Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–8, 2018

www.elsevier.com/locate/jbiosc

Development of a perfusable 3D liver cell cultivation system via bundling-up assembly of cell-laden microfibers

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Received 12 November 2017; accepted 31 January 2018
Available online xxx

Although the reconstruction of functional 3D liver tissue models *in vitro* presents numerous challenges, it is in great demand for drug development, regenerative medicine, and physiological studies. Here we propose a new approach to perform perfusion cultivation of liver cells by assembling cell-laden hydrogel microfibers. HepG2 cells were densely packed into the core of sandwich-type anisotropic microfibers, which were produced using microfluidic devices. The obtained microfibers were bundled up and packed into a perfusion chamber, and perfusion cultivation was performed. We evaluated cell viability and functions, and also monitored the oxygen consumption. Furthermore, fibers covered with vascular endothelial cells were united during the perfusion culture, to form vascular network-like conduits between fibers. The presented technique can structurally mimic the hepatic lobule *in vivo* and could prove to be a useful model for various biomedical research applications.

[**Key words:** Hepatocyte; Perfusion culture; Hydrogel; Alginate; Coculture; Vascular network; Tissue engineering]

Hepatocytes, liver parenchymal cells, play central roles in the liver and have many physiological functions such as drug metabolism, blood detoxification, and protein synthesis. Both primary hepatocytes and hepatoma cell lines including HepG2 and HepaRG cells, have widely been employed to predict drug efficacy and toxicity before conducting animal/human trials (1,2). For this purpose, new cultivation techniques for liver cells that improve hepatic functions and viability, and reproduce liver-specific physicochemical environments are of great importance in conducting experiments using models with physiologically relevant liver architectures.

Until now, researchers have proposed various approaches to culture hepatocytes *in vitro* in order to maintain hepatocyte function and viability. The collagen-gel sandwich method recaptures the microenvironments surrounding hepatocytes *in vivo*, ensuring the formation of both cell–matrix and cell–cell interactions. The preparation of multicellular spheroids using non-cell-adhesive culture dishes is a popular technique to generate 3D cellular clusters (3,4). Additionally, attempts to introduce collagen microparticles into clusters of hepatocytes or HepG2 cells have recently been reported (5,6). Heterotypic cell-coculture systems, such as cell micropatterning (7), formation of linear organoids in hydrogel fibers (8), and stacking of multiple cell sheets (9,10), have also been reported. These methods are advantageous as they are highly efficient in forming cell–cell interactions and capable of improving hepatic functions. However, in order to provide microenvironments that closely mimic physiological conditions, a culture system for liver cells that satisfies the following points is more desirable: (i) uniform and effective supply of oxygen and nutrients to the cells, even in high-density cell clusters; (ii) ability to dynamically tune dissolved oxygen (DO) concentration, as the expression of several important genes of liver cells is associated with partial oxygen pressure (11–13); (iii) hierarchical assembly of multiple types of cells as in the case of *in vivo* tissues; and (iv) accessibility to sufficient amounts of cell sources ($\sim 10^6$ cells) for biochemical analysis of the hepatocyte functions.

The use of perfusion cultures is a reasonable strategy to satisfy some of these requirements. Compared to static cultivation, perfusion cultures enable the efficient supply of oxygen and nutrients to the cells, as well as the rapid removal of wastes from the system. Additionally, it is also possible to precisely control the DO concentration simply by controlling the perfusion flow rates and/or the partial O_2 pressure at the inlet (14,15). Until now, microfluidic systems that employ porous membranes (16,17), micropillar arrays (18,19), and microwells (20,21) have been developed for perfusion cultivation of liver cells. Hollow fibers with encapsulated cells (22–24) and decellularized organs (25) have also been used for perfusion culture, enabling efficient molecular transport to the cells. These studies have demonstrated the high promise of perfusion culture platforms in reproducing the physiological behaviors of liver cells *in vitro*.

In our previous study, we developed a strategy to produce hepatic micro-organoids in hydrogel microfibers or sheets using microfluidic systems (8,26). Liver cells (primary hepatocytes or hepatoma cells) and non-parenchymal cells (fibroblasts) were encapsulated in the hydrogel matrices at high densities, in which the cell positions were precisely controlled. One of the most remarkable advantages of this liver cells culture process is its ability to align cells into linear regions within a width of several tens of micrometers, structurally mimicking the linear hepatocyte assemblies *in vivo*, called hepatic cords. We speculated that these

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1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.01.022

hydrogel fibers were particularly suitable as unit structures in creating large-sized perfusable liver tissue equivalents because such fibers could be easily bundled-up and packed in a perfusion chamber. Such a platform would enable the dynamic control of the culture environment of liver cells while achieving uniform and effective oxygen and nutrient delivery to the cells because the void spaces between the fibers could work as conduits. Moreover, vascularized tissues could be produced by preparing and uniting endothelial cell (EC)-covered fibers, forming a structure that highly resembled the hepatic lobule, which is the unit structure of the liver.

Based on this concept, we propose a new perfusion system for liver cell cultivation. Fig. 1 demonstrates the process of fabricating and assembling hydrogel microfibers. Sandwich-type barium alginate (BaAlg) hydrogel microfibers, in which HepG2 cells as model were encapsulated in the core, were prepared using a microfluidic laminar flow system. The obtained fibers were bundled up through the recovery using a roller and packed into a perfusion chamber, followed by perfusion cultivation. We investigated the effects of the perfusion flow rate on liver cell-specific functions and cell viabilities. Furthermore, HepG2 cells were co-cultured with vascular ECs to structurally mimic the 3D hepatic lobules and the sinusoidal structure of the liver.

MATERIALS AND METHODS

Cell culture HepG2 cells (human hepatocellular carcinoma cell line, obtained from RIKEN BRC, Ibaraki, Japan) and HH cells (bovine carotid artery normal endothelial cell, obtained from JCRB Cell Bank, Osaka, Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, MO, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, MA, USA), 100 unit/mL penicillin, and 0.1 mg/mL streptomycin (Sigma–Aldrich) in a CO_2 incubator (APM-30D; ASTEC, Fukuoka, Japan) at 37 °C with 5% CO_2 under a humidified atmosphere. Confluent cells were harvested using trypsin/EDTA (Sigma–Aldrich) and suspended in saline. The cell suspensions were filtered through a cell strainer (BD Biosciences, CA, USA) with a mesh size of 70 μm to remove larger cell aggregates before use in experiments.

Fabrication of the polydimethylsiloxane microfluidic device We fabricated two types of polydimethylsiloxane (PDMS) microfluidic devices; one to produce cell-laden microfibers (Fig. 2A), and the other for perfusion cultivation, using standard soft lithography and replica molding techniques (27). The widths of the inlet and gelation channels were 100 and 400 μm, respectively, and the length of the gelation channel was 50 mm. The depth of the entire channel was uniformly at 200 μm. For perfusion cultivation, a PDMS fluidic channel with a width and depth of 1 mm and a length of 15 mm was also designed and fabricated.

Preparation of the cell-laden hydrogel microfibers In this study, alginate polymer, conjugated with GRGDSP peptide (NaAlg; NOVATACH MVG GRGDSP, FMC Health and Nutrition, Philadelphia, PA, USA), was used for all experiments. HepG2 cells were suspended in an aqueous solution of 0.7% NaAlg supplemented with 0.9% NaCl (Wako Pure Chemical Industries, Osaka, Japan), 1% bovine serum albumin (BSA; Rockland, Limerick, PA, USA), and 10 mM HEPES (Thermo Fisher) at a concentration of 3×10^8 cells/mL. The same solution, with a higher concentration of NaAlg (1.0%), was used to prepare the shell regions of the sandwich-type fiber. The shell regions mechanically reinforce the fiber and support the core region with densely packed cells. An aqueous solution of 10% dextran (Mw of 450,000–650,000, obtained from *Leuconostoc* spp.; Sigma–Aldrich), 0.9% NaCl, and 10 mM HEPES was used as the buffer solution, whereas the same solution with 20 mM $BaCl_2$ (Wako) and a lower concentration of NaCl (0.72%) was used as the gelation solution. These solutions were continuously introduced into the microchannel using syringe pumps (KDS200; KD Scientific, Holliston, MA, USA). The flow rates of the alginate solution with HepG2 cells (Q_1), alginate solution for the shell region ($Q_2 = Q_2'$), buffer solution ($Q_3 = Q_3'$), and gelation solution ($Q_4 = Q_4'$) were 10, 12.5, 2.5, and 50 μL/min, respectively.

Bundling of cell-laden microfibers and perfusion cultivation Cell-laden hydrogel microfibers, formed in and extruded from the microfluidic device, were recovered using a roller with a diameter of ~55 mm, which was partially dipped in a bath containing an aqueous solution of 20 mM $BaCl_2$, 0.72% NaCl, and 10 mM HEPES. The rotation speed of the roller was ~35 rpm, with a corresponding recovery speed of ~6 m/min. On the roller, ~120 fibers were tied up at one point using a thin nylon wire to obtain a fiber bundle. After washing with HEPES-buffered saline, the bundle was packed in the open-air perfusion chamber. The open chamber packed with the bundle was sealed with a flat PDMS plate, and these two PDMS plates were tightly fixed by pressing them together using poly(methyl methacrylate) (PMMA) plates and stainless-steel jigs. The perfusion chamber was then put in a CO_2 incubator, and perfusion cultivation was performed. We used a cell culture medium with low serum concentration (1% FBS) to modulate the proliferation of HepG2 cells and enhance the liver-specific functions. This medium was continuously introduced into the perfusion chamber using a syringe pump at a constant flow rate of 1 or 15 μL/min.

To examine the flow distribution to the inter-fiber spaces, O.C.T. compound (Sakura Finetek, Tokyo, Japan) with blue-colored tracer particles (0.5 μm; FluoroMax Dyed Blue Aqueous Fluorescent Particles, Thermo Fisher, concentration of ~2×10^{10} particles/mL) was introduced into the perfusion chamber at a flow rate of 15 μL/min for ~10 min. HepG2 cells were stained with a green colored dye (PKH67 Green Fluorescent Cell Linker Kit, Sigma–Aldrich) beforehand. Frozen sections, with a thickness of 5 μm, were prepared using a cryostat (Leica CM1510S, Leica Biosystems, Wetzlar, Germany), and the distribution of the tracer particles was observed.

To form capillary networks between the fibers, we employed fibers covered with ECs (HH cells). ECs were stained red using the PKH26 Red Fluorescent Cell Linker Kit (Sigma–Aldrich) beforehand according to the manufacturer's protocol. HepG2 cell-

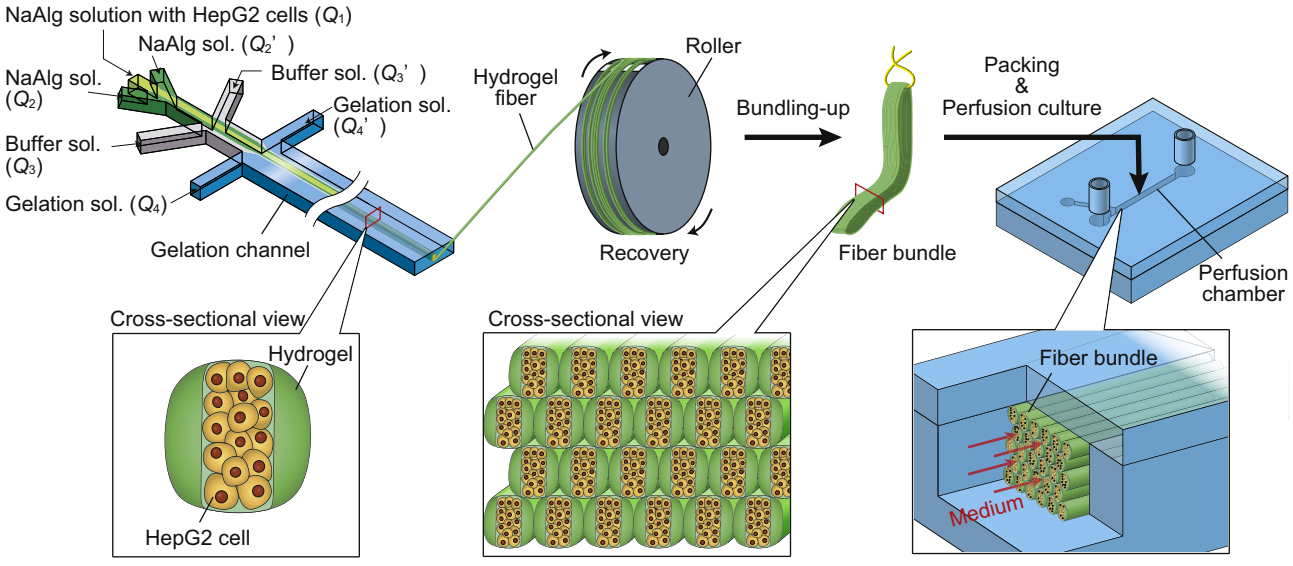

FIG. 1. Schematic showing the bundling-up assembly of the cell-laden hydrogel microfibers. Liver cell (HepG2 cell)-embedding microfibers were prepared using microfluidic devices. The microfibers produced were then recovered using a roller, and tied-up at one point to form a fiber bundle. The obtained bundle was packed into a perfusion chamber and perfusion cultivation was performed by introducing a cell culture medium.

Please cite this article in press as: Yajima, Y., et al., Development of a perfusable 3D liver cell cultivation system via bundling-up assembly of cell-laden microfibers, J. Biosci. Bioeng., (2018), https://doi.org/10.1016/j.jbiosc.2018.01.022