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Vascular-like network prepared using hollow hydrogel microfibers

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One major challenge in the field of tissue engineering was the creation of volumetric tissues and organs *in vitro*. To achieve this goal, the development of a three-dimensional vascular-like network that extended throughout the tissue-engineered construct was essential to supply sufficient oxygen and nutrients to all of the cells in the constructs. For sufficient oxygenation and nutrition of the tissue-engineered constructs, the distance between each microvessel-like channel in the network should ideally be within 100–200 μm. In addition, the medium or blood should be perfused through the microchannels as soon as possible after the seeding of cells into the templates (scaffolds) of the constructs. In the present study, we proposed a novel technique for fabricating an engineered vascular-like network that satisfied these two requirements. The network comprised assembled hollow alginate hydrogel microfibers with mammalian cells enclosed in the gel portions. We controlled the distance between each flow microchannel (hollow core portions and interspace of the microfibers) to be within 150 μm by using microfibers with a gel thickness of approximately 50 μm. Furthermore, we confirmed that medium could be perfused into the flow channels quickly (within 10 min) after immobilization of the cells in the assembly. A human hepatoblastoma cell line (HepG2) proliferated in the gel portions of the microfibers and maintained their specific function during perfusion culture for 7 days. These results showed that the novel vascular-like networks fabricated here had the potential to allow the creation of volumetric tissues *in vitro*.

[**Key words:** Hollow fiber; Vasculature; Alginate; Organ; Tissue engineering]

Recent advances in the field of tissue engineering have enabled the *in vitro* creation of thin tissues and those with low oxygen demands such as skin and cartilage (1,2). The next goal is the creation of volumetric tissues and organs *in vitro*. To achieve it, the development of a three-dimensional vascular-like network throughout the constructs is essential for supplying oxygen and nutrients to all of the cells in the constructs. Without such a network, the cells in the distal regions of the constructs inevitably undergo necrosis caused by insufficient delivery of oxygen and nutrients to the cells by passive diffusion from the surrounding environment, which limits the creation of thick tissues. To provide sufficient oxygen and nutrients to the tissue-engineered constructs, the distance between each microvessel-like channel in the network should ideally be within 100–200 μm (3,4). A second important requirement for the creation of volumetric tissues *in vitro* is the time until perfusion. Mature artificial tissues should be created as quickly as possible to be more useful to patients. To reduce the maturation time, cells are seeded into the templates (scaffolds) of the artificial tissues at a high cell density. To keep these seeded cells alive, medium or blood should be perfused into the microchannels as soon as possible after seeding. Although several techniques (e.g., those using microfabricated channels, decellularized tissue matrices, the spontaneous tube-forming activity of vascular endothelial cells, and sacrificial materials) have been reported for the fabrication of vascular-like networks (5–14), to the best of our knowledge, there are no methods that satisfy both of the requirements.

In the present study, we propose a novel vascular-like network that (i) can be perfused with liquid quickly after the seeding of cells and (ii) has a distance between channels of less than 150 μm. One key material for preparation of the network is assembled hollow alginate hydrogel microfibers that enclose mammalian cells (Fig. 1A). We used both the hollow core portions and the interspace of the fibers as flow channels for the medium and blood. The fabrication procedure is detailed as follows. The cell-enclosing hollow alginate microfibers with a gel thickness of approximately 50 μm are prepared, and then the fibers are assembled into bundles. In the bundles, the distance between each flow channel (hollow core portions and the interspace of the fibers) would be less than 150 μm. Strict control of diameter of the microfibers is essential to achieve this distance. Furthermore, the rapid formation of the hollow microfibers is also necessary to allow liquid perfusion into the flow channels soon after bundle preparation. We have already developed techniques to fabricate both solid-core and hollow-core alginate microfibers, based on the liquid–liquid co-flowing stream technique, and we have applied those materials to

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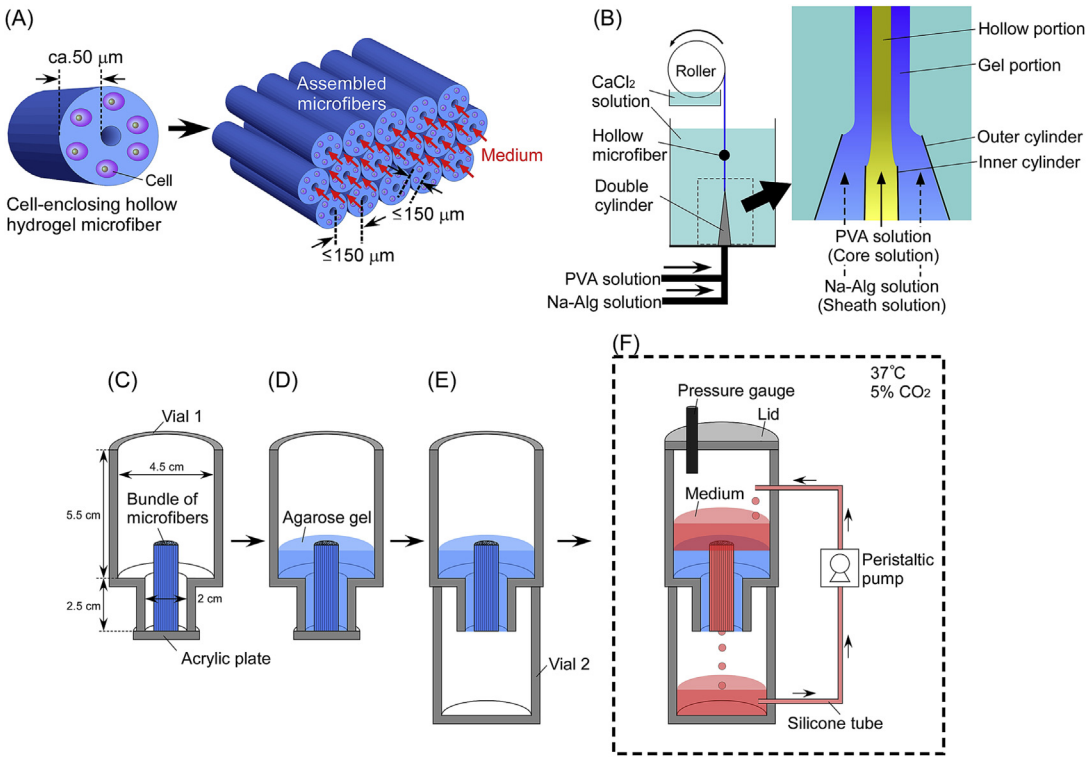

FIG. 1. (A, B) Schematics showing the fabrication of the engineered vascular-like networks (A) and microfiber formation (B). (C–F) The setup for perfusing media into a bundle of microfibers seeded with HepG2 cells.

creation of tissue-engineered constructs (10,15–17). In those reports, we demonstrated that the size of the hollow fibers could be strictly controlled and that the fibers were formed rapidly, while avoiding microfiber tangling during the fiber preparation process (15,17).

The aim of this study was to evaluate the feasibility of our method. Specifically, we first optimized the preparation conditions for the microfibers to fabricate them with a gel thickness of approximately 50 μm. Next, we examined whether liquid could be perfused into the flow channels soon after preparation of the bundles. Finally, proliferation of the cells immobilized in the gel portion of the fibers and the cellular albumin expression were examined during perfusion culture.

MATERIALS AND METHODS

Materials Sodium alginate (Na-Alg; Kimica I-1G; molecular weight: 70,000; glucuronic acid residues: 61%) was kindly donated by Kimica Co. (Tokyo, Japan). Poly (vinyl alcohol) (PVA; degree of saponification: ≥96%; degree of polymerization: 400–600) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Agarose (SeaPlaque agarose) was obtained from Cambrex Bio Science Rockland Inc. (Rockland, ME, USA). Porcine gelatin (type A) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). A human albumin ELISA kit was purchased from Bethyl Laboratories (Montgomery, TX, USA). A human hepatoblastoma cell line (HepG2, RCB1648) was purchased from the Riken Cell Bank (Tsukuba, Japan). The cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 incubator.

Preparation of hollow alginate hydrogel microfibers Hollow microfibers were prepared using our previously reported technique (Fig. 1B) (17). Briefly, a coaxial double cylinder was constructed from a stainless steel needle cut perpendicular to its axial direction (inner cylinder, inner diameter: 410 μm) and a tapered plastic tube (outer cylinder, tip inner diameter: 610 μm). The double cylinder was placed at the bottom of a bath containing a 100 mM $CaCl_2$ solution. Aqueous solutions of 10% (w/v) PVA (core solution) and 1% (w/v) Na-Alg (sheath solution) were simultaneously extruded into the $CaCl_2$ solution from the inner and outer cylinders using syringe pumps, respectively. The total volumetric flow rate of the core and sheath solutions was constant at 2.0 mL/min. Only the sheath solution rapidly gelled through the uptake of Ca^{2+} from the $CaCl_2$ solution. The resultant hollow microfibers were collected with a roller. The pulling rate of microfibers by the roller (=rotation speed of the roller) was fixed to 28.8 cm/s. The microfibers were then incubated in a large volume of a 100 mM $CaCl_2$ solution for 24 h to induce further gelation. The mean inner and outer diameters of the microfibers were measured on approximately 100 randomly-selected samples using an optical microscope (DM IL LED, Leica, Tokyo, Japan).

The hollow microfibers with enclosed HepG2 cells were prepared as follows. The cells collected from cell culture dishes by trypsinization were passed through a filter (mesh size: 40 μm, Corning Inc., NY, USA) for removal of large cell aggregates. The cells were added to a solution of 1% (w/v) Na-Alg in a calcium-free Krebs–Ringer HEPES-buffered solution (CF-KRH; pH 7.4, 1.0×10^6 cells/mL) and carefully dispersed for preparation of uniformly cell-laden microfibers. The microfibers were prepared using the Na-Alg solution, a solution of 10% (w/v) PVA in CF-KRH, and a solution of 100 mM $CaCl_2$ with 10 mM HEPES (pH 7.4, 4°C) under otherwise identical conditions. Bundles of 200 microfibers (length: 3.5 cm; 3.8×10^5 cells/bundle) were collected by cutting the microfibers on the roller.

Perfusion of medium A bundle of 200 microfibers was placed on an acrylic plate set at the bottom of an acrylic vial (vial 1) (Fig. 1C). A 35-mL solution of 2% (w/v) agarose in CF-KRH at 30°C was poured into vial 1 and cooled to 4°C to induce gelation (Fig. 1D). The acrylic plate placed at the bottom of vial 1 was then removed, and vial 1 was placed on top of another acrylic vial (vial 2) (Fig. 1E). DMEM (20 mL) containing 10% (v/v) FBS was poured onto the agarose gel in Vial 1, which was then completely sealed with an acrylic lid. Vials 1 and 2 were connected by silicone tube and placed in an incubator (37°C, 5% CO_2) (Fig. 1F). The medium in vial 2, which fell in drops from vial 1 through the microfiber bundle, was returned to vial 1 via the tube that passed through a peristaltic pump. The flow rate of the medium in the bundle was maintained constant at 0.6 mL/min by increasing the pressure in vial 1 (pressure gauge: PM-281; As One Co., Tokyo, Japan). The pressure was increased by returning the medium to the completely-sealed vial 1. The perfusion of medium through the bundle was continued for 7 days. During the perfusion, the medium was not replaced with a fresh medium. Small samples of medium were collected at predetermined intervals. The concentration of albumin in the samples was determined using a human albumin ELISA kit. The bundle was removed from vial 1 and fixed in a 10% neutral-buffered formalin solution. The bundles were then embedded in paraffin, sectioned at 90° to their axial direction (≥3 sections per bundle), and stained with hematoxylin and eosin (H&E). The cell numbers in the bundles were calculated based on the ratio of cellular area to bundle area in the H&E-stained slices.

Statistical analysis Data are presented as mean ± standard deviation. Student's unpaired *t*-tests were used to test the significance of differences between two groups.