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Plasma-Patterned Polydimethylsiloxane Surface With Single-Step Coating with a Mixture of Vitronectin and Albumin Enables the Formation of Small Discs and Spheroids of Human Induced Pluripotent Stem Cells

Ryotaro Yamada,[a] Koji Hattori,[b] Motohiro Tagaya,[c] Toru Sasaki,[d] Daisuke Miyamoto,[e] Kohji Nakazawa,[e] Shinji Sugiura,[b] Toshiyuki Kanamori,[b] and Kiyoshi Ohnuma[a,*]

[a]Department of Bioengineering, Nagaoka University of Technology, Nagaoka, Niigata, Japan;[b]Research Center for Stem Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki, Japan; [c]Department of Materials Science and Technology, Nagaoka University of Technology, Nagaoka, Niigata, Japan;[d]Department of Electrical Engineering, Nagaoka University of Technology, Nagaoka, Niigata, Japan;[e]Faculty of Environmental Engineering, The University of Kitakyushu, Kitakyushu, Fukuoka, Japan

***Address all correspondence to:** Kiyoshi Ohnuma, Department of Bioengineering, Nagaoka University of Technology, 1603-1 Kamitomioka-cho, Nagaoka, Niigata 940-2188, Japan; Tel.: +81-258-47-9454; kohnuma@vos.nagaokaut.ac.jp.

ABSTRACT: Plasma treatment is an easy method of cleaning and hydrophilizing glass and polymer surfaces to facilitate adsorption of cell-adhesive proteins. Thus a patterned plasma treatment is useful for fabricating cell adhesion patterns. We previously succeeded in producing human induced pluripotent stem cell (hiPSC) discs (2 mm in diameter) on plasma-patterned polydimethylsiloxane (PDMS) by single-step coating of a mixture of 2 ubiquitous proteins, vitronectin and γ-globulin. Under serum-free and feeder-free conditions, without any undefined cell adhesion molecules, γ-globulin blocked hiPSC adhesion on surfaces not treated with plasma. However, γ-globulin has low cost-effectiveness and availability, and the resulting disc diameter was too large for cell-based assays. We demonstrate that bovine serum albumin (BSA) can also be used to block hiPSC adhesion on plasma-untreated PDMS surfaces coated with vitronectin. We succeeded in creating small hiPSC discs (200 μm in diameter) using single-step coating of a mixture of vitronectin and BSA. The hiPSCs proliferated without escaping from the patterned area and finally spontaneously detached from the discs to form spheroids. We believe that our method for generating hiPSC discs and spheroids will be useful for developing new bioengineering devices to enhance cell differentiation and to test drug safety for human embryonic development, contributing to future medical applications.

KEY WORDS: low-pressure plasma, microenvironment control, serum- and feeder-free culture, micro patterning, embryo

I. INTRODUCTION

Human induced pluripotent stem cells (hiPSCs) exhibit an infinite self-renewal capacity and pluripotency, and they can be generated from somatic cells and thus contain the donor's genetic information.[1,2] They can be a good cell source for re-

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jection-free transplantation of tissues and patient-specific drug screening. The adhesion of hiPSCs needs to be carefully controlled because the cells cannot survive or maintain their pluripotency without adhesion.[3,4] Moreover, spherical cell aggregates, otherwise known as embryoid bodies, are conventionally used for cell differentiation because cell–cell interactions affect hiPSC differentiation.[5] Thus, the regulation of hiPSC adhesion and the control of the shape and size of hiPSCs are important for maintaining hiPSC pluripotency and for inducing differentiation into special types of cells. To facilitate cell adhesion on glass and polymer surfaces, plasma treatment (in this article, "plasma" refers to low-pressure plasma, not blood plasma, unless otherwise stated) is one of the easiest methods because plasma treatment can be uniformly and reproducibly applied on a wide area.[6] Thus, applying plasma to control cell adhesion is a promising method for the development of new medical applications of hiPSCs.

The patterning of cells, including hiPSCs, can be easily performed by masked plasma oxidation, which uses patterned physical masks to partially prevent plasma treatment.[7–10] Using masked plasma oxidation, we succeeded in creating hiPSC patterns (2-mm-diameter discs) on polydimethylsiloxane (PDMS) surfaces under culture conditions defined in a previous study.[9] PDMS is one of the most popular biocompatible materials for research and development of small cell culture devices because this elastomer is nontoxic, chemically inert, transparent, and gas permeable.[11–13] Plasma treatment on PDMS oxidized ≡Si–CH_3 to generate ≡Si–O–Si≡ groups suggests that hydrophilic and siliceous layers were formed on the surface[9,11] (Fig. 1). Vitronectin mediates the adhesion of cells, including hiPSCs, to hydrophilic and siliceous (glass) surfaces, as reflected in the protein's name (*vitro*, meaning "glass," and *nectin*, meaning "cell adhesion molecule").[14–17] On the other hand, the untreated PDMS surface was hydrophobic, on which γ-globulin was adsorbed to block hiPSC adhesion.[9,11,18,19] Defined culture conditions, which do not contain any undefined cell adhesion molecules, enabled us to evaluate hiPSC adhesion and to form hiPSC patterns.[10,20,21] The advantage of our method was that the patterned surfaces were easy to produce: patterned plasma treatment was performed with a perforated mask followed by single-step coating with a mixture of vitronectin and γ-globulin (compare with multistep coating[22]). However, there were two issues. One was the high cost and limited availability of γ-globulin. The other was the size of the patterned hiPSCs (2 mm in diameter), which were too large. In addition, we did not try to generate 3-dimensional hiPSC aggregates (spheroids) in our previous study.[9]

Here, we attempted to produce small (200–1000 μm in diameter), 2-dimensional hiPSC discs using bovine serum albumin (BSA). BSA is more readily available and less expensive than γ-globulin. Moreover, like γ-globulin, BSA does not mediate cell adhesion and is adsorbed into untreated PDMS surfaces.[23] Thus BSA might be a good substitute for γ-globulin to block vitronectin adsorption on untreated PDMS surfaces. To fabricate smaller (200–1000 μm in diameter) perforated masks, PMMA sheets with a thickness of 200 μm were drilled to create holes. Furthermore, we tried to harvest the resulting hiPSC discs to form spheroids.

II. MATERIALS AND METHODS

A. Culture and Subculture of hiPSCs

The hiPSC cell line 201B7[1] was obtained from RIKEN BRC Cell Bank (Tsukuba, Japan) through the National BioResource Project for the Ministry of Education, Culture, Sports, Science and Technology, Japan. For all experiments, hiPSCs cultured in knockout serum replacement–based medium on mouse embryonic fibroblast feeder cells were transferred to serum-free and feeder-free culture conditions in hESF9a medium[20,24] on dishes coated with 2 μg/mL fibronectin from bovine blood plasma (F-1141; Sigma-Aldrich, St. Louis, MO) and were passaged at least once before use.[21,24] For subculturing, the cells were detached from the culture dish using 0.2–0.5 U/mL dispase (17105-041; Life Technologies, Grand Island, NY) in hESF9a medium and replated on hESF9a medium with 5 μM Rho-associated coiled-coil kinase (ROCK) inhibitor (Y-27632; Wako Pure Chemical Industries, Osaka, Japan), which blocks dissociation-induced apoptosis of hiPSCs.[4] The hESF9a medium was changed daily. For the adhesion experiments, hiPSCs were dissociated into single cells by incubation and trituration in 0.02% (w/w) ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS)−/− and then plated in hESF9a solution with 5 μM ROCK inhibitor.

B. Preparation and Plasma Treatment of PDMS Surfaces

The PDMS prepolymer and curing agent (Sylgard 184; Dow Corning, Midland, MI) were thoroughly mixed at a 10:1 weight ratio. To create PDMS sheets, we poured the mixture between 2 polyethylene terephthalate films separated with 2-mm rubber spacers and cured it in an oven at 120°C for 2 hours. The PDMS sheets were rinsed with ethanol and sterilized at 160°C for 2 hours. The PMMA perforated masks were drilled using a numerical control milling machine (Micro MC-2-Light; PMT Corporation, Fukuoka, Japan) and rinsed and sterilized with ethanol. The sizes of the perforated holes are listed in Table 1.

TABLE 1: Sizes of Perforated Masks

Hole Diameter (μm)	Pitch (μm)	Hole Depth (μm)	Holes (n)
200	200	200	110
500	550	200	195
1000	2200	200	90

PDMS sheets with or without a perforated mask were hydrophilized by treatment with low-pressure air plasma for 60 seconds (20 kHz, 6.8 kV, 12.6 mA, YHS-R; SAKIGAKE-Semiconductor Co., Kyoto, Japan) after 5-minutes of evacuation with a vacuum pump (pumping speed: 40.8 L/min, achievable pressure: 2 Pa, TA150XA; Tasco, Osaka, Japan). Between 30 minutes and 1 hour after plasma treatment, during which the hydrophilicity of the PDMS surface was stable,[9] the surface of the PDMS sheet was coated with 5.5 mg/mL rabbit γ-globulin (011-000-002; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), 10 mg/mL BSA (free of fatty acids, A8806; Sigma), 0.6 μg/cm^2 human blood plasma-derived vitronectin (2349-VN; R&D Systems), or a combination of these proteins, and incubated overnight at 37°C. If the perforated mask was used, it was removed before coating with the proteins. The coating proteins were removed immediately before use.

C. Cell Attachment Assay and Immunocytochemistry

Attached living cells were stained with 1 μM calcein AM (an acetoxymethyl ester of calcein, Dojindo, Kumamoto, Japan), a fluorescent dye that can be transported into living cells, for 20 minutes at 37°C. For immunocytochemistry, hiPSCs plated on conventional culture dishes were rinsed with PBS+/+, fixed in 4% formaldehyde with 0.5 mmol/L magnesium chloride and 0.5 mmol/L calcium chloride, permeabilized, blocked with PBS+/+ containing 0.2% Triton X-100 and 10 mg/mL BSA, and reacted with primary antibodies, which were visualized with secondary antibodies. The antibodies were diluted in PBS+/+ containing 0.2% Triton X-100 and 10 mg/mL BSA. The antibody information is listed in Table 2. Nuclei were stained with 0.4 μM 4′,6-diamidino-2-phenylindole (Wako). Fluorescence micrographs were obtained with a BZ-8100 fluorescence microscope (Keyence, Osaka, Japan) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

TABLE 2. Antibodies

Marker	Primary Antibody	Secondary Antibody
SSEA4	Antihuman SSEA4 (MC-813-70) Mouse monoclonal IgG3, MAB4304[a] Dilution 1:1000	Goat antimouse IgG3 (γ3) Alexa Fluor 488, A21151[b] Dilution 1:500
Oct3/4	Antihuman Oct3/4 (H-134) Rabbit polyclonal IgG, sc-9081[c] Dilution 1:500	Goat antirabbit IgG (H + L) Alexa Fluor 546, A11035[b] Dilution 1:500

[a]Chemicon (Millipore), Billerica, MA.
[b]Life Technologies, Carlsbad, CA.
[c]Santa Cruz Biotechnology, Dallas, TX.

D. Data Analysis

Images were analyzed with ImageJ software (National Institutes of Health). Statistical analyses were performed with Toraneko software (Ogura M. In Excel simple multiple comparison (KS Science Textbooks). Tokyo, Japan: Kodansha; 2012.).

III. RESULTS

A. Adhesion of hiPSCs to Plasma-Treated and Untreated PDMS Surfaces Coated With a Mixture of Vitronectin and BSA

We hypothesized that, like γ-globulin, BSA can be used to prevent hiPSC adhesion on plasma-untreated PDMS surfaces by blocking the adsorption of vitronectin. BSA is a major component of fetal bovine serum and is widely used for blocking nonspecific binding of proteins. To test our working hypothesis, we coated untreated and plasma-treated PDMS surfaces with a mixture of vitronectin and BSA or a mixture of vitronectin and γ-globulin overnight and plated single-cell dissociated hiPSCs in ESF9a medium with 5 μM ROCK inhibitor, which blocks dissociation-induced apoptosis of hiPSCs. One day after plating, hiPSCs were stained with a live cell dye, calcein AM, to visualize living cells. Human iPSCs did not adhere to the untreated surface but did adhere to the plasma-treated surface coated with a mixture of vitronectin and BSA (Fig.

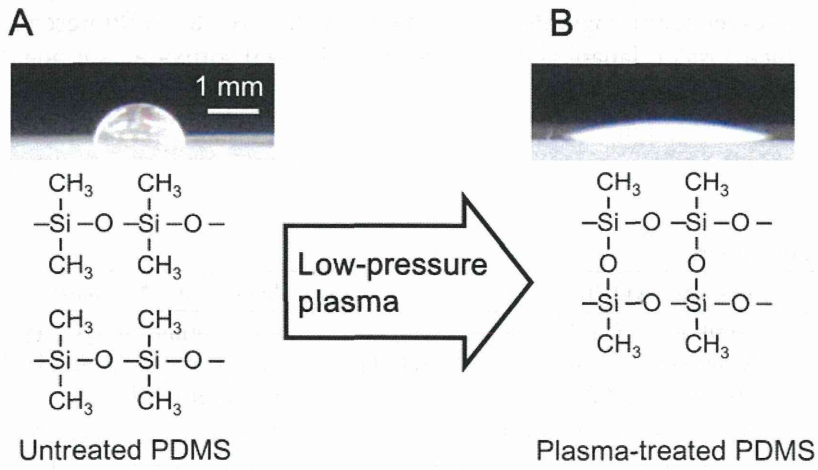

FIG. 1: Schematics of plasma treatment on a polydimethylsiloxane (PDMS) surface. Low-pressure (air) plasma coverts the PDMS surface from a hydrophobic surface **(a)** into a hydrophilic siliceous surface **(b)**.

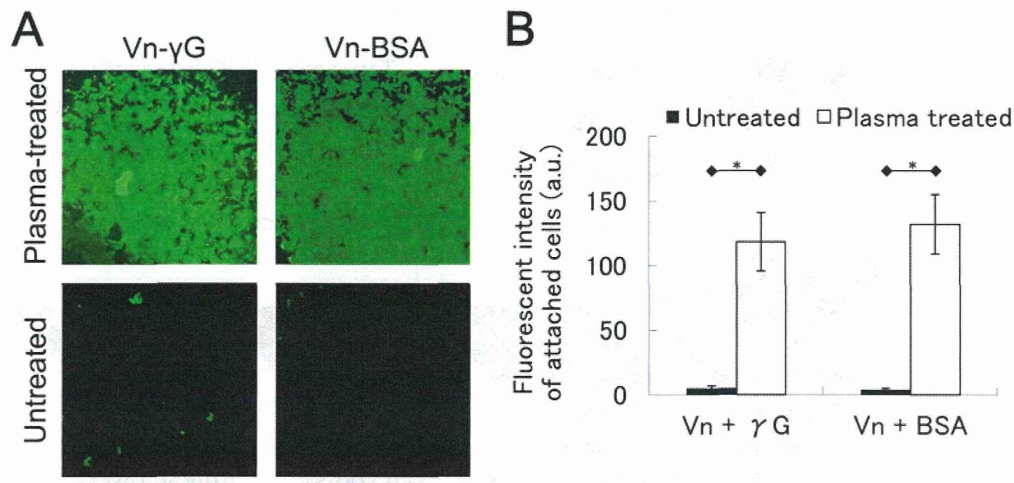

FIG. 2: Adhesion of human induced pluripotent stem cells (hiPSCs) to plasma-treated and untreated polydimethylsiloxane (PDMS) surfaces. Fluorescence images of hiPSCs stained by calcein AM (**a**) and average fluorescent intensities (**b**) of plasma-treated and untreated PDMS surfaces coated with a mixture of vitronectin and γ-globulin or vitronectin and bovine serum albumin (BSA). Data are presented as mean ± standard error (n = 4). $^*P < 0.05$, Holm's multiple comparisons.

2). The results were the same for the surface coated with a mixture of vitronectin and γ-globulin (Fig. 2), which supported our working hypothesis.

B. Disc Formation of hiPSCs: Masked Plasma Oxidation of a PDMS Surface Followed by Single-Step Coating with Vitronectin and BSA

Based on the results, we tried to generate hiPSC discs on PDMS surfaces by masked plasma oxidation and single-step coating with a mixture of vitronectin and BSA. We previously succeeded in creating hiPSC discs (2 mm in diameter) on PDMS surfaces using masked plasma oxidation with PDMS mask (1 mm in thickness) perforated with a hole-punch.[9] To create smaller hiPSC patterns, PMMA sheets (200 μm in thickness) were used to generate masks, which were perforated with holes with diameters of 1000, 500, or 200 μm (Fig. 3A and B). PDMS surfaces covered with the perforated PMMA masks were treated with plasma to form hydrophilic and siliceous spots (Fig. 3C). After removal of the masks, the PDMS surfaces were coated with a mixture of vitronectin and BSA to create vitronectin spots surrounded by BSA, and hiPSCs were plated (Fig. 3C). Discs of hiPSCs formed on the circular plasma-treated areas of all 3 sizes. Immunostaining of undifferentiated cell markers, OCT3/4 and SSEA4, revealed that the hiPSCs kept

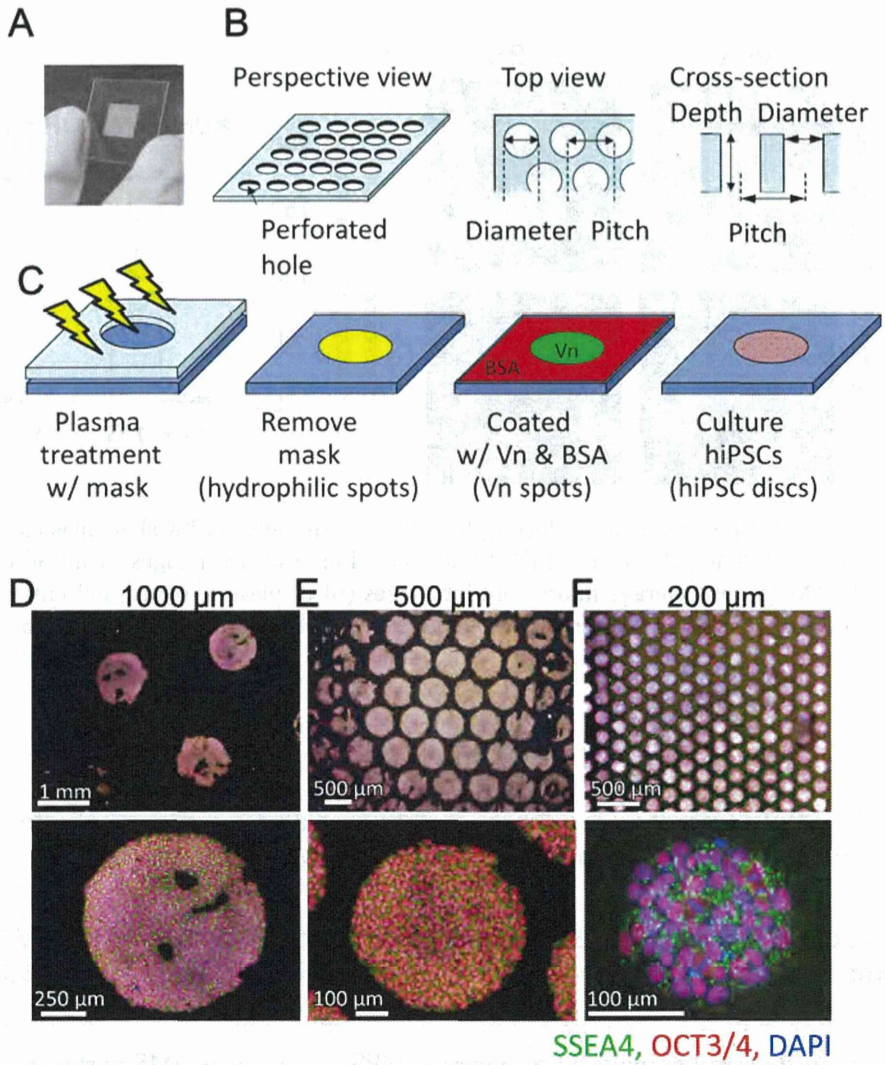

FIG. 3: Discs of human induced pluripotent stem cells (hiPSCs) created by plasma patterning of polydimethylsiloxane (PDMS) surfaces and single-step coating of vitronectin and bovine serum albumin (BSA). Photograph (**a**) and schematic (**b**) of perforated poly(methyl methacrylate) (PMMA) masks. (**c**) Schematic of preparation of plasma-patterned, protein-coated PDMS. PDMS sheets covered with a perforated PMMA mask were treated with plasma and then coated with a mixture of BSA and vitronectin. (**d–f:** Immunostaining for undifferentiated cell marker antibodies, anti-SSEA4 antibody (green) and anti-OCT3/4 antibody (red), 4 days after cell plating. The diameters of perforated masks were 1000 (**d**), 500 (**e**), and 200 μm (**f**). Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). The lower panels are the magnified images of the upper panels.

an undifferentiated state (Fig. 3D–F). Notably, the small and homogeneous discs were created in a wide area (Fig. 3F). These results suggest that our method could be used to prepare equally sized, small hiPSC discs on plasma-patterned PDMS surfaces.

C. Spheroid Formation of hiPSCs: Spontaneous Detachment of hiPSC Aggregates

Next, we tried to form spheroids, which are spherical aggregates of hiPSCs. We previously reported that the edges of the hiPSC discs became thick (multilayer) in several days because the cells continued to proliferate without escaping from the plasma-treated area.[9] Ohnuma et al.[10] also reported that hiPSC discs could be harvested without dissociating cells via incubation in a protease-free medium with Ca^{2+} and without Mg^{2+},

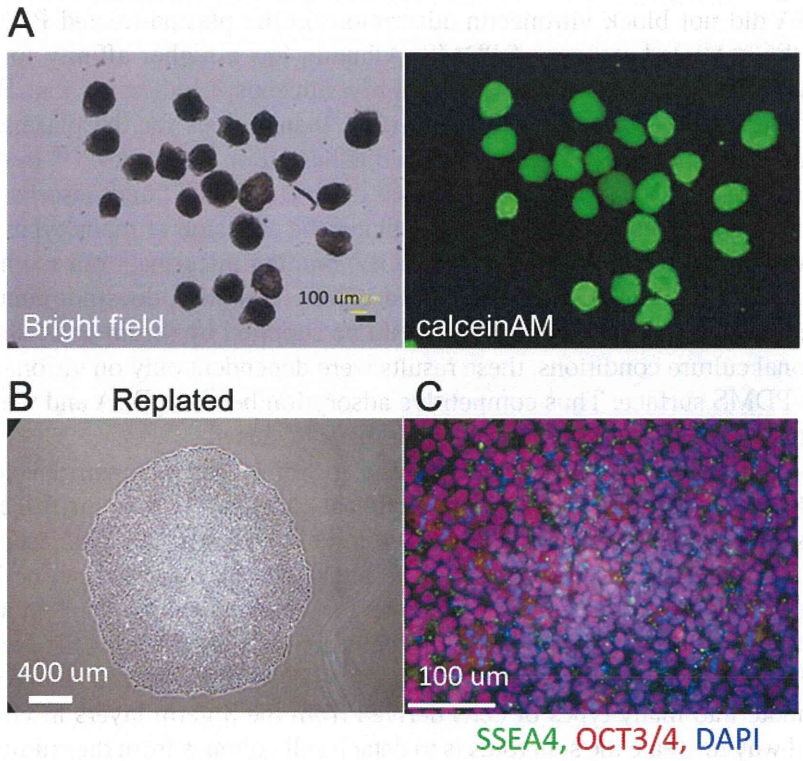

FIG. 4: Spontaneous human induced pluripotent stem cell (hiPSC) spheroid formation. **a:** Bright field (left) and live cell fluorescence image (calcein AM, right) of hiPSCs 1 week after hiPSCs were plated on plasma-patterned polydimethylsiloxane (500 μm in diameter) and coated with bovine serum albumin and vitronectin. **b, c:** Replated spheroid. Phase-contrast image (**b**) and immunostaining for the undifferentiated-cell marker antibodies anti-SSEA4 antibody (green) and anti-OCT3/4 antibody (red) (**c**). Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue).

followed by pipetting. Thus we expected the hiPSC discs to become hemispherical after several days in culture, and then become spheroids after the harvest of cell aggregates using the protease-free medium. The discs became hemispheres, as expected; however, the hemispheres spontaneously detached from PDMS 1 week after culture, without any harvesting procedure (Fig. 4A). The spheroids consisted of living cells because the spontaneously detached hiPSC spheroids were calcein AM positive and reattached on fibronectin-coated polystyrene dishes (Fig. 4AB), suggesting that the hiPSCs were not dead when spheroids formed and detached. Immunostaining of undifferentiated cell markers revealed that the reattached cells kept an undifferentiated state (Fig. 4C). These results suggest that our method can be used to prepare equally sized hiPSC spheroids.

IV. DISCUSSION

BSA, like γ-globulin, prevented hiPSC adhesion on untreated PDMS surfaces, suggesting that BSA did not block vitronectin adsorption on the plasma-treated PDMS area but did on the untreated regions of PDMS. Albumin has a higher affinity for PDMS surfaces than it does for quartz, which is highly siliceous,[23] indicating that BSA has a higher affinity for the untreated PDMS surface than it does for the plasma-treated siliceous PDMS surface. BSA also inhibits cell adhesion on substrates.[22,25] In contrast, vitronectin mediates hiPSC adhesion on tissue culture dishes[16,17] and adsorbs well on siliceous surfaces.[14,15] Because vitronectin mediates the adhesion of many types of cells that express arginine-glycine-aspartic acid (RGD) binding integrins,[26] our methods can be applied to these cells. Because our defined culture condition does not contain any undefined cell-adhesive molecules, which could be supplied by serum and feeder cells in conventional culture conditions, these results were dependent only on vitronectin and BSA on the PDMS surface. Thus competitive adsorption between BSA and vitronectin on plasma-treated and untreated surfaces might have occurred.[25,27,28]

Discs and spheroids of hiPSCs can be used to mimic early human development, which is difficult to investigate because of technical and ethical problems. Like human embryonic stem cells, hiPSCs have an epiblast-type nature. The human epiblast is a disc-shaped cell aggregate that will give rise to the fetus. The hiPSC discs can be harvested noninvasively using proteinase-free solutions for further experiments.[10] In addition, spheroids of embryonic stem/induced pluripotent stem cells, which are called embryoid bodies, are frequently used for testing pluripotency because the cells in an embryoid body can differentiate into many types of cells derived from the 3 germ layers in vitro.[5] The conventional way to create the spheroids is to detach cell colonies from the culture dishes and replate them onto nonadherent culture dishes to allow the formation of cell aggregates. Equally sized spheroids spontaneously detached using our method. Therefore our method is an easy and cost-effective way to mimic early human embryonic development.

The hiPSC spheroids detaching spontaneously from the PDMS surface was unexpected. The adhesion force of hiPSCs on substrates is fairly weak.[29] Moreover, in normal hiPSC cultures, in the absence of patterning, multilayered parts of the colonies