for tissue strands fabrication.

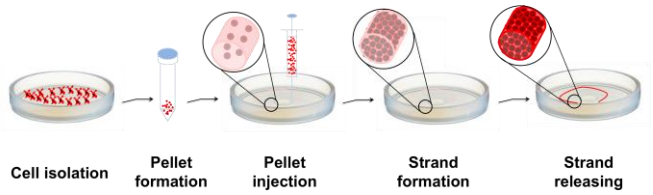

Figure 1. Procedure for tissue strand fabrication process.

Cell viability was tested at different time points (Day 1, 4, 7, and10) for tissue strands as previously described [9]. Briefly, each sample was washed with PBS and underwent 30 minutes fluorescence staining with LIVE/DEAD staining kit (Life Technologies, Carlsbad, CA), and directly imaged using a Leica fluorescent microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA). Images were collected from six different locations randomly chosen from each sample. *ImageJ* software (National Institutes of Health, Bethesda, MD, USA) was used for automated intensity calculation of red- and green-stained tissue strands, and percentages of viable signal were calculated by dividing green fluorescent intensity with sum of red fluorescent intensity and green fluorescent intensity.

To evaluate tissue specific protein expression, immunofluorescence staining was used for bTC3 cell specific markers: C-peptide, a precursor for insulin, and insulin. Samples were imaged with confocal microscope (Zeiss LSM 710, Berlin, Oberkochen, Germany). Antibodies for C-peptide, and insulin were purchased from Santa Cruz Biotechnology, Inc. (Dallas, USA.), and Abcam (Cambridge, USA) and applied per the instruction of the manufacturers.

D. Hybrid Tissue Fabrication

Matured tissue strands were tailored as 5mm long building unit, and deposited layer by layer, with a vascular structure embedded in the center to mimic the vascularized tissue. Firstly, 6 tissue strands each with a length of 5mm were closely assembled as the first two base layers, following by a third layer of tissue strands with a vascular conduits in the center with the length of 20mm. Finally, another two layers of tissue strands were laid on top to enclose the vasculature completely. Upon fabrication, the hybrid structure was kept in a customized polycarpolactone (PCL) mold to ensure initial integrity, and incubated at 37ºC with 5% CO_2. Figure 2 shows a schematic figure for the process.

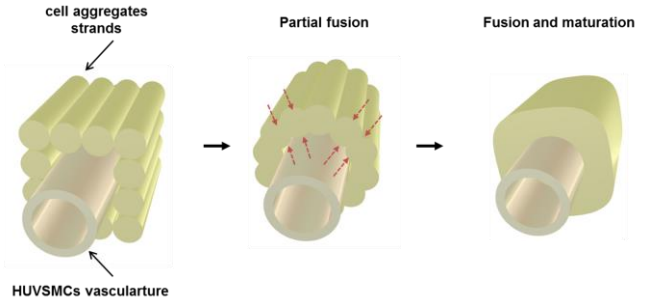

Figure 2. Schematic representation of hybrid tissue fabrication.

III. Results

A. Fabrication and Characterization of Semi-permeable Conduits

Tubular conduits were successfully printed with continuous uniform structure, as presented in Figure 3A. The average lumen diameter and vasculature diameter of fabricated conduits were 709 ± 15.9 µm and 1248.5 ± 37.2 µm, respectively (Figure 3B). Cells were individually encapsulated and uniformly distributed in vascular conduits (Figure 3C). Upon 4 weeks incubation in HUVSMC cell-specific media, vascular conduits were enriched with extra cellular matrix while maintaining hollow lumen (Figure 3D). Matured vasculature conduits were kept in culture until they were used in hybrid fabrication with tissue strands.

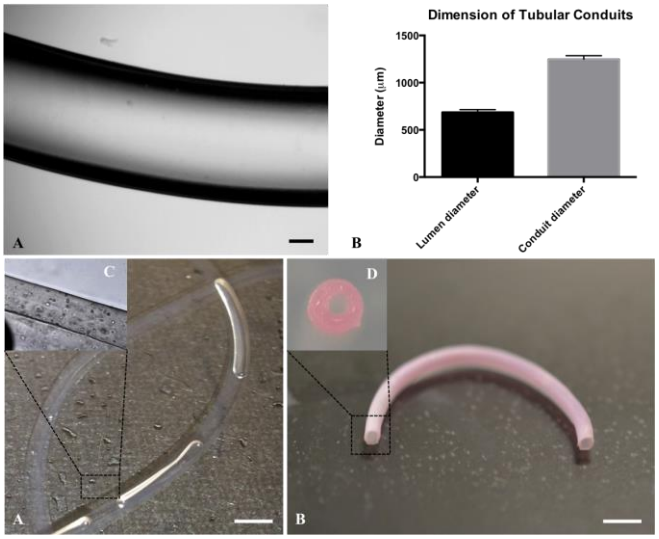

Figure 3. Printed tubular conduits. (A) Tubular conduit with lumenal center and uniform wall thickness (scale bar: 200µm); (B) dimension of tubular conduit and its lumen; (C) cellular conduit; (D) a long-term cultured vascular conduit (scale bar: 2mm)

B. Tissue Strands Fabrication and Maturation

Cell pellet made from 2.0 × 10^8 was successfully transferred into about 150mm long micro-tubular system (Figure 4A, C, D). Tissue strands were formed with reasonable integrity and mechanical strength upon de-crosslinking of conduits after 4 days of in vitro incubation (Figure 4B, E). The average diameter of tissue strands was about 639 ± 47 µm (Figure 4G). Cell viability was maintained upon fabrication as well as during culture (Figure 4F). The average viability on day 1 post fabrication was 75 ± 0.5%, and gradually increased to 77 ± 0.5%, and finally reached to 87 ± 3% on day 7 (Figure 4H).

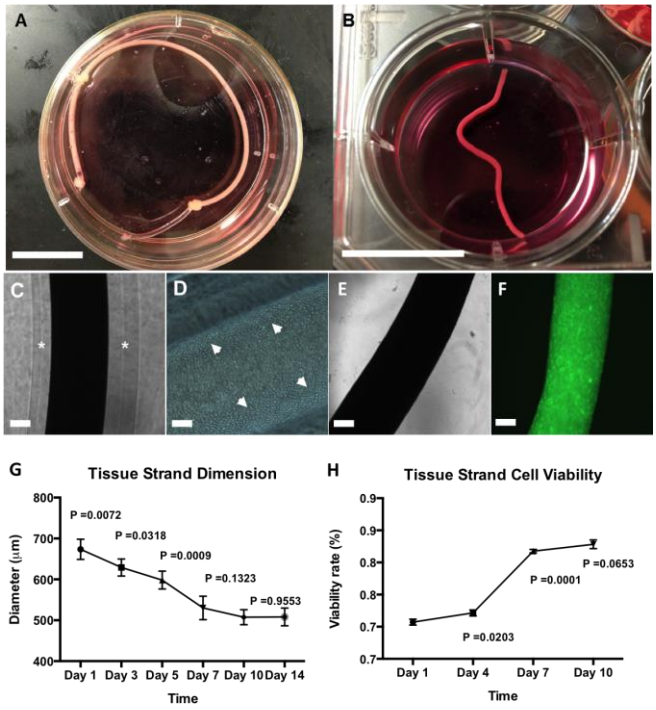

Figure 4. Tissue strand fabrication and characterization. (A, C) the cell pellet was encapsulated into a tubular conduit; (B, E) a tissue strand released from the tubular conduit; (D) cells were encapsulated with high density; (F) viable cells labeled with green fluorescence; (G) dimension of tissue strands at different time point; (H) viability of tissue strands over time. (Scale bars for A & B: 1mm; for C-F: 200 µm)

To test the fusion potential of fabricated tissue strands, two individual strands were placed onto 150mm petri dish with contact. Fusion started as early as 24h post fabrication during incubation (Figure 5A1, B1, C1), and further fused with more cell migration and ingrowth into each other between two strands on day 4 (Figure 5A2, B2, C2). At day 7, two strands were almost completely fused into one large strand due to interfacial tension, with contracted morphology, and no visible gap between each other (Figure 5A3, B3, C3).

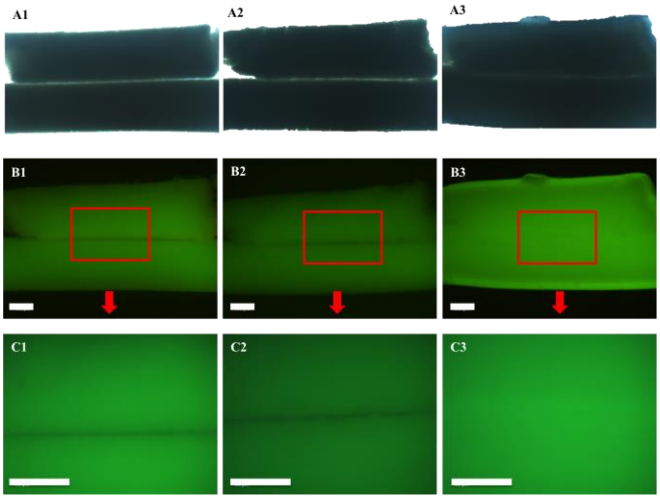

Figure 5. Self-assembly of tissue strands. (scale bar: 200µm)

To evaluate the expression of tissue specific proteins, two markers were used for immunofluorescence labeling with specific antibodies. As shown in Figure 6, substantial amount of cells were positively stained with C-peptide (A, C, stained red), as well as insulin (B, D, stained green) in tissue sections after fabrication and in vitro culture. This indicates that cells within tissue strands still maintained their insulin expression. Notably, positive staining was not only observed around cell nucleus, but also present in the intercellular space (Figure 6C, D)

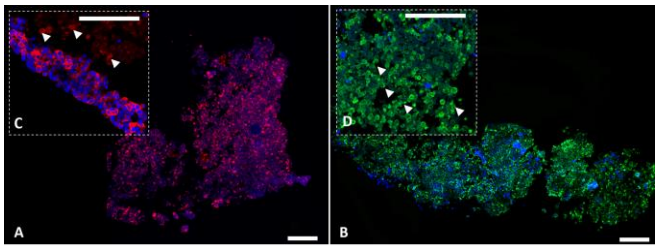

Figure 6. Immunofluorescence staining of bTC3 cell markers: (A, C) C-peptide staining (red); (B, D) insulin staining (green). (blue represents cell nuclear staining by DAPI, scale bar: 200μm)

C. *Fabrication of Hybrid Tissue Constructs*

In this work, macro-vasculature tissues were successfully fabricated. Although evident gaps were observed between tissue strands immediately after fabrication, the gradual fusion between strands held them as an entire unit during in vitro incubation (Figure 7A). The hybrid structure was able to present itself as an integrated structure in culture media, with notable fusion, and diminished gaps between strands on day 3 (Figure 7B). The hybrid tissue continued to fuse, remodel and mature in prolonged culture, and presented itself as a seamless vascularized tissue (Figure 7C). Also, cell migration and ingrowth into the vascular wall were also observed at the interfaces of tissue strands and vasculature (Figure 7D, E). A histological image also displays fused tissue strands around the vasculature (Figure 7F).

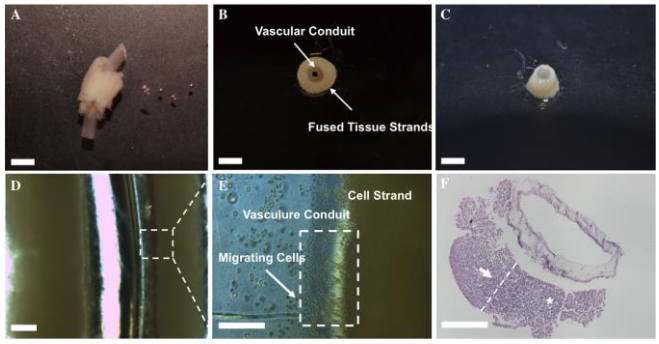

Figure 7. Hybrid macro-vascularized tissue fabrication. Upper pannel: schematic representation of hybrid tissue fabrication process; (A-C) fusion of tissue strands with vasculature and maturation of hybrid tissue; (D, E) Integration of tissue strands with vasculature through cell migration; (F) histological evidence of fusion.

IV. Discussion and Conclusion

In this study, we developed a scalable tissue strand fabrication technique, which can be used as a "bioink" to facilitate scale-up organ printing process. We modified traditional tissue spheroid fabrication technique, by applying tissue strands made of pure cells for tissue or organ fabrication with assistance of robotic printer. Our micro-tubular conduits served as a great reservoir for fabricating tissue strands, which demonstrated nearly 90% cell viability, fast fusion, and maintained functionality with tissue specific markers expression. The capability of tissue strands as "bioink" for robotic bioprinting has been successfully demonstrated in the hybrid tissue fabrication, where bTC3 tissue strands were deposited together with HUVSMC laden vascular conduits, mimicking a vascularized pancreatic tissue. In the future, multiple cell types, like endothelial cells and fibroblasts could be included in vascular conduits and to further resemble the natural counterpart. Also, endothelial cells could be co-cultured with bTC3 cells to make self-vascularized tissue strands, and used for scale-up tissue printing. In that way, we could envision the vascularized tissue strands may connect with the main cellular conduits in 3D, forming a fully vascularized tissue analog.

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