RESEARCH ARTICLE

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 roles 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 Seftonin 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[27]. 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[28]. 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

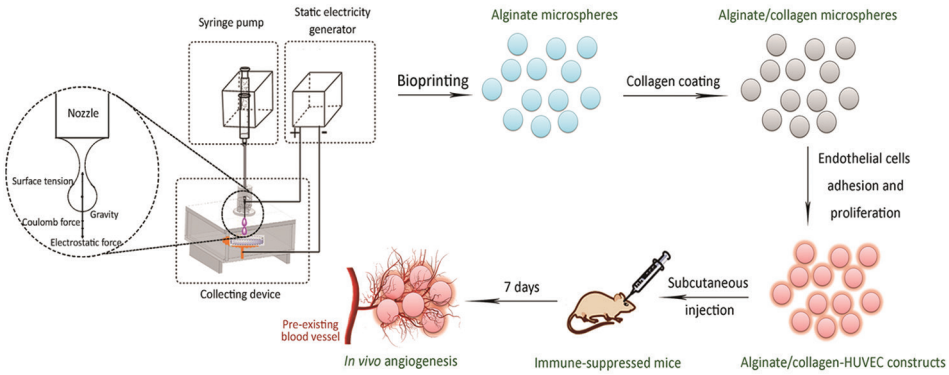

Figure 1. Schematic presentation of this study. Alginate microspheres with tunable properties were fabricated through the electro-assisted inkjet printing technology and coated with different concentrations of collagen fibrils. Human umbilical vein endothelial cells (HUVECs) adhered and proliferated 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.

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

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% CO_2 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 10^6 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-TGCACCACCAACTGCTTAGC, reverse-GGCATGGACTGTGGTCATGAG; CD31, forward-AACAGTGTTGACATGAAGAGCC, reverse-TGTAAAACAGCACGTCATCCTT; VE-Cadherin, forward-TTGGAACCAGATGCACATTGAT,reverse-TCTTGCGACTCACGCTTGAC; vWF, forward-CCGATGCAGCCTTTTCGGA, reverse-TCCCCAAGATACACGGAGAGG; hypoxia-inducible factor1a (HIF1a), forward-GCCGCTGGAGACACAATCAT,reverse-TCTGTGTCGTTGCTGCCAAA; VEGF, forward-AGGAGGAGGGCAGAATCATC, reverse-GGCACACAGGATGGCTTGAA; VEGFR,forward-CAGAAGGGCTCTGTGGAAAGT, reverse-GAGGTTCCTTGAACAGTGAGGTAT. 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-HUVECs 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 ×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[29]. 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[18]. 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.*[30]. This property made the microsphere modules especially favorable for bioengineering construct formation and further minimal-invasive applications.

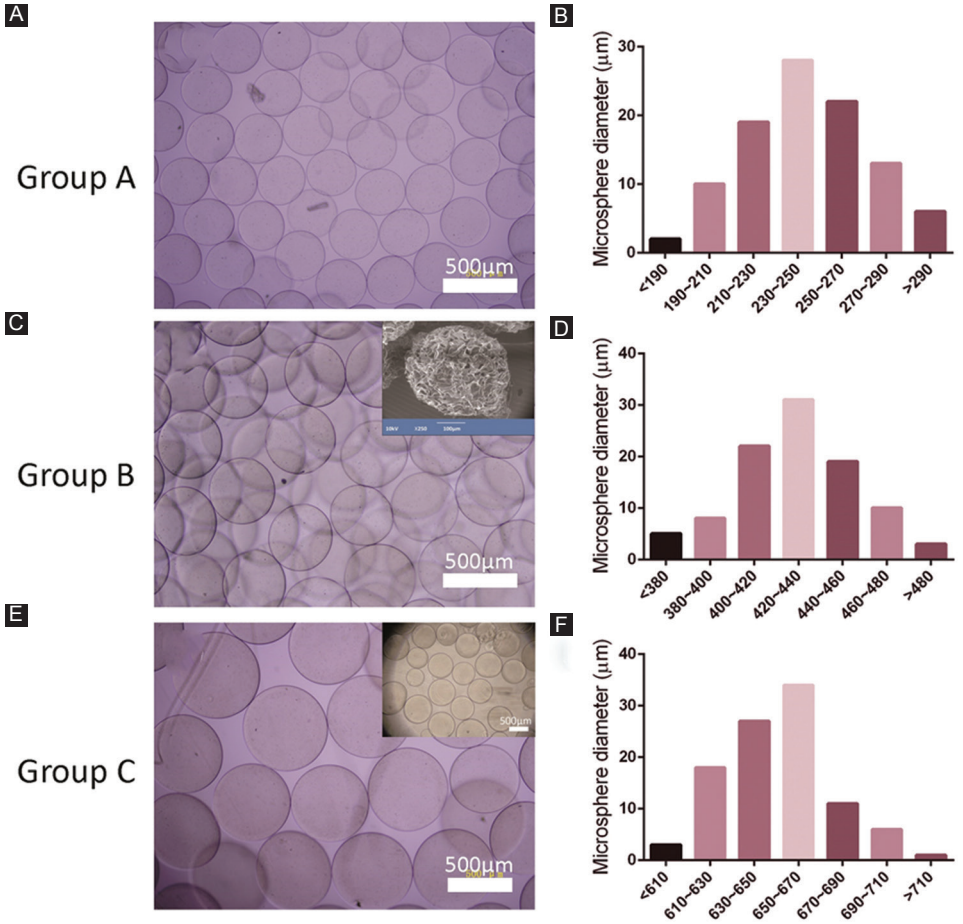

Figure 2. Characteristics of three groups of alginate microspheres. (**A**) Morphology of Group A alginate microspheres. (**B**) Diameter distribution of Group A alginate microspheres. (**C**) Morphology of Group B alginate microspheres. The magnified image showed the microstructure of microspheres examined 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.

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[31,32]. Furthermore, alginate has been approved by the regulatory authorities for applications such as wound dressing, dental impression, and as a food supplement[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[34], fibronectin[35], and collagen[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[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).

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[38]. 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 pores 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 immunofluorescence 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[2]. HIF1a, a member of the HIF family, regulates a wide range of angiogenic genes under hypoxic condition, including induction of VEGF expression[39]. 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

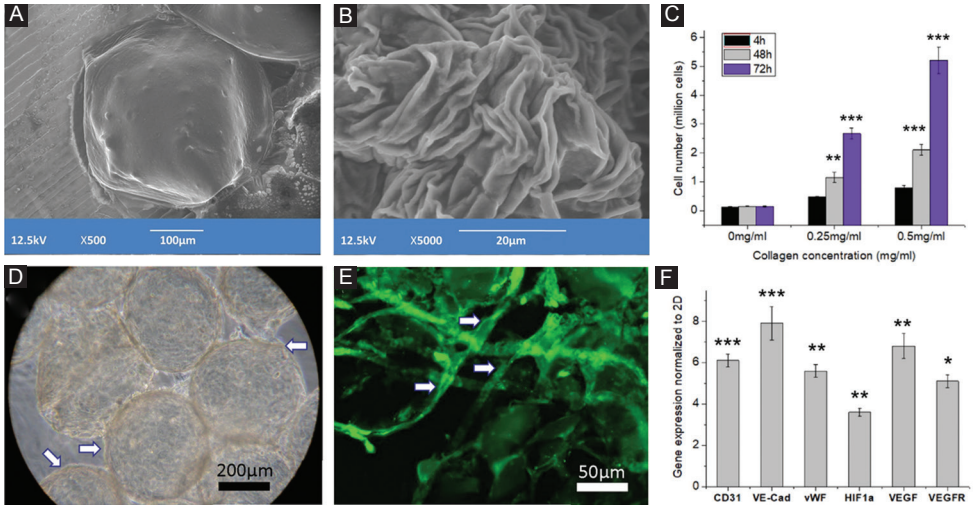

Figure 3. Collagen coating, human umbilical vein endothelial cells (HUVECs) seeding, and *in vitro* prevascularized tissue formation of Group B microspheres. (**A**) scanning electron microscopy (SEM) examination showing overall morphology of alginate/collagen microspheres. (**B**) Magnified SEM image showing collagen fibril coating on the alginate microspheres. (**C**) HUVECs adhesion and proliferation of alginate/collagen microspheres determined by cell viability testing. Data of test groups were compared with the control group where no collagen was added. (**D**) Optic microscope image of alginate/collagen-HUVECs constructs 72 h after HUVECs seeding, where arrows indicate HUVECs on the microsphere surface. (**E**) Anti-human PECAM-1/CD31 immunofluorescence staining demonstrating prevascularized tissue formation 72 h after HUVECs seeding, where arrows indicated lumen-like HUVECs structure. (**F**) Endothelial gene (CD31, VE-Cadherin, vWF, hypoxia-inducible factor1a, vascular endothelial growth factor [VEGF], VEGF-releasing) expression of HUVECs in the three-dimensional construct compared with 2D culture. *indicates $P<0.5$, **indicates $P<0.01$, ***indicates $P<0.001$.

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/mm^2. This number decreased with the increase of microsphere diameter, with Group C constructs regenerated about 31 microvessels/mm^2. Both control groups showed little new blood vessel ingrowth, with vessel density of about 4.4 and 3.3/mm^2, 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[42]. 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

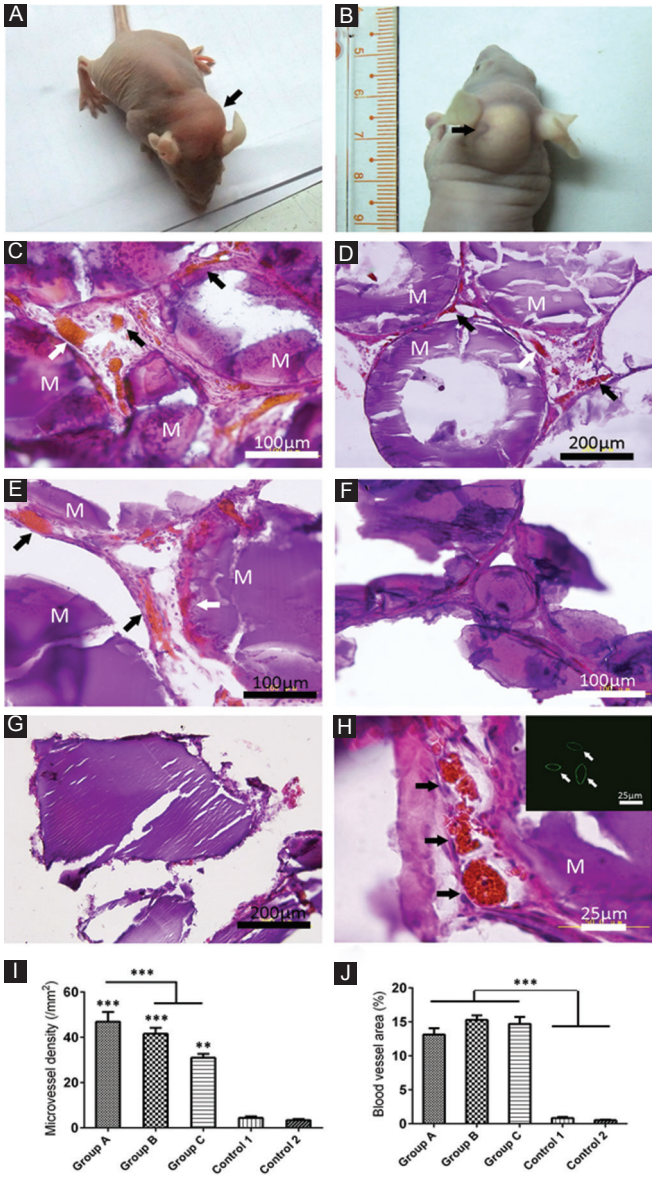

Figure 4. Subcutaneous injection and *in vivo* angiogenesis. (**A**) The appearance of immune-deficient mice immediately after injection. The black arrow indicates the injection site. (**B**) The appearance of injection site 7 days after injection. The black arrow indicates blood vessel ingrowth into 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 $P<0.001$.

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[43]. Our practice utilized the inosculation strategy, by constructing prevascularized hydrogel microspheres *in vitro* and then performed *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

integrate the microspheres into a bulk structure, i.e., by hydrogel embedding, to facilitate minimally invasive injection *in vivo*, 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/mm^2 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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