Sensors and Actuators B 198 (2014) 249–254

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Fabrication of microchannel networks in multicellular spheroids

Nobuhiko Kojima[a,b,c,*], Shoji Takeuchi[b,c], Yasuyuki Sakai[b,c]

[a] Graduate School of Nanobioscience, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan
[b] Institute of Industrial Science, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan
[c] Life BEANS Center, BEANS Project, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan

ARTICLE INFO

Article history:
Received 9 December 2013
Received in revised form 10 February 2014
Accepted 28 February 2014
Available online 19 March 2014

Keywords:
Alginate
Hepatocyte
Hydrogel
Liver
Microsphere
Microstructure

ABSTRACT

Multicellular spheroids are indispensable in cell biology as three-dimensional environments, in tissue engineering for achieving higher functions, and in drug validation by pharmaceutical companies. However, spheroids have limited nutrient exchange because they lack microvasculature. In this study, we established a method for fabricating microchannel networks in multicellular spheroids to overcome this limitation. Alginate hydrogel beads with almost the same size as animal cells were prepared by discharging alginate solution using an inkjet system. Using these beads, we produced "heterospheroids" that comprised the same number of cells and hydrogel beads based on the rapid cell/particle aggregation method. The hydrogel beads occupied the branched, connected spaces in the heterospheroids, which resembled microvasculature. The connectivity of the microchannel networks was confirmed using confocal laser microscopy and staining cells with 1-μm polystyrene beads after digesting the hydrogel beads using alginate lyase solution. The microchannel networks improved the albumin secretion rate and suppressed the expression of hypoxia-inducible factor-1α in Hep G2 cells. Experiments with rat primary hepatocytes demonstrated that the branched luminal-like structures overcame the limitations of albumin secretion and ammonia clearance. These findings suggest that it is possible to fabricate microchannel networks that can effectively maintain cellular functions by enhancing material exchange.

1. Introduction

Multicellular spheroids are useful tools for simulating cell behavior in a three-dimensional (3D) environment. They have been used to study chemo- and radiosensitivity of solid tumors [1], brain tumor invasion [2], and tumor angiogenesis [3]. Spheroid formation by hepatocytes has been demonstrated to maintain differentiated functions [4] and is more representative of the liver environment in vivo during the determination of drug toxicity [5]. In addition, spheroid formation with rat primary hepatocytes has been reported to reconstitute cell polarity and bile canaliculi structures [6].

Multicellular spheroids composed of hepatocytes have smooth surfaces enclosed by extracellular matrix and inner structures with tightly packed cells. Although they are well-organized tissues, the dense structures prevent the effective exchange of gas and nutrients from the outer environment. Thus, the insides of larger spheroids tend to undergo necrotic cell death because of limited supply of oxygen, glucose, and other substrates. The thickness of the viable rims of cells surrounding the necrotic centers of spheroids is 100–220 μm in most types of human tumor cells [1] because of limited supply of oxygen and/or nutrients, accumulation of lactate products, and low pH [7–9]. Several approaches have been developed to address these limitations. For example, agitation of suspended spheroids in a flask [10] or perfusion of 3D liver tissues in a device [11] have been used to enhance gas and nutrient exchange. Another option is to apply gas-permeable materials to culture dishes to improve the oxygen supply. The use of these materials has been reported to enhance hepatic functions (e.g., albumin secretion) in rat primary hepatocytes and the human hepatoma cell line Hep G2 [12,13]. Necrosis can also be prevented by dividing a spheroid into smaller compartments using porous fibers, which can supply oxygen and nutrients [14]. All of these approaches aim to improve the gas and nutrient supply by changing the surrounding environment of spheroids. However, because of the tight cell-to-cell adhesion in the spheroid, it is still difficult to expose the inner cells to physiologically active substances with higher molecular weight. Therefore, it is necessary to provide spheroids with inner spaces that facilitate the exchange of gas and nutrients.

* Corresponding author at: Graduate School of Nanobioscience, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan.
Tel.: +81 45 787 2214; fax: +81 45 787 2413.
E-mail addresses: nobuhiko@yokohama-cu.ac.jp, nobuhiko@iis.u-tokyo.ac.jp (N. Kojima).

http://dx.doi.org/10.1016/j.snb.2014.02.099
0925-4005/

We previously studied "spheroid engineering" using two types of rapid cell aggregation systems [15,16]. In particular, the aggregation method using a high molecular weight material medium (e.g., methylcellulose medium) allowed us to collect living cells and polystyrene particles that measured between 100 nm and 100 μm in diameter [16]. These results strongly suggested that any material that includes hydrogel beads can be aggregated to form hybrid multicellular spheroids. When we used the same number and size of cells and hydrogel beads, the alignment of the cells and gel beads had a similar appearance to an interlocked network. Relatively small nutrients/proteins (<20 kDa) can diffuse freely through alginate hydrogel beads [17,18]; therefore, they can be used as a "spacer" to reduce the cell density of the spheroid. Another study mentioned that alginate hydrogel membranes have an exclusion limit of 21–25 kDa for dextran and 78–103 kDa for protein [19]. Lanza et al. also concluded that alginate hydrogel microspheres are permeable to molecules with a molecular weight of ≥600 kDa, such as immunoglobulin G [20]. If necessary, the alginate hydrogel can be digested by alginate lyase [21], which would allow larger molecules to access the center of spheroids.

In the present study, we describe the production of hybrid spheroids of Hep G2, a human hepatoma cell line, cells and alginate hydrogel beads with a diameter of approximately 20 μm. Furthermore, we confirm the connectivity of the microchannel networks in engineered spheroids and discuss the functional differences between conventional and engineered spheroids.

2. Materials and methods

2.1. Cell line and rat primary hepatocytes

Hep G2 cell line was obtained from the Japanese Center Research Bank and grown in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Life Technologies, Carlsbad, CA, USA). Cells were maintained at a subconfluent density, thereby allowing recovery every 2 or 3 days. Hep G2 cells were stained with PKH67 (Sigma–Aldrich) as required. Cells were observed using a confocal laser (LSM 7 DUO, Carl Zeiss, Oberkochen, Germany) or fluorescence microscope (Axio Observer. A1, Carl Zeiss).

Rat primary hepatocytes were isolated from the liver of male Wister rats (Sankyo Lab Service, Tokyo, Japan), weighing 200–300 g, using the collagenase perfusion method, as previously described [22]. Cell pellets were resuspended in DMEM with 5% FBS, 1 mM dexamethasone (Wako, Osaka, Japan), 1× insulin–transferrin–selenium (BD Biosciences, San Jose, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). Viability was determined using the trypan blue exclusion method. We only used cells with viability >85%. Animal studies were conducted according to the protocol approved by the Animal Care Committee at the University of Tokyo.

2.2. Preparation of alginate hydrogel beads

To prepare alginate hydrogel beads, 1.5% sodium alginate solution (Sigma) was discharged into 5% calcium chloride (Sigma) solution using an inkjet system with 25, 40, or 60 μm-diameter nozzles (WaveBuilder and PulseInjector, Cluster Technology, Osaka, Japan). The beads were incubated in a FITC-conjugated poly-L-lysine (P3543, Sigma) solution to facilitate fluorescent labeling, as necessary.

2.3. Assembly and culture of heterospheroids composed of cells and hydrogel beads

We established a rapid aggregation system that allowed the simultaneous accumulation of 100 nm to 100 μm particles using 3% methylcellulose (MC; M0512, Sigma) medium [16]. The MC medium was poured into a Petri dish with a positive-displacement pipette (Microman; Gilson, Middleton, WI, US) because the 3% MC medium was highly viscous. The concentration of Hep G2 cells or alginate hydrogel beads in normal culture medium (without MC) was adjusted to 2×10^6 cells/ml or 2×10^6 beads/ml, respectively. We mixed equal volumes of cells and beads and injected 1 μl of the mixture into the MC medium to assemble heterospheroids that comprised cells and hydrogel beads.

2.4. Visualization of actin filaments and nuclei

To isolate spheroids from the 3% MC medium, we added 5 U/ml of cellulase solution (Onozuka RS; Yakult Pharmaceutical Industry, Tokyo, Japan) to the MC medium and incubated the mixture for 30 min at 37 °C to digest the high weight molecular structures of MC. These spheroids were fixed with 4% paraformaldehyde (PFA; Sigma) for 1 h at 4 °C and permeabilized with 0.025% saponin (Sigma) solution for 30 min at room temperature. After treatment with a blocking buffer (4% FBS in PBS) for 30 min at room temperature, we incubated the spheroids in a medium that included rhodamine-conjugated phalloidin (0.5 μg/ml; Life technologies) and Hoechst 33342 (Dojindo, Kumamoto, Japan) for 2–4 h, which was followed by washing with phosphate-buffered saline (PBS; Life Technologies).

2.5. Geometrical analysis

Confocal images of spheroids were binarized (Fig. S1) and analyzed using the NIH ImageJ program. The void ratio was calculated by dividing the channel area with the spheroid area (including both cell and channel areas). Circularity ($4\pi S/L^2$) was analyzed based on the perimeter (L) including the edges of holes inside spheroids and the cell area (S). Student t-tests were used to compare samples, and differences were considered significant at $p < 0.05$.

Supplementary Fig. S1 associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2014.02.099.

2.6. Connectivity assay

To form pores in the heterospheroids composed of Hep G2 cells and hydrogel beads by digesting the alginate hydrogel beads, we incubated the heterospheroids in 200 μg/ml of alginate lyase (Sigma–Aldrich) solution for 10 min at 37 °C. The cells were pre-stained with PKH67, green fluorescent dye. To visualize the connectivity of spheroids, they were incubated in normal culture medium with 1 μm red fluorescence beads (Polysciences, Warrington, PA, USA) for 15 min at 37 °C. The excess beads were washed away with PBS and spheroids were observed by confocal microscopy.

2.7. Hypoxia-inducible factor (HIF)-1α expression

cDNA was amplified directly from aggregates using a CellAmp Whole Transpcriptome Amplification kit (Real Time) Ver. 2 (Takara Bio, Shiga, Japan). To detect the level of HIF-1α expression, we used the TaqMan real-time polymerase chain reaction (PCR) system, and StepOnePlus with TaqMan primers for human HIF-1α detection (Assay ID: Hs00153153_m1) and human GAPDH detection (an endogenous control, 4333764F, Life Technologies). All values were