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Cellular building unit integrated with microstrand-shaped bacterial cellulose

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ABSTRACT

In bottom-up tissue engineering, a method to integrate a pathway of nutrition and oxygen into the resulting macroscopic tissue has been highly desired, but yet to be established. This paper presents a cellular building unit made from microstrand-shaped bacterial cellulose (BC microstrand) covered with mammalian cells. The BC microstrands are fabricated by encapsulating *Acetobacter xylinum* with a calcium alginate hydrogel microtube using a double co-axial microfluidic device. The mechanical strength and porous property of the BC microstrands can be regulated by changing the initial density of the bacteria. By folding or reeling the building unit, we demonstrated the multiple shapes of millimeter-scale cellular constructs such as coiled and ball-of-yarn-shaped structures. Histological analysis of the cellular constructs indicated that the BC microstrand served as a pathway of nutrition and oxygen to feed the cells in the central region. These findings suggest that our approach facilitates creating functional macroscopic tissue used in various fields such as drug screening, wound healing, and plastic surgery.

1. Introduction

The fabrication of functional macroscopic tissues with high cellular density has been a target of tissue engineering [1–4]. A major approach to construct such tissues is one based on bottom-up tissue engineering that creates tissues by assembling building units composed of cells and other biomaterials [1,5]. However, the resulting macroscopic tissue often suffers cell death in the central region of the tissue; one of the reasons is that the building units lack a pathway of nutrition and oxygen.

In this work, we present a cellular building unit that is made from a microstrand-shaped porous nanofibrous scaffold covered with cells. This building unit can be transformed into millimeter-scale cellular constructs such as coiled and ball-of-yarn-shaped structures (Fig. 1). In the constructs, the microstrand-shaped scaffold serves as a pathway that facilitates the diffusion of biologically essential substances into the central region. Consequently, nutrition and oxygen reach the cells in the central region of the macroscopic cellular constructs to keep them alive.

As the microstrand-shaped scaffold, we use bacterial cellulose (BC), which is a nanofibrous cellulosic material produced by the bacteria, *Acetobacter xylinum* (*A. xylinum*). This material has been suggested to be suitable as a scaffold because 1) BC supports cell adherence and growth [6–10], 2) BC has sufficient mechanical strength and stability to retain its structure during cell cultivation [7,11], 3) BC has interconnected pores allowing the diffusion of molecules through the scaffold [12,13], 4) BC is not degradable by mammalian cells, and 5) BC shows high biocompatibility [12,14]. To fabricate microstrand-shaped BC, we use a double co-axial microfluidic device (Fig. 1A and B) and form a core–shell microfiber in which the bacteria are encapsulated within the core covered with the shell of a calcium alginate hydrogel. After the formation of the BC microstrand by the encapsulated bacteria, we remove the calcium alginate gel and the bacteria to purify the BC microstrand (Fig. 1C–E). Cells are then seeded on BC microstrands to produce cellular building units that can be reeled or folded into millimeter-scale cellular constructs (Fig. 1F–H). Here, we first measure mechanical strength, density of cellulose nanofibrils, and cell affinity of the BC microstrands fabricated by our system. We then examine the potency of our cellular building unit to form various

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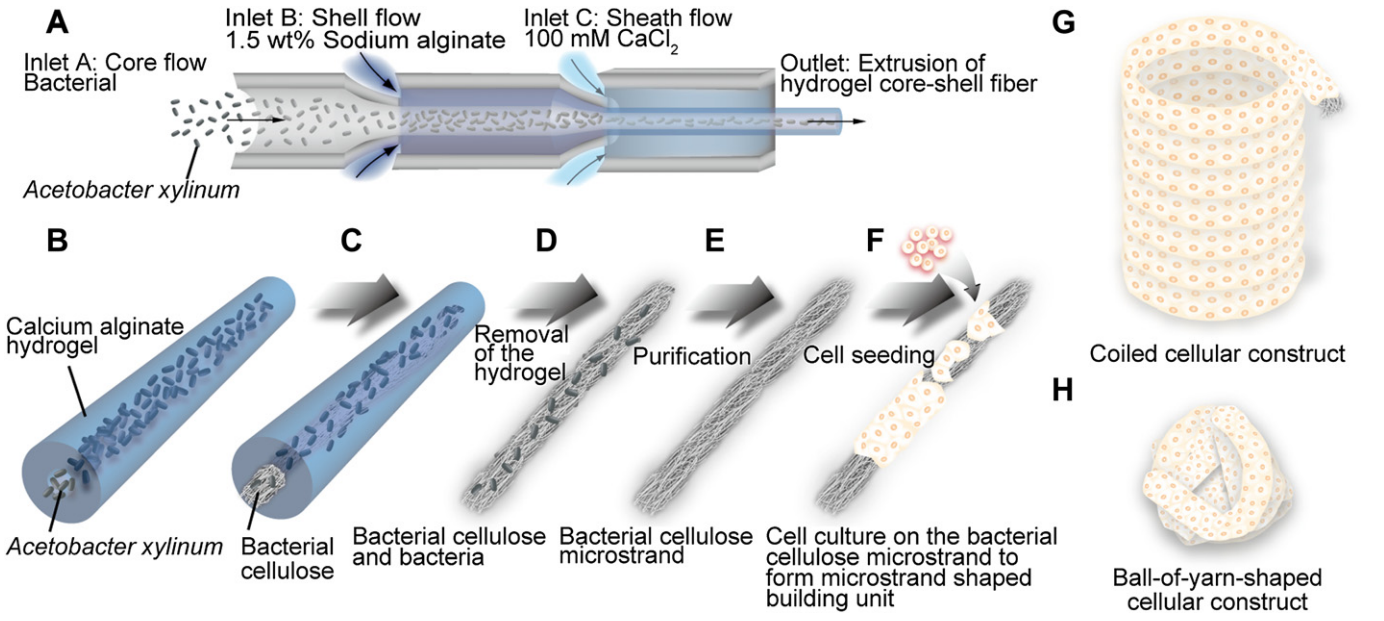

Fig. 1. Schematic illustration of the process to fabricate BC microstrands and BC microstrand-based cellular constructs. (A) Fabrication process of a bacterial/alginate core–shell hydrogel fiber. The bacteria encapsulating calcium alginate hydrogel fiber is rapidly fabricated by a double co-axial laminar flow device. (B) The bacterial suspension is confined within the core of bacteria/alginate hydrogel core–shell fiber. The bacteria are allowed to produce BC within the core by culturing the hydrogel fiber. (C) The bacteria form cellulose network within the core of the hydrogel fiber. (D) Calcium alginate hydrogel is dissolved by citric acid solution. BC maintain its structure without the shell. (E) The BC microstrands are obtained after the removal of the bacteria by NaOH treatment. (F) NIH-3T3 cells are seeded onto the BC microstrand. (G, H) The BC microstrand-based building units are able to be transformed into a coiled cellular construct (G) and a ball-of-yarn-shaped cellular construct.

shapes of macroscopic cellular constructs having a pathway that feeds the cells even in the central region with nutrition and oxygen.

2. Materials and methods

2.1. Bacterial strains and growth medium

The bacterial strain we used was *A. xylinum* subsp. sucrofermentas BPR2001 provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The bacteria were pre-cultured on plates composed of 50.0 g/l glucose (10017-00, Kanto Chemical, Japan), 12.5 g/l $CaCO_3$ (Kanto Chemical), 5.0 g/l yeast extract (Becton, Dickinson and Company, USA), and 1.5 wt% agar (Wako Pure Chemical Industries, Japan). For production of BC microstrands, the bacteria were cultured in 20 ml/l corn steep liquor (CSL, Showa Sangyo Co., Ltd, Japan), 80 g/l glucose, 1.0 g/l KH_2PO_4 (Wako), 3.3 g/l $(NH_4)_2SO_4$ (Wako), 0.25 g/l $MgSO_4{\cdot}7H_2O$ (Nacalai tesque, Japan), and 1.11 g/l $CaCl_2$ (Wako) dissolved in deionized water. The pH of the growth medium was adjusted to 6.0. The density of the bacterial cell was calculated as colony formation unit (CFU). CFU was determined as followed: the bacterial suspensions were serially diluted 10^4-fold with the growth medium, and 100 μl of the diluted solutions was spread over the culture plates. The culture plates were incubated at 37 °C for 4 days and the numbers of colonies were counted to calculate the original concentration of the bacteria.

2.2. Double co-axial laminar flow microfluidic device

The device was fabricated as previously described [15,16]. A photograph of the device is shown in Fig. S1. Briefly, the device is constituted from glass tubes and connectors. Two cylindrical glass tubes (outer diameter: 1.0 mm; inner diameter: 600 μm, Narishige, Japan) were pulled using a puller (PC-10, Narishige) and the tip of the glass tube was adjusted to about 230 μm and 450 μm in diameter. The pulled glass tubes were inserted to a square glass tubes (outer diameter: 1.4 mm, inner diameter: 1 mm, Vitrocom Inc., NJ, USA) to align the central axis of the two glass tubes. The pulled glass tubes with the tip diameter of 230 μm functioned as the first channel that guide the core flow and the other pulled glass tube functioned as the second channel that guide the shell flow (Fig. 1A).

2.3. Fabrication of BC microstrands

The bacterial suspensions for the fabrication of BC microstrands were prepared as follows; colonies of *A. xylinum* formed on the plates were collected into deionized water, mixed well by pipetting, and then filtered through a membrane (pore size: 5 μm). The bacterial suspension, 1.5 wt% sodium alginate solution, 100 mM $CaCl_2$ solution were introduced into the double co-axial microfluidic device from the inlet A, B, and C at a flow rate of 75 μl/min, 250 μl/min, and 3.6 ml/min, respectively (Fig. 1A). The fabricated core–shell fibers were cultured in the culture medium statically at 30 °C for 24 h to produce bacterial cellulose in the core and autoclaved (120 °C, 15 min, 1 bar) to stop culturing. To purify the BC microstrands, the shell of the calcium alginate gels was dissolved by immersing into 100 mM citric acid solution (Wako) and then treated with 1 M NaOH solution at 75 °C for 3 h.

2.4. Scanning electron microscopy

To obtain scanning electron microscope (SEM) images, BC microstrands were lyophilized using freeze drier (FDU-1200, Eyela, Japan). The lyophilized samples were coated with Osmium using HPC-30W plasma coater machine (Vacuum Device Inc., Japan). The SEM images were captured using SU8000 (Hitachi High-Technologies Corporation, Japan) operating at 15 kV.

2.5. Analysis of average distance between cellulose nanofibrils

The SEM images of BC microstrands were analyzed to obtain average distance between cellulose nanofibrils by using Image J software (National Institute of Health, USA). The average distance between cellulose nanofibrils was calculated from the intensity profiles of the binarized SEM images. We applied "Remove Outliers" option prior to binarization in order to reduce noises in the SEM images. The SEM images obtained at ×2000 magnification were binarized with dark background using auto-threshold option. In this way, the distance between the intensity peaks corresponds to the distance between cellulose nanofibrils. The intensity profile was obtained along a line parallel to the horizontal axis of the image. The average distance for each condition was calculated as a mean value of 27 intensity profiles in randomly selected 9 areas of the images from three independent samples.

2.6. Analysis of tensile strength

We measured the tensile strength of BC microstrands as previously described [17]. Briefly, we used a glass capillary (quarts, inner diameter: 0.6 mm, outer diameter: 0.62 mm, length: 70 mm, Quarzkapillaren, Glas Müller, Germany) as a force sensing cantilever and a glass tube (inner diameter: 0.6 mm, outer diameter: 1.0 mm, G-1, Narishige, Japan) to fix and pull the one end of BC microstrand. The cantilever and the glass tube were connected to manipulators, and placed in parallel to each other. Each end of a BC microstrand was fixed to the cantilever and the glass tube perpendicularly by glue (Cyanoacrylate-based adhesive, Aronalpha, TOAGOUSEI, Japan). The fixed BC microstrand was pulled by controlling the manipulator of the glass tube. All of these experiments were performed in water. For detailed description, the force applying to the BC microstrand, F, was expressed as $F = k\,\Delta x$,