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Application of Ultra-Water-Repellent Surface to Cell Culture

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In this study, we applied an ultra-water-repellent film to cell culture. We cultured cells in droplets on the film and fabricated cell aggregates. Furthermore, we allocated cells on micropatterned surfaces consisting of ultra-water-repellent regions and cell culture-treated regions. The results show that the material is useful for cell culture.

[**Key words:** cell culture, ultra-water-repellent surface, embryonic stem cells, vascular structure, cell patterning]

Ultra-water-repellent films have a water contact angle of more than 150°. The ultra-water-repellency is governed by the chemical composition and geometrical structure of the surface (1, 2). Water droplets on ultra-water-repellent films roll over and over. This characteristic is expected to be applicable for the engineering of products such as eyeglasses, lenses and automobile windows (3). In the field of cell culture, ultra-water-repellent surfaces might have a potential use in that no mammalian cells adhere on them owing their ultrahydrophobicity. In this study, we applied an ultra-water-repellent surface to cell culture, and developed two novel culture methods, namely, a droplet cell culture and a cell patterning culture.

Cell replacement therapy is a promising approach for the treatment of loss of tissue functions. It is, however, challenged by a limited supply of appropriate cells. Recently, embryonic stem (ES) cells and mesenchymal stem cells (MSCs) have been investigated and are expected to be the most potential cell sources for regenerative medicine. These cells can differentiate into appropriate cells by applying stimuli such as growth factors, extracellular matrixes and cell interactions (4). For example, the cell aggregation of ES cells (known as embryonic bodies; EBs) causes the cells to differentiate into cardiomyocytes (5). Hanging drop culture and micromass pellet cultures are generally used for the generation of functional cardiomyocytes from ES cells or the construction of cartilage from MSCs (4–6). However, it takes considerable effort. In this study, we developed a droplet cell culture as an easy method of producing ES cell or MSC aggregates using an ultra-water-repellent surface.

Cells in tissue architecture are allocated precisely *in vivo*. For example, angiogenesis begins from the cell lining. Therefore, cell patterning methods are important for constructing *in vivo* like-tissue organs such as vascular structures. In addition to droplet cell culture, in this study, we allocated several kinds of cell using a micropatterned surface consisting of ultra-water-repellent regions and cell-culture-treated regions.

Mouse ES cells 129/Sv were purchased from Dainippon Sumitomo Pharma (Osaka) and cultured on feeder-free gelatin-coated plates in CultiCell medium for ES cells (Dainippon Sumitomo Pharma) supplemented with 500 U/ml recombinant murine leukemia inhibitory factor (mLIF; ESGRO, Chemicon, Temecula, CA, USA). Human MSCs were purchased from Cambrex Bio Science (Walkersville, MD, USA) and cultured in MSC growth medium (MSCGS; Cambrex Bio Science). Human umbilical vein endothelial cells (HUVECs) were purchased from Kurabo (Osaka) and cultured in Humedia-EG2 (Kurabo). Mouse 3T3 cells (American Type Culture Collection, Manassas, VA, USA) were cultured using Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.2 mg/ml streptomycin sulfate, and 100 U/ml potassium penicillin G. These cells were cultured at 37°C in a humidified atmosphere of CO_2 and 95% air.

Ultra-water-repellent surfaces were fabricated on substrates using the following process. Thin films of approximately 300 nm thickness were deposited using microwave-plasma-enhanced chemical vapor deposition (MPECVD) to form a nanotextured surface. The precursor in the MPECVD was trimethylmethoxysilane (TMMOS; $(CH_3)_3Si(OCH_3)$; Sigma Aldrich, St. Louis, MO, USA), which was used in an Ar gas atmosphere. Detailed preparation procedures were reported previously (7). The chemical composition of the films was analyzed by Fourier transform infrared spectroscopy (FTIR; FTS7000, Varian, Palo Alto, CA, USA). Furthermore, the roughness of the surfaces was measured using an atomic

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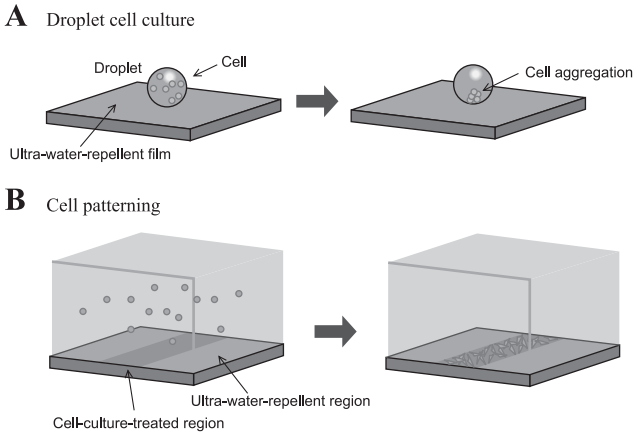

FIG. 1. Schemes of cell culture using ultra-water-repellent films. (A) Droplet cell culture. A small amount of a cell suspension was poured onto the film and a droplet formed. The cells were then cultured and allowed to form aggregates within the droplet. (B) Cell patterning. A micropatterned surface consisting of ultra-water-repellent surface regions and cell-culture-treated regions (hydrophilic regions or tissue culture surface regions) was used. A cell suspension was added onto the surface and cultured for several days. The cells adhered on the cell-culture-treated regions and proliferated.

force microscope (AFM) (SPA300HV+SPI3800N; Seiko Instruments, Chiba). Water contact angles were measured with goniometers (KRUSS DSA10-Mk2). The method of aggregate formation is illustrated in Fig. 1A. ES cells or MSCs were trypsinized and suspended in EB medium, which consisted of DMEM with 15% knock-out serum replacement (KSR; Gibco, Gaithersburg, MD, USA), 0.1 mM 2-mercaptoethanol (Gibco), 1% nonessential amino acids (Gibco), 25 U/ml penicillin G and 25 mg/ml streptomycin or chondrogenic induction medium for MSCs (Cambrex), respectively. These suspensions (20 and 250 μl) were poured onto the ultra-water-repellent film, to form droplets. Approximately 5 ml of the medium was placed outside the film to prevent the distillation of the medium from the droplets. We referred to this method as the droplet cell culture.

The method of cell patterning is illustrated in Fig. 1B. To fabricate a hydrophobic/hydrophilic pattern, we applied vacuum ultraviolet (VUV) lithography (8). Briefly, the ultra-water-repellent surface was locally exposed to VUV light (an excimer lamp, Ushio Electric, UER20-172V, $\lambda = 172$ nm and 10 mW/cm^2, was used) through a photomask for 20 min under a reduced pressure of 10 Pa. Chemical functional groups of -CH_3 on the ultra-water-repellent film were oxidized by the VUV irradiation, and as a result, the sample surface was covered with -COOH and -OH groups (7). Consequently, its surface became hydrophilic (water contact angle, $\theta < 10°$) (7). To fabricate a line pattern consisting of ultra-water-repellent regions and tissue culture surface regions, a mask or scratching method was used. A wire (width, 20 μm) was placed on the 35-mm-culture dish (Asahi Techno Glass, Chiba); then, ultra-water-repellent films were deposited on the dish by MPECVD; finally, the wire was picked up, or after depositing the films on the 35-mm-culture dish, the surface was scratched to remove the films (line width of tissue culture surface, 20 μm). HUVECs (1.0×10^4 cells) were seeded onto the surface and cultured to allocate the cells in a line (width, 20 μm). Furthermore, we transferred the patterned HUVECs onto a biological gel: the cells were seeded onto the surface and cultured for 3 d; then, Matrigel (Becton Dickinson, Bedford, MA, USA) was added at 50 μl/cm^2 and incubated at 37°C to be gelated; after the culture, the Matrigel was harvested using tweezers.

For electron microscopy, samples were fixed by culturing in a 4% glutaraldehyde solution for 12 h at 4°C. The fixed cells were then further fixed with osmium tetroxide for 30 min at room temperature. The samples were dried with t-butylalcohol using VFD vacuum evaporator (Shinku, Mito). The dried samples were coated with osmium tetroxide using an osmium plasma coater (Nippon Laser & Electronics Lab, Aichi). The processed cells were observed under an S-800 electron microscope (Hitachi, Tokyo).

Figure 2A shows that the ultra-water-repellent surface was found to consist of mainly C-H, Si-H, Si-CH_3, Si-CH_2-Si, and Si-O-Si chemical species. No substantial absorption bands corresponding to hydroxyl groups (OH) were observed. Figure 2B shows the surface roughness measured by atomic force microscopy. The surfaces had rough microstructures consisting of a piled layer of granular particles with a diameter of approximately 140 nm. Most of these particles were prepared in a clustering process in the gas phase and deposited on the structure. The clusters led to the irregular surface topography composed of granular particles, resulting in the superhydrophobic surface characteristics and the contact angle of water on the film being over 150° (Fig. 2C). Such compositions and topography provided ultra-water repellency to the surface (9–11).

When a small amount of the ES cell or MSC suspension was poured onto the ultra-water-repellent surfaces, these cell suspensions formed a liquid sphere as shown in Fig. 2C. As culture time increases, in general, the contact angle on the film decreases and the droplets spread on the film depending on the change in the properties of the film, which

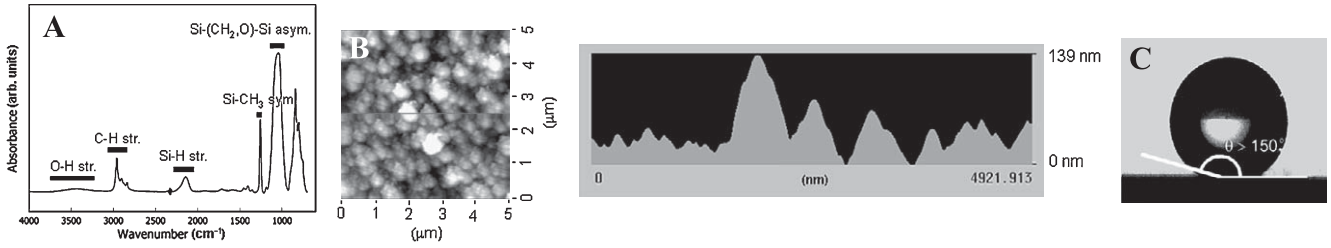

FIG. 2. Characteristics of ultra-water-repellent films. (A) The chemical composition of the films was analyzed by FTIP spectroscopy. (B) The roughness was measured by AFM. (C) Photograph of the water droplet poured onto the ultra-water-repellent films.