Rapid and efficient *in vivo* angiogenesis directed by electro-assisted bioprinting of alginate/collagen microspheres with human umbilical vein endothelial cell coating layer

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Abstract: Rapid reconstruction of functional microvasculature is the urgent challenge of regenerative medicine and ischemia therapy development. The purpose of this study was to provide an alternative solution for obtaining functional blood vessel networks *in vivo*, through assessing whether hydrogel-based microspheres coated by human umbilical vein endothelial cells (HUVECs) can direct rapid and efficient *in vivo* angiogenesis without the addition of exogenous growth factors or other supporting cells. Uniform alginate microspheres with adjustable diameter were biofabricated by electro-assisted bioprinting technology. Collagen fibrils were evenly coated on the surface of alginate microspheres through simple self-assembly procedure, and collagen concentration is optimized to achieve the highest HUVECs adhesion and proliferation. Immunofluorescence staining and gene analysis confirmed the formation of the prevascularized tubular structure and significantly enhanced endothelial gene expression. HUVECs-coated hydrogel microspheres with different diameters were subcutaneously injected in immune-deficient mice, which demonstrated rapid blood vessel regeneration and functional anastomosis with host blood vessels within 1 week. Besides, microsphere diameter demonstrated influence on blood vessel density with statistical differences but showed no obvious influence on the area occupied by blood vessels. This study provided a powerful tool for rapid and minimal-invasion angiogenesis of bioprinting constructs and a potential method for vascularized tissue regeneration and ischemia treatment with clinically relevant dimensions.

Keywords: Angiogenesis; Human umbilical vein endothelial cells; Microspheres; Minimal-invasive; Vascular tissue engineering

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1. Introduction

One of the bottleneck problems of tissue repair and regeneration is still the establishment of functional blood vessels in vivo, which are essential for supporting long-term survival and functioning of newly regenerated tissues\(^1\). Angiogenesis is commonly accepted as new blood vessel formation from growth and sprouting of existing blood vessels leading by proliferation and migration of endothelial cells\(^2\). These new vessels play an important role in the repair of damaged tissues and are of special importance for the clinical applications of large-scale three-dimensional (3D) tissue constructs, because oxygen and nutrient demands of cells in the center cannot be met by diffusion reaction from host tissues but only by blood vessel perfusion\(^3\). During the past decades, several approaches have been established to promote vasculogenesis and angiogenesis. One commonly explored technology is the delivery of angiogenic growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor to stimulate endothelial cell recruitment and/or to facilitate neovessel stability\(^4\). However, this approach is limited by poor stability of growth factors, short half-lives, rapid diffusion, and complexity of growth factor delivery system\(^5\).

Modular tissue engineering, which is proposed by McGuigan and Sefton in 2006, is a bottom-up approach featured by sub-millimeter sized, cylindrical collagen “modules” covered with endothelial cell layer\(^6,7\). This technology enables minimal-invasive injection of engineered constructs in vivo, rapid blood perfusion through the interconnected passageway among the modules, and efficient generation of highly vascularized tissues\(^8,9\), while preventing thrombosis by expression and secretion of several modulating molecules\(^10\). Recently, researchers have further developed this technology by utilizing spheroid microbeads instead of cylindrical modules since they demonstrate better mechanical properties and would form more uniform interconnect channels during perfusion\(^11\). Chan et al. have fabricated alginate microsphere by focused air-jet stream technology and generated in vitro capillary beds in microfluidic systems\(^12\). Zhang et al. utilized gelatin Cultispher S microspheres from Sigma as mesenchymal stem cells and human umbilical vein endothelial cells (HUVECs) microcarriers in spinner flasks, and studied the communication mechanisms of cocultured cells\(^13\). Rioja et al. have fabricated agarose-hydroxyapatite-fibrinogen microspheres encapsulating human HUVECs and fibroblasts through a water-in-oil emulsification process and demonstrate the formation of injectable pre-vascularized microtissues in vitro\(^14\).

Even though emulsification technology is widely used in microsphere generation, the complex process and oil phase sometimes limit its application in biomedical researches. Inkjet bioprinting, as the earliest invented cell printing technology, is first reported in 2004 to achieve drop-on-demand patterning of live cells\(^15,16\). Inspired by electrohydrodynamic printing technology for electronic product applications\(^17\), electro-assisted inkjet printing is developed to fabricate hydrogel microspheres\(^18,19\), cell-laden microspheres\(^20\), and 3D tissue engineering scaffolds\(^21,22\). Our and other groups’ previous studies have confirmed many advantages of this technology, including high throughput, high cell viability, round shape and adjustable diameter of droplet formation, as well as high printing precision, good shape fidelity and induced adipose, and bone and nerve tissue formation\(^23-26\). As to vascularization study, our previous study demonstrated that the interconnected gaps among the adipocyte-laden microspheres, endothelial cells, and collagen layer could serve as the starting point of in vivo vascularization, thus regenerating human vascularized adipose tissue in immune-deficient mice and achieved the maintenance of tissue weight and volume for up to 12 weeks\(^25\). Zhang et al. have fabricated VEGF-releasing (VEGFR) alginate microspheres through electro-assisted inkjet printing. Then, they have a surface coated the alginate microspheres with chitosan, mixed with HUVECs-laden collagen gel and demonstrated the importance of alginate-chitosan microspheres for supporting and guidance of alignment of HUVECs in vitro\(^18\).

Inspiration by previous researches, the aim of the present study is to provide an alternative solution for obtaining functional blood vessel networks in vivo. To achieve this goal, we assessed whether prevascularized microsphere-based construct fabricated by electro-assisted inkjet printing can direct rapid and efficient in vivo angiogenesis without additional growth factors or other supporting cells. The electro-assisted inkjet printing process enabled high-throughput biofabrication of alginate microsphere with spheroid shape and adjustable diameter (245 µm, 430 µm, and 657 µm). Collagen fibrils were surface coated to alginate microspheres as a biomimetic extracellular matrix (ECM) for HUVECs adhesion, proliferation, and function maintenance. Supported by the optimized collagen concentration, HUVECs proliferation, enhancement of endothelial gene expression, and prevascularized tissue formation were observed. Then, in vitro constructs with different microsphere diameters were subcutaneously injected into immune-deficient mice, which demonstrated rapid blood vessel regeneration within 7 days. These newly generated vessels demonstrated functional anastomosis with host blood vessels without additional growth factors or other supporting cells. More interestingly, microsphere diameter demonstrated an obvious influence on blood vessel density, but not the area occupied by blood vessels. To the
best of our knowledge, this is the first attempt to utilize electro-assisted inkjet printing microspheres for in vivo angiogenesis study. This approach provided a powerful tool for rapid and minimal-invasion angiogenesis based on bioprinting constructs and a potential method for vascularized tissue regeneration and ischemia treatment with clinically relevant sizes.

2. Methods and Materials

2.1 Cell Culture

HUVECs (Invitrogen) were cultured in M200 medium supplemented with low serum growth factors according to the provider’s instructions. The culture medium was changed every 2-3 days. HUVECs from passage 2 to 3 were used in this study.

2.2 Material Preparation

Sodium alginate (Sigma, A0682) was dissolved in physiological saline solution at 4% (w/v) by repeated heating for 3 times in a stove (80°C). Bacteria and mycoplasma detection (YEASEN, 40601ES20) were performed to avoid any contaminations. Type I collagen from rat tail (Sigma, 4 mg/mL) was diluted in M200 medium to concentrations of 0.25 mg/mL and 0.5 mg/mL, respectively. The collagen solution was adjusted to pH 7.2 by 0.1 mol/L NaOH solution and immediately used after preparation.

2.3 Electro-assisted Inkjet Printing

We established electro-assisted inkjet printing device with a static electricity power supply (SA167-Y, Tianjin, China), a syringe pump (Longer Pump Ltd.), and a grounded collecting device, as demonstrated in Figure 1. Microspheres were fabricated based on the principle of electrohydrodynamic atomization, where the tip of the alginate solution jet from a capillary nozzle was disintegrated into micron-sized droplets under the joint action of electrostatic force, Coulomb force, gravity, and surface tension. The droplets quickly fall into the solidifying solution-100 mM calcium chloride, and round shaped hydrogel microspheres were fabricated. Based on this principle, many parameters can be used to control the roundness and diameter of the microspheres, including nozzle diameter, voltage, electrode distance, push speed, and the concentration of solidifying solution. Three groups of alginate microspheres, namely Group A, Group B, and Group C, with spheroid shape and different diameters were generated by the parameter combinations demonstrated in Table 1. Size of the alginate microspheres was analyzed by Image-pro Plus 5.0 (Media Cybernetics, USA). Three independent samples and ten random pictures (more than 200 microspheres) were analyzed for each group.

2.4 Elasticity Testing

To test the elasticity property of the obtained alginate microspheres, Group A, Group B, and Group C

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Table 1. Parameter combinations for microsphere fabrication.

| Group | Needle gauge-inner diameter (µm) | Voltage (kV) | Electrode distance (mm) | Push speed (mL/h) | Microsphere diameter (µm) |
|-------|---------------------------------|-------------|-------------------------|------------------|--------------------------|
| A     | 27-191                          | 7           | 25                      | 15               | 245 ± 26                 |
| B     | 24-292                          | 8           | 30                      | 25               | 430 ± 37                 |
| C     | 24-292                          | 6           | 25                      | 20               | 657 ± 46                 |

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Figure 1. Schematic presentation of this study. Alginate microspheres with tunable properties were fabricated through the electro-assisted inkjet printing technology and coated with different concentrations of collagen fibrils. Human umbilical vein endothelial cells (HUVECs) adhered and proliferated on the surface of alginate/collagen microspheres to form an endothelial cell layer. Alginate/collagen-HUVECs constructs were subcutaneously injected into immune-deficient mice. After 7 days, implants were harvested and in vivo angiogenesis was evaluated.
microspheres were extruded through syringe needles with different gauge size. Clinically used disposable syringe needles of 16-gauge (inner diameter 1194 µm), 22-gauge (inner diameter 394 µm), and 27-gauge (inner diameter 394 µm) were used to extrude three groups of microspheres. The integrity of the alginate microspheres after extrusion was accorded by an optical microscope (Nikon). Elastic deformation was calculated by needle inner diameter/average microsphere diameter ×100% when all the microspheres were intact after extrusion.

2.5 Collagen Coating

Alginate microspheres were evenly mixed with collagen solutions with volume proportion of about 1:2. The mixture was placed at 37°C in the atmosphere of 5% CO₂ for 24 h to allow the self-assembly of collagen fibrils. After collagen coating, microspheres were collected and washed for 3 times by PBS for further studies. We referred to the collagen-coated alginate microspheres as alginate/collagen microspheres in this research.

2.6 Scanning Electron Microscopy (SEM) Analysis

Morphology of alginate microspheres before and after collagen coating was examined by SEM analysis. Briefly, samples were fixed in 1 mol/L sodium cacodylate buffer with 2% glutaraldehyde, 3% paraformaldehyde, 5% sucrose, and went through critical point drying. Before scanning, samples were mounted on aluminum supports, sputter-coated with gold and examined under FEI Quanta 200 SEM from Holland.

2.7 Cell Seeding

HUVECs were collected by standard trypsin-EDTA treatment and suspended in culture media at a concentration of 10⁶ cells/mL. 1 mL of HUVECs suspension was evenly mixed with about 200 µL of alginate/collagen microspheres and cultured in non-adhesive plates for 3 days to facilitate cell adhesion to the microsphere surface. Culture media were changed every day. We refer to this construct as alginate/collagen-HUVECs microspheres in this research.

2.8 Cell Proliferation and Viability Analysis

HUVECs adhesion and proliferation on collagen-coated alginate microspheres were determined by CellTiter-Blue Cell Viability Assay (Promega, Fitchburg, WI) according to manufacturer’s instructions. Briefly, 4 h, 48 h, and 72 h after cell seeding, the alginate/collagen-HUVECs microspheres were transferred into a new well. 200 µL of assay reagent and 1 mL of culture medium were added. After 4 h incubation, the supernatant was read by fluorescence with excitation 560 nm and emission 590 nm filter pair (SpectraMax M2, Molecular Device, Sunnyvale, CA). Alginate/collagen microspheres without cells were subjected to the same process and data were used as the blank. All the data were normalized to cell number by the standard curve. Three samples were tested for each group.

2.9 Immunofluorescence Staining of in vitro Samples

Alginate/collagen-HUVECs microspheres were fixed in 4% paraformaldehyde, embedded in optimal cutting temperature (OCT) compound, and followed by cryosectioning into 10 µm slices (HD- 1800, HuiDa). The slice samples were permeabilized with 0.1% Triton-X, blocked with blocking buffer and incubated with anti-human PECAM-1/CD31 (R and D Systems) and corresponding secondary antibody. Controls were carried out by replacing the primary antibodies with immunoglobulin G antibodies. Specimens were observed under a fluorescence microscope (OLYMPUS BX51, JAP). Image acquisition was performed using Applied DP-Controller system (OLYMPUS, JAP).

2.10 Real-Time Reverse Transcription-polymerase Chain Reaction (RT-PCR)

Quantified gene analysis was carried out to determine the gene expression level of HUVECs in the bioprinting construct. Total RNA was extracted and reverse transcribed using cDNA synthesis kit (Takara) according to the manufacturer’s instruction. Real-time RT-PCR using SsoFastTM Eva-Green Supermix (Bio-Rad) on Bio-Rad CFX96 RT-PCR platform was performed. The sequences of the specific primers were as follows: Housekeeping gene-GAPDH, forward-TG CACACAACTGCTTAGC, reverse-GGCAATGGA CTGTGGTGATGAG; CD31, forward-AACAGTGGT- GACATGAAGAGCC, reverse-TGTAAACAGC ACATCATT; VE-Cadherin, forward-TTG GAACCAGATGCATGAT, reverse-CTTGTGGACT CAGCCTTGAC; vWF, forward-CCGATGCAGCCTT TTCGGA, reverse-TCCCCCAAGATACCGGAGGAG; hypoxia-inducible factor1α (HIF1α), forward-GGCCCT GGAGACACAATCAT, reverse-TCTGTGGTGTTGGCT CGCAA; VEGF, forward-AGGAGGAGGGCAGAACATC, reverse-GGCAACAGGAGTGGCCTTGA; VEGFR, forward-CAGAGGAGCCTGCTGGAAGAT, reverse-GAGGTTCCCTTGAACAGTGGATT. After amplification, the melting curves were analyzed and the value of a specific gene was given by normalization to the house-keeping gene in each sample. Fold change was calculated by dividing the gene value of the alginate/collagen-HUVECs microspheres constructs with two-dimensional (2D) cultured HUVECs.
2.11 Subcutaneous Injection

Eight weeks old female immune-deficient BALB/c nude mice \( (n=3) \) were purchased from the Department of Laboratory Animal Science, Peking University Health Science Center. Guidelines established by Peking University Health Science Center for the care and use of laboratory animals were followed. 1 mL of three groups of alginate/collagen-HUVEC's constructs (about 150,000 microspheres for Group A, 25,000 microspheres for Group B, and 8000 microspheres for Group C) were subcutaneously injected in nude mice by 16-gauge needles to investigate angiogenesis efficiency. Groups A, B, and C constructs were composed of Groups A, B, and C alginate microspheres, respectively. The site of implantation was chosen because it was appropriate to hold the implants with volume as large as 1 ml. Collagen coating density for all the groups was 0.5 mg/mL. HUVECs were cultured with collagen-coated alginate microspheres for 3 days before the injection. Two control groups were fabricated and in vivo injected under the same condition as test groups. Control Group 1 was set as pure alginate microspheres (Group B microspheres) without collagen and HUVECs layer. Control Group 2 was set as the simple mixture of alginate, collagen, and HUVECs without microsphere structure. 7 days after injection, animals were killed and the remaining injections were retrieved. In vivo angiogenesis was determined by histological and immunohistological evaluations. Schematic of this research is shown in Figure 1.

2.12 Histological Analysis

Harvested tissues were fixed in 10% neutral buffered formalin, embedded in OCT compound and frozen at 70°C. Frozen samples were sliced into 10 μm sections using a cryostat sectioning (HD- 1800, HuiDa). The slices were washed and then stained with hematoxylin-eosin (HE) working solution according to the manufacturer’s instructions. Specimens were observed under a fluorescence microscope (OLYMPUS BX51, JAP).

2.13 Immunohistological Evaluation and Vascularity Analysis

Besides histological analysis, we also performed immunohistological evaluation of in vivo harvested tissues. The sample slices from cryostat sectioning were permeabilized, blocked, and stained with anti-human PECAM-1/CD31 antibody. Controls were carried out by replacing the primary antibodies with immunoglobulin G antibodies. Specimens were observed under a fluorescence microscope (OLYMPUS BX51, JAP). To determine blood vessel density, five images were taken from three nonconsecutive sections of each sample, and the number of blood vessels was counted manually at \( \times 200 \). To examine the percentage of area occupied by blood vessels, a minimum of 10 individual images and at least 150 blood vessels were analyzed as previously described\(^{39}\). Image analysis was performed using Image-Pro Plus 5.0 (Media Cybernetics, USA).

2.14 Statistical Analysis

Statistical analysis was performed by GraphPad Prism 6 using two-way analysis of variance in conjugation with Tukey’s test, pairwise multiple comparison procedures. Differences were considered statistically significant when \( P<0.05 \). All the data were presented as mean values ± standard deviation.

3. Results and Discussion

3.1 Biofabrication of Alginate Microspheres

In the previous work, we manufactured an electro-assisted bioprinting device for microsphere generating, which facilitated the creation of a sterile environment and easy adjustment of microsphere properties, such as diameter, porosity, and swelling behavior\(^{38}\). Here, alginate microspheres with an average diameter of 257 μm (Group A), 430 μm (Group B), and 657 μm (Group C) were fabricated by adjusting the parameter combinations, as shown in Table 1. Group A microspheres showed integrated and spheroid contour (Figure 2A). Image analysis demonstrated Gauss distribution of microsphere diameter, as shown in Figure 2B, where 69% of the Group A microspheres ranged between 210–270 μm. Group B microspheres also showed spheroid contour and uniform size, with SEM examination showed the porous structure of the microspheres, which would facilitate mass transfer and nutrient supply for possible cell encapsulation applications (Figure 2C). Image analysis demonstrated Gauss distribution of microsphere diameter, with more than 70% of the microspheres ranged between 400–460 μm (Figure 2D). Similar phenomena in morphology and size distribution were observed in Group C (Figure 2E and F), suggesting that uniform microspheres with tunable size were fabricated by this technology. We tested the mechanical strength and elasticity of the alginate microspheres through extrusion with gauges of different inner diameter. As demonstrated in Figure 2E, 100% of Group C microspheres maintained their spheroid shape and integrity after extrusion through all the gauge sizes, the smallest being 27 gauge with an inner diameter of 394 μm. The highest elastic deformation was 60%, suggesting excellent elasticity. These results were similar to the previous studies by Chui et al.\(^{39}\). This property made the microsphere modules especially favorable for bioengineering construct formation and further minimal-invasive applications.
Alginate has been widely used as a matrix for bioprinting and tissue engineering applications due to mild physiological gelling condition, controllable mechanical properties, porous structure, proper swelling behavior, and degradation properties, drug loading capacity, and sound biocompatibility\cite{31,32}. Furthermore, alginate has been approved by the regulatory authorities for applications such as wound dressing, dental impression, and as a food supplement\cite{33}. In this research, we fabricated highly elastic alginate microspheres to serve as modular of the injectable constructs. Microspheres of three different diameters were fabricated to further study possible relationships between microsphere diameter and angiogenesis efficiency. However, alginate is a relatively inert biomaterial that lacks adherent interaction with mammalian cells. One common approach to provide cell adhesion is to involve cellular adhesion molecules such as laminin\cite{34}, fibronectin\cite{35}, and collagen\cite{36}. In this study, collagen was coated on the surface of alginate microspheres to facilitate endothelial cells adhesion, proliferation, and biofunction maintenance.

### 3.2 Collagen Coating Facilitates HUVECs Adhesion and Proliferation

Naturally, collagen fibril formation is a self-assembly process largely determined by intrinsic properties of collagen molecules. This process can be replicated in vitro under proper conditions without adding chemicals that may be harmful to cells, which is especially favorable for cell-involving bioengineered devices\cite{24}. In this study, a layer of collagen fibril coating on the surface of alginate microspheres was achieved by utilizing self-assembly mechanism. In this study, we mixed the alginate microspheres and collagen solutions with the volume proportion of about 1:2, and incubated in 37°C for 24 h to allow complete self-assembly of collagen fibrils and to ensure full coverage of collagen on the microsphere surface, which was indicated by SEM examination (Figure 3A).

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**Figure 2.** Characteristics of three groups of alginate microspheres. (A) Morphology of Group A alginate microspheres. (B) Diameter distribution of Group A alginate microspheres. (C) Morphology of Group B alginate microspheres. The magnified image showed the microstructure of microspheres examined by SEM (scale bar: 100 µm). (D) Diameter distribution of Group B alginate microspheres. (E) Morphology of Group C alginate microspheres. The magnified image showed microsphere morphology after extrusion through a 27-gauge needle (scale bar: 500 µm). (F) Diameter distribution of Group C alginate microspheres.
SEM image of higher magnification clearly showed the collagen fibrils on the surface of microspheres (Figure 3B). Collagen concentration was optimized based on HUVECs behavior in a period of 72 h (Figure 3C). When no collagen was coated on alginate microspheres (control group), only about 12% of seeded HUVECs adhered to the microspheres on 4 h and showed no obvious proliferation on 72 h. The cell adhesion rate on 4 h was 47% and 79% for 0.25 mg/mL and 0.5 mg/mL collagen, respectively. Moreover, the cell proliferation rate on 72 h was 5.6 times and 6.6 times, respectively. Compared with the control group, these results showed significantly enhanced HUVECs adhesion as well as proliferation. We demonstrated the results of Group B since its size was in the middle range. Regardless of the diameter of microspheres, the similar trend was observed in all the three groups of alginate microspheres. Since starting from 48 h, there were statistical differences between 0.25 mg/mL and 0.5 mg/mL collagen, the optimized collagen concentration was 0.5 mg/mL.

ECM has always played an important role in vascularization since it provides support and inductive cues to endothelial cells during angiogenesis/vasculogenesis. Type I collagen, fibrin, and Matrigel are the most widely used ECM for vascularization applications. Type I collagen is the most abundant proteins in mammals, and many types of cells have been incorporated in collagen containing bio-engineered constructs for vascular tissue regeneration purposes, and more effective results were reported compared with the use of synthetic polymers[37,38]. Our results demonstrated an outstanding supporting effect of collagen on the behavior regulation of endothelial cells, which were in accordance with previous reports[9,10].

Even though collagen is an ideal substrate for cell attachment, communication, and signaling, it shows the inherent weakness of mechanical strength. Without timely cell-mediated reconstruction, the cellularized collagen gel could not support the long-term structural stability and/or physical loading of the hemodynamic environment[39]. In this study, we utilized alginate microspheres, which demonstrated excellent mechanical properties, as a basic element of the construct. Collagen was added into the construct by a surface coating on the alginate microspheres. This design showed merits in three aspects: (1) Involvement of collagen would not influence the overall mechanical properties of the construct; (2) collagen was presented on the surface of microspheres to direct mediated cell-matrix interaction and communication, thus achieving dramatically improved HUVEC adhesion and proliferation; (3) collagen layer instead of commonly reported collagen gel was distribute on the surface of microspheres, thus enabled the formation of interconnected channels in the range of tens to hundreds of microns among the gaps of the microspheres. These interconnected channels were larger compared with the

poles in collagen gels, which are mostly in the range of tens of microns, and were of significant importance for cell migration and prevascularized tissue formation.

### 3.3 Prevascularized Construct Formation in vitro

After HUVECs seeding for 3 days, HUVECs formed a layer-like structure on the surface of alginate/collagen microspheres, as shown in Figure 3D. The marker protein of HUVECs, anti-human CD31 immuno-fluorescence staining demonstrated the formation of prevascularized tissue in vitro, indicated by the presence of lumen-like structure (Figure 3E). Interestingly, lumen-like structures resembling natural vessels were usually seen among the gaps of microspheres. Compared with 2D cultured HUVECs, all the endothelial marker genes tested showed significantly improved expression, with the highest of 7.9 times (VE-Cadherin) and the lowest of 3.6 times (HIF1a), which suggested greatly-enhanced endothelial phenotype (Figure 3F). The similar trend was observed for all the three groups of microspheres. We demonstrated the results of Group B since their diameter was in the middle range, and the results were considered representative. Based on these results, collagen coating concentration of 0.5 mg/mL and HUVECs seeding for 72 h was chosen for subsequent in vivo studies.

HUVECs are one of the most commonly used endothelial cell sources since human umbilical veins are more accessible than other blood vessels. As a result, this cell type is made into standardized products in many bio-companies. HUVECs express many important endothelial markers, among which, CD31 and VE-Cadherin are pro-angiogenic cell adhesion proteins in nature, whereas vWF is an anti-angiogenic intracellularly expressed protein[39]. HIF1a, a member of the HIF family, regulates a wide range of angiogenic genes under hypoxic condition, including induction of VEGF expression[40]. VEGF and its receptor VEGFR are pro-angiogenic factors facilitating the proliferation, differentiation, and migration of endothelial cells[40]. Numerous studies have demonstrated the formation of in vitro prevascularized structure resembling natural microvascular networks when HUVECs are cultured under proper conditions[41]. We assume the prevascularized construct formation in our study was facilitated by bioactive and angiogenic cues provided by collagen fibrils, the interconnected channels formed by surrounding microsphere for HUVEC proliferation and migration, and the inherent biological properties of HUVEC, i.e., proliferation, secretion of growth factors and ECM, microenvironment remodeling, and responsiveness to external stimuli.

### 3.4 In vivo Angiogenesis

In vitro, prevascularized constructs were subcutaneously injected in immune-deficient mice (Figure 4A). The
animals showed a healthy condition with normal weight growth during the experimental period of 7 days. The injections retained the original site and shape, and blood vessel formation was observed in the injection sites, indicated by arrows in Figure 4B. HE staining demonstrated biomaterial degradation to a certain degree, while still maintaining the spheroid shape of injected microspheres. Neo-blood vessel formation was evident by red color staining, indicating red blood cells and functional anastomosis with host blood vessel network in all the three test groups (Figure 4C-E). Most of the newly generated vessels were distributed among gaps of microspheres, as demonstrated by arrows. In the control Group 1, where pure alginate microspheres without collagen and HUVECs layer, and control Group 2, where the same amount of cells and biomaterials without microsphere structure were injected, little angiogenesis was observed (Figure 4F and G). HE staining only demonstrate the leftover of biomaterials, where purple color indicate alginate and red clusters indicate collagen.

Magnified HE staining and PECAM-1 staining of test groups demonstrated functional blood vessel formation indicated by the presence of red blood cells and blood vessel lumens (Figure 4H). Microvessel density was counted by image analysis, as demonstrated in Figure 4I. Group A constructs with microsphere average diameter of 245 µm resulted in about 47 microvessels/mm². This number decreased with the increase of microsphere diameter, with Group C constructs regenerated about 31 microvessels/mm². Both control groups showed little new blood vessel ingrowth, with vessel density of about 4.4 and 3.3/mm², respectively. All the test groups showed significantly increased blood vessel density compared with the control group. The three test groups also showed significant differences among each other, suggesting the important influence of microsphere diameter on neo blood vessel density. Besides vessel density, another important factor related to angiogenesis efficiency is the area occupied by blood vessels. Figure 4J demonstrated that despite differences in microsphere diameters, area covered by blood vessels were all around 14% with no significant differences among the three groups of injections. In accordance with other analysis, the control groups showed little angiogenesis.

It is now well accepted that new blood vessel ingrowth should be timely and sufficient to ensure the survival of whole bio-engineered device. However, spontaneous vascularization due to the inflammatory response and endogenous release of angiogenic growth factors from cells within the implants are often too slow to provide adequate nutrient and oxygen support to cells in the center of the transplanted tissue. Two principals of vascularization strategies have emerged in the past decades: The first one focuses on the ingrowth of blood vessels into the implants from the surrounding host tissue stimulated by angiogenic cues. However, a rapid blood supply reconstruction may be achieved by inosculation, when microvascular networks are generated
within engineered constructs before implantation. In this case, these networks only need to develop functional anastomosis with the host vessel network to ensure fully perfusion within a short period of time\(^4\). Our practice utilized the inosculation strategy, by constructing prevascularized hydrogel microspheres \textit{in vitro} and then performed \textit{in vivo} implantation, and demonstrated the powerful potential of this methodology.

In this study, we designed alginate microspheres with a diameter of about 200 µm to 600 µm as basic units of the constructs. The microspheres demonstrated high elasticity to ensure formation and maintenance of interconnected channels after injection as well as under stress of the host skin. These gaps and channels were natural space for host vessel ingrowth and anastomosis with pre-existing vascularized structures. Besides, we chose not to

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4}
\caption{Subcutaneous injection and \textit{in vivo} angiogenesis. (A) The appearance of immune-deficient mice immediately after injection. The black arrow indicates the injection site. (B) The appearance of injection site 7 days after injection. The black arrow indicates blood vessel ingrowth into the implants. (C) Hematoxylin-eosin (HE) staining of Group A implants 7 days after injection. Black arrows indicate functional blood vessels. “M” indicates non-degraded microspheres. (D) HE staining of Group B implants 7 days after injection. (E) HE staining of Group C implants 7 days after injection. (F) HE staining of the control Group 1. (G) HE staining of the control Group 2. (H) High-magnification image of HE staining, where arrows indicated functional blood vessels indicated by red blood cells. The magnified image showed anti-human PECAM-1 immunofluorescence staining of harvested tissue. White arrows indicated HUVECs lumen structure. Scale Bar: 25 µm. (I) Blood vessel density of the test groups and control groups. (J) Percentage of the area occupied by blood vessels of the test groups and control groups. **indicates \(P<0.01\), ***indicates \(P<0.001\).}
\end{figure}
Electro-assisted bioprinting for angiogenesis in vivo, 1(6):2963-9. DOI, which was especially favorable when it comes to clinical applications. Even though in vitro experimentation demonstrated the structural integrity of microspheres by extrusion through 27-gauge needle, we chose to perform in vivo injection by 16-gauge needle, which is commonly used in the clinic for minimally invasive operations, to avoid mechanical disturbance to the largest extent. There may be some disruptive effect on the prevascularized structure during the injection procedure, which is the common dilemma of minimally invasive operation of cell-laden constructs, but our results demonstrated rapid and efficient in vivo angiogenesis with functional anastomosis with host vessel network, indicated by the presence of abundant of red blood cells and lumen structures. These data were comparable to the blood vessel density of human subcutaneous adipose tissue, which is about 150-350/mm² depending on the body mass index of individuals[44]. Further efforts should be paid to explore the detailed mechanism and the interface of in vitro construction and in vivo injection of engineered constructs.

Due to the versatility of bioprinting technology, we fabricated microspheres with distinct diameters and closely studied the influence of microsphere size on angiogenesis effect. The results showed that increased microspheres resulted in reduced blood vessel number but did not largely influence area occupied by functional blood vessels. A possible reason was that a certain amount of blood should be perfused to ensure the survival of a certain volume of implants, which was 1 mL in this study. Both control groups showed only a little angiogenesis, which demonstrated the significant importance of collagen layer and endothelial cells for angiogenesis, and were in accordance with Koike N’s report that the simple combination of collagen gel and endothelial cells could not achieve sustainable and functional blood vessels[45]. Our future researches will focus on the exploration of angiogenesis mechanism and secondary transplantation, i.e., harvest of functional blood vessel network by gentle removal of undegraded biomaterials under physiological conditions, and incorporation of the harvested blood vessel network with other tissue engineered constructs for vascularized tissue regeneration. Our observations provide a versatile strategy as well as detailed instructions for construct design and fabrication for investigations of vascularized tissue regeneration.

4. Conclusion

This study proposed a strategy for bioprinting fabrication of injectable constructs to achieve rapid and efficient in vivo angiogenesis. Alginate microspheres with porous structure, integrated and regular shape, high elasticity, and tunable diameters were fabricated through electro-assisted inkjet printing technology. Collagen fibrils were surface coated on alginate microspheres to facilitate HUVEC adhesion, proliferation, and prevascularized tissue formation. After subcutaneous injection in mice, the constructs were retained in the injection site by the tension of the skin. The interconnected channels among the microspheres were maintained. Host blood vessels established timely and efficient angiogenesis and perfused the whole implants within 7 days. Blood vessel density, but not area occupied by blood vessels, was influenced by the microsphere diameter. This research provided a novel and versatile strategy for the fabrication of engineered constructs with clinically relevant dimensions.

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References

1. Nih LR, Gojgini S, Carmicharel ST, et al., 2018, Dual-function Injectable Angiogenic Biomaterial for the Repair of Brain Tissue Following Stroke. Nat Mater, 17(7):642-51. DOI 10.1038/s41563-018-0083-8.
2. Risau W, 1997, Mechanisms of Angiogenesis. Nature, 386(6626):671-4.
3. Carmeliet P, 2003, Angiogenesis in Health and Disease. Nat Med, 9(6):653-60.
4. Pal A, Vernon BL, Nikkhah M, 2018, Therapeutic Neovascularization Promoted by Injectable Hydrogels. Bioact Mater, 3(4):389-400. DOI 10.1016/j.bioactmat.2018.05.002.
5. De Witte TM, Fratila-Apachitei LE, Zadpoor AA, et al., 2018, Bone Tissue Engineering Via Growth Factor Delivery: From Scaffolds to Complex Matrices. Regen Biomater, 5(4):197-211. DOI 10.1093/rb/rby013.
6. McGuigan AP, Sefton MV, 2006, Vascularized Organoid Engineered by Modular Assembly Enables Blood Perfusion. Proc Natl Acad Sci U S A, 103(31):11461-6. DOI 10.1073/pnas.0602740103.
7. McGuigan AP, Leung B, Sefton MV, 2006, Fabrication of Cells Containing Gel Modules to Assemble Modular Tissueengineered Constructs. Nat Protoc, 1(6):2963-9. DOI 10.1038/nprot.2006.443.
8. West MED, Sefton EJB, Sefton MV, 2018, Bone Marrow-Derived Macrophages Enhance Vessel Stability in Modular
Engineered Tissues. *Tissue Eng Part A*. DOI 10.1089/teta.2018.0222.

9. Cooper TP, Sefton MV, 2011, Fibronectin Coating of Collagen Modules Increases in vivo HUVEC Survival and Vessel Formation in SCID Mice. *Acta Biomater*, 7(3):1072-83. DOI 10.1016/j.actbio.2010.11.008.

10. McGuigan AP, Sefton MV, 2008, The Thrombogenicity of Human Umbilical Vein Endothelial Cell Seeded Collagen Modules. *Biomaterials*, 29(16):2453-63. DOI 10.1016/j.biomaterials.2008.02.010.

11. Leong W, Wang DA, 2015, Cell-laden Polymeric Microspheres for Biomedical Applications. *Trends Biotechnol*, 33(11):653-66. DOI 10.1016/j.tibtech.2015.09.003.

12. Chan JM, Zervantonakis IK, Rimchala T, et al., 2012, Engineering of in vitro 3D Capillary Beds by Self-Directed Angiogenic Sprouting. *PLoS One*, 7(12):e50582. DOI 10.1371/journal.pone.0050582.

13. Zhang S, Zhou M, Ye Z, et al., 2017, Fabrication of Viable and Functional Pre-vascularized Modular Bone Tissues by Coculturing MSCs and HUVECs on Microwriters in Spinner Flasks. *Biotecnol J*, 12(8):1700008. DOI 10.1002/biot.201700008.

14. Rioja AV, Daley ELH, Habif JC, et al., 2017, Distributed Vasculogenesis from Modular Agarose-hydroxypatite-Fibrinogen Microbeads. *Acta Biomater*, 55:144-52. DOI 10.1016/j.actbio.2017.03.050.

15. Ding S, Feng L, Wu J, et al., 2018, Bioprinting of Stem Cells: Interplay of Bioprinting Process, Bioinks, and Stem Cell Properties. *ACS Biomaterials Science and Engineering*. *ACS Biomater Sci Eng*, 4(9):3018-124. DOI 10.1021/acsbiomaterials.8b00399.

16. Roth EA, Xu T, Das M, et al., 2004, Inkjet Printing for High-throughput Cell Patterning. *Biomaterials*, 25(17):3707-15. DOI 10.1016/j.biomaterials.2003.10.052.

17. An BW, Kim K, Lee H, et al., 2015, High-resolution Printing of 3D Structures Using an Electrohydrodynamic Inkjet with Multiple Functional Inks. *Adv. Mater.*, 27(29):4322-8. DOI 10.1002/adma.201502092.

18. Zhang W, Choi JK, He X, 2017, Engineering Microvascularized 3D Tissue Using Alginate-Chitosan Micropapules. *J Biomater Tissue Eng*, 7(2):170-3. DOI 10.1166/jbte.2017.1547.

19. Xie M, Gao Q, Zhao H, et al., 2019, Electro-Assisted Bioprinting of Low-Concentration GelMA Microdroplets. *Small*, 15(4):e1804216. DOI 10.1002/smll.201804216.

20. Yao R, Zhang R, Luan J, et al., 2012, Alginate and Alginate/Gelatin Microspheres for Human Adipose-derived Stem Cell Encapsulation and Differentiation. *Biofabrication*, 4(2):25007. DOI 10.1088/1758-5082/4/2/025007.

21. Wu B, Takeshita N, Wu Y, et al., 2018, Pluronic F127 Blended Polycaprolactone Scaffolds via e-jetting for Esophageal Tissue Engineering. *J Mater Sci Mater Med*, 29(9):140. DOI 10.1007/s10856-018-6148-z.

22. Kim M, Yun HS, Kim GH, 2017, Electric-field Assisted 3D-fibrous Bioceramic-based Scaffolds for Bone Tissue Regeneration: Fabrication, Characterization, and in vitro Cellular Activities. *Sci Rep*, 7(1):3166. DOI 10.1038/s41598-017-03461-x.

23. Wu Y, Han Y, Wong YS, et al., 2018, Fibre-based Scaffolding Techniques for Tendon Tissue Engineering. *J Tissue Eng Regen Med*, 12(7):1798-821. DOI 10.1002/tcrm.2701.

24. Li XR, Liang H, Sun J, et al., 2015, Electrospun Collagen Fibers with Spatial Patterning of SDF1α for the Guidance of Neural Stem Cells. *Adv Healthc Mater*, 4(12):1869-76. DOI 10.1002/adhm.201500271.

25. Yao R, Zhang R, Lin F, et al., 2013, Biomimetic Injectable HUVEC-adipocytes/Collagen/Alginate Microsphere cocultures for Adipose Tissue Engineering. *Biotechnol Bioeng*, 110(5):1430-43. DOI 10.1002/bit.24784.

26. Yao R, Zhang R, Lin F, et al., 2012, Injectable Cell/Hydrogel Microspheres Induce the Formation of fat Lobule-like Microtissues and Vascularized Adipose Tissue Regeneration. *Biofabrication*, 4(4):45003. DOI 10.1088/1758-5082/4/4/045003.

27. Jayasinghe SN, Townsend-Nicholson A, 2006, Stable Electric-field Driven Cone-jetting of Concentrated Biosuspensions. *Lab Chip*, 6(8):1086-90. DOI 10.1039/b606508m.

28. Xie YB, Castracane J, 2009, High-Voltage, Electric Field-Driven Micro/Nanofabrication for Polymeric Drug Delivery Systems Electrostatic Techniques and Biomedical Application. *IEEE Eng Med Biol*, 28(1):23-30. DOI 10.1109/memb.2008.931013.

29. Richardson TP, Peters MC, Ennett AB, et al., 2001, Polymeric System for Dual Growth Factor Delivery. *Nat Biotechnol*, 19(11):1029-34. DOI 10.1038/nbt1029-1029.

30. Chui CY, Bonilla-Brunner A, Seifert J, et al., 2019, Atomic Force Microscopy-indentation Demonstrates that Alginate Beads are Mechanically Stable Under Cell Culture Conditions. *J Mech Behav Biomed Mater*, 93:61-9. DOI 10.1016/j.jmbbm.2019.01.019.

31. Augst AD, Kong HJ, Mooney DJ, 2006, Alginate Hydrogels as Biomaterials. *Macromol Biosci.*, 6(8):623-33. DOI 10.1002/mabi.20060069.

32. Uludag H, De Vos P, Tresco PA, 2000, Technology of
Mammalian Cell Encapsulation. *Adv Drug Deliver Rev*, 42(1-2):29-64. DOI 10.1016/s0169-409x(00)00053-3.

33. Glicklis R, Shapiro L, Agbaria R, *et al.*, 2000, Hepatocyte Behavior within Three-dimensional Porous Alginate Scaffolds. *Biotechnol Bioeng*, 67(3):344-53. DOI 10.1002/(sici)1097-0290(20000205)67:3<344::aid-bit11>3.0.co;2-2.

34. Dhoot NO, Tobias CA, Fischer I, *et al.*, 2004, Peptide-modified Alginate Surfaces as a Growth Permissive Substrate for Neurite Outgrowth. *J Biomed Mater Res A*, 71(2):191-200. DOI 10.1002/jbm.a.30103.

35. Mosahebi A, Wiberg M, Terenghi G, 2003, Addition of Fibronectin to Alginate Matrix Improves Peripheral Nerve Regeneration in Tissue-engineered Conduits. *Tissue Eng*, 9(2):209-18. DOI 10.1089/ten.2003.9.209.

36. Prang P, Muller R, Eljaouhari A, *et al.*, 2006, The Promotion of Oriented Axonal Regrowth in the Injured Spinal Cord by Alginate-based Anisotropic Capillary Hydrogels. *Biomaterials*, 27(19):3560-9. DOI 10.1016/j. biomaterials.2006.01.053.

37. Nerem RM, Seliktar D, 2001, Vascular Tissue Engineering. *Annu Rev Biomed Eng*, 3:225-43. DOI 10.1146/annurev. bioeng.3.1.225.

38. Weinberg CB, Bell E, 1986, A Blood-vessel Model Constructed from Collagen and Cultured Vascular Cells. *Science*, 231(4736):397-400. DOI 10.1126/science.2934816.

39. Krock BL, Skuli N, Simon MC, 2011, Hypoxia-induced Angiogenesis: Good and Evil. *Genes Cancer*, 2(12):1117-33. DOI 10.1177/1947619111423654.

40. Ferrara N, Gerber HP, LeCouter J, 2003, The Biology of VEGF and its Receptors. *Nat. Med*, 9(6):669-76. DOI 10.1038/nm0603-669.

41. Hauser S, Jung F, Pietzsch J, 2017, Human Endothelial Cell Models in Biomaterial Research. *Trends Biotechnol*, 35(3):265-77. DOI 10.1016/j.tibtech.2016.09.007.

42. Laschke MW, Harder Y, Amon M, *et al.*, 2006, Angiogenesis in Tissue Engineering: Breathing Life into Constructed Tissue Substitutes. *Tissue Eng*, 12(8):2093-104. DOI 10.1089/ten.2006.12.2093.

43. Laschke MW, Menger MD, 2012, Vascularization in Tissue Engineering: Angiogenesis Versus Inosculation. *Eur Surg Res*, 48(2):85-92. DOI 10.1159/000336876.

44. Ledoux S, Queguiner I, Msika S, *et al.*, 2008, Angiogenesis Associated With Visceral and Subcutaneous Adipose Tissue in Severe Human Obesity. *Diabetes*, 57(12):3247-57. DOI 10.2337/db07-1812.

45. Koike N, Fukumura D, Gralla O, *et al.*, 2004, Creation of Long-lasting Blood Vessels. *Nature*, 428(6979):138-9. DOI 10.1038/428138a.