

Thermoresponsive poly(*N*-isopropylacrylamide) hydrogel substrates micropatterned with poly(ethylene glycol) hydrogel for adipose mesenchymal stem cell spheroid formation and retrieval

Gyeongju Kim^a, Yuna Jung^a, Kanghee Cho^a, Hyun Jong Lee^{b,*}, Won-Gun Koh^{a,*}

^a Department of Chemical and Biomolecular Engineering, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea

^b Department of Chemical and Biological Engineering, Gachon University, Seongnam-si, Gyeonggi-do 13120, Republic of Korea

ARTICLE INFO

Keywords:

Adipose mesenchymal stem cell
Thermoresponsive polymer
Cell spheroid
Retrieval

ABSTRACT

Cell spheroid formation is necessary to develop three-dimensional (3D) cellular environments that provide appropriate cell-cell and cell-matrix interactions similar to *in vivo* environments without additional substrates. Although some methods including stirring culture, low adhesion plate culture, hanging drop, and microfluidics are used to construct cell spheroids, there is no method to fulfill all of the mass production of uniform spheroids, simple media change, and easy retrievability. Here, bulk poly(*N*-isopropylacrylamide) (PNIPAAm) hydrogel substrate (PHS) was used to fabricate, culture, and retrieve cell spheroids. Adipose-derived stem cells (ASCs) were cultured on bulk PHS to form spheroids. ASCs formed cell spheroids directly on substrates without additional manipulation. These spheroids adhered to the semi-adhesive substrate, while the spheroids fabricated using the nonadhesive surface method floated without getting fixed to the surface. Bulk PHS stiffness was evaluated using the compressive test (compressive modulus: 153 ± 11 kPa). A poly(ethylene glycol) (PEG) hydrogel microwell pattern was created on PHS to control the spheroid size, forming uniform ASC spheroids between 100 and 150 μm in diameter on 200 and 300 μm well-patterned substrates. Cell-cell interactions in the resulting ASC spheroids were evaluated based on fibronectin and laminin expression; fluorescence intensities of fibronectin- and laminin-immunostained images of ASC spheroids were 10.9 and 7.3 times higher than those of ASCs cultured on the tissue culture plate, respectively. ASC spheroids were detached following incubation at 4 °C for 10 min (retrieval efficiency: $74 \pm 19\%$). Retrieved spheroid cell viability was over 97.5%. The PEG hydrogel microwell-patterned PHS is a convenient spheroid fabrication and retrieval platform that can increase cell spheroid usage.

1. Introduction

Over the last few decades, two-dimensional (2D) culture systems have been used for a wide range of fundamental cell studies due to simple culturing conditions and facilitation of microscopic analysis [1]. However, it has been demonstrated that many cell types lose their original properties such as proliferation, differentiation, and function in 2D culture conditions, and thus, many efforts have been made to develop three-dimensional (3D) culture conditions [2,3]. Cell-cell interaction is a crucial element in 3D cell culture conditions, and it is a factor that distinguishes it from 2D cultures. Cell spheroid formation is a 3D cell culture method to increase cell-cell interaction, and it replicates *in vivo* cell-cell and cell-extracellular matrix (ECM) interactions without additional substrates [4,5]. It was recently demonstrated that the differentiation efficiency of MSCs was successfully increased in 3D culture

systems compared to that in conventional 2D culture systems [6–8].

Some methods such as stirring culture, low adhesion plate culture, hanging drop, and microfluidics can be used to construct cell spheroids, but they do not satisfy all of the formations of uniform-sized spheroids, media exchange, and easy retrieval of spheroids [3–5,9–11]. Another method is a microwell platform system, which is a simple way to generate uniform-sized spheroids in large quantities [12–17]. However, it was not easy to retrieve the spheroids from conventional microwell platforms because the substrates provide only cell-adhesive surfaces. The retrieval of cell spheroids is essential for increasing clinical and biomedical usability for disease pathophysiology, tissue engineering, and regeneration [18]. Therefore, a new cell spheroid formation system with easy retrievability is required.

Here, we present a new thermoresponsive microwell platform system to achieve retrieval and mass production of uniform-sized

* Corresponding authors.

E-mail addresses: hjlee2@gachon.ac.kr (H.J. Lee), wongun@yonsei.ac.kr (W.-G. Koh).

<https://doi.org/10.1016/j.msec.2020.111128>

Received 4 November 2019; Received in revised form 6 April 2020; Accepted 25 May 2020

Available online 26 May 2020

0928-4931/ © 2020 Elsevier B.V. All rights reserved.

spheroids. Poly(*N*-isopropylacrylamide) (PNIPAAm) hydrogel, which is a widely used thermoresponsive polymer, was used as a substrate. The cell attachment and detachment using the temperature-sensitive property of PNIPAAm have been elaborated in previous studies [19–22]. However, there was no direct spheroid formation and retrieval on the thermoresponsive surface, and some studies tried to generate spheroids from cell sheet engineering [23–25]. We applied bulk PNIPAAm hydrogel substrate (PHS) to form and retrieve cell spheroids directly on the substrate. The polymerization process from *N*-isopropylacrylamide (NIPAAm) monomer to PNIPAAm hydrogel was modified so that the substrate had an appropriate property for the construction of cell spheroids at the temperature above the lower critical solution temperature (LCST), and the spheroids were disassembled from the substrate when the temperature was below LCST.

The stiffness of fabricated PHS was evaluated by a compressive modulus. The fabricated bulk PHS provides a semi-adhesive surface that permits cells to form spheroids while cells adhere and spread as a monolayer in the general PNIPAAm surface [22,26,27]. The thermoresponsive property of adjusted PHS was evaluated by transparency, volume changes, and water contact angles. The uniform size control of spheroids was accomplished using the poly(ethylene glycol) (PEG) hydrogel microwell pattern. Adipose-derived stem cells (ASCs), which are mesenchymal stem cells (MSCs) derived from adipose tissue, were used in this study as a cell material for spheroid formation. The uniformity of ASC spheroid size was evaluated via microwell pattern size, and fibronectin and laminin expressions were observed to examine the effect on ECM proteins. ASC spheroids were detached and retrieved from the thermoresponsive substrate using temperature change, and the retrieval efficiency was evaluated using cell viability.

2. Experimental section

2.1. Materials

Unless indicated otherwise, all chemicals and solvents were of analytical grade and used as provided by the manufacturers. *N*-isopropylacrylamide (NIPAAm), *N,N'*-methylenebis(acrylamide) (MBA), ammonium persulfate (APS), *N,N,N',N'*-tetramethylethylenediamine (TMEDA), benzophenone, poly(ethylene glycol) diacrylate (PEG-DA; MW 575 Da), 2-hydroxy-2-methylpropiophenone (HOMPP), benzyl alcohol, sodium periodate, dimethyl sulfoxide (DMSO), ethanol (99.9 v/v %), collagenase, bovine serum albumin (BSA), and triton X-100 were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Silicone rubber sheets (3 mm) were purchased from Sungjin (Seoul, KOR). Photolithography photomasks were designed using AutoCAD software and printed on transparencies using a standard laser jet printer (LaserWriter 16/600 PS, Apple Inc., Cupertino, CA, USA). Trypsin-EDTA, penicillin/streptomycin, and phosphate-buffered saline (PBS, pH 7.4, 1×) were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Cellsera (Rutherford, NSW, AUS). Dulbecco's-modified Eagle's medium (DMEM/low glucose) and DPBS (modified, 1×) were purchased from HyClone Laboratories Inc. (South Logan, UT, USA). LIVE/DEAD viability/cytotoxicity kit, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), paraformaldehyde, and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Mouse anti-fibronectin antibody-Alexa Fluor 488 conjugate and mouse anti-laminin α -1 antibody-Alexa Fluor 488 conjugate were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.2. Micropatterned thermoresponsive hydrogel fabrication

PNIPAAm hydrogel substrate (PHS) was prepared using free radical polymerization. NIPAAm monomer solution was prepared by dissolving 100 mg of NIPAAm, 2 mg of MBA, and 30 mg of APS in 1 mL of

deionized water as the monomer, crosslinker, and initiator, respectively. To prepare molds for polymerization, squares (18-mm squares) were punched out from silicone rubber sheets, and the punched sheets were attached to slide glasses. After the mold was filled with 600 μ L of NIPAAm monomer solution, 15 μ L of TMEDA was dropped onto the solution in the mold. Polymerization was done at 4 °C for 24 h. PHS was taken from the mold and washed extensively with deionized water.

Compressive tests were performed using an Instron 3340 at RT. A swollen disc was clamped between the parallel plates, and the compressive strain was applied at a rate of 10 mm/min until the disc fractured. The modulus was obtained from the slope of the linear region of the stress-strain curve.

Photoinduced graft polymerization of PEG-DA was utilized to modify the PHS surface. The prepared PHS was immersed in 10 wt% benzophenone in ethanol for 3 min and dried in a fume hood to evaporate ethanol. PEG hydrogel precursor solution was prepared by dissolving PEG-DA (50 wt%), HOMPP (2 v/v%), benzyl alcohol (0.5 wt%), and sodium periodate (0.5 mM) in deionized water. The benzophenone immobilized PHS was coated with 200 μ L of precursor solution and covered with a photomask containing the microwell pattern (squares of 200, 300, or 500 μ m, and gap distances of 200 μ m between squares). The PHS was exposed to 365 nm, 300 mW/cm² UV light (EFOS Ultracure 100ss Plus, UV spot lamp, Mississauga, ON, Canada) for 5 s to crosslink, and the PEG microwell-pattern was grafted onto the PHS. The microwell-patterned PHS with pattern sizes of 200, 300, and 500 μ m were named 200-PHS, 300-PHS, and 500-PHS, respectively. After the photomask was removed, the fabricated microwell-patterned PHS was rinsed with deionized water for 5 min to remove unreacted PEG hydrogel precursor solution. Next, it was washed extensively with ethanol to remove unreacted benzophenone and to sterilize for subsequent cell culture. The fabricated hydrogel with removed residuals was kept in PBS for cell culture experiments, or it was stored in deionized water for other experiments.

The images of non-patterned PHS and microwell-patterned PHS (200-PHS, 300-PHS, and 500-PHS) were obtained using a fluorescence microscope (IX 51, Olympus, Tokyo, Japan). The images of PHS at swollen state in PBS below the LCST were observed using the fluorescence microscope.

To demonstrate that thermoresponsive PHS was intact after patterning, quantum dots (QDs, 1 v/v%) were mixed with PEG hydrogel precursor. The patterning process was done in the same manner. The images of the QD-entrapped region were obtained using a laser scanning confocal microscope (LSM 880, Carl Zeiss, Oberkochen, Germany). PHS before and after patterning and PEG hydrogel after patterning were analyzed using X-ray photoelectron spectroscopy (XPS) (K-alpha, Thermo VG, U.K.) with monochromated Al X-ray sources (Al K α line: 1486.6 eV). The samples were kept in deionized water before freeze-drying.

2.3. Characterization of the thermoresponsive property of PHS

To evaluate the thermoresponsive property of PEG hydrogel patterned PHS, the transparency and volume of substrates depending on temperature were observed. The water temperature was altered after immersing 200-PHS in deionized water. The transparency and volume of 200-PHS were observed at 4, 25, 28, 31, 34, and 37 °C. Photographs of 200-PHS at different temperatures were obtained, and length, width, and thickness were measured using ImageJ software. The volume ratio of the sample was calculated as follows:

$$\text{Volume ratio (\%)} = \frac{(\text{volume at a certain temperature})}{(\text{volume at 4}^\circ\text{C})} \times 100$$

The water contact angle (DSA100, Kruss, Hamburg, Germany) was measured to confirm changes in PHS hydrophobicity at Smart Materials Research Center for IoT at Gachon University.

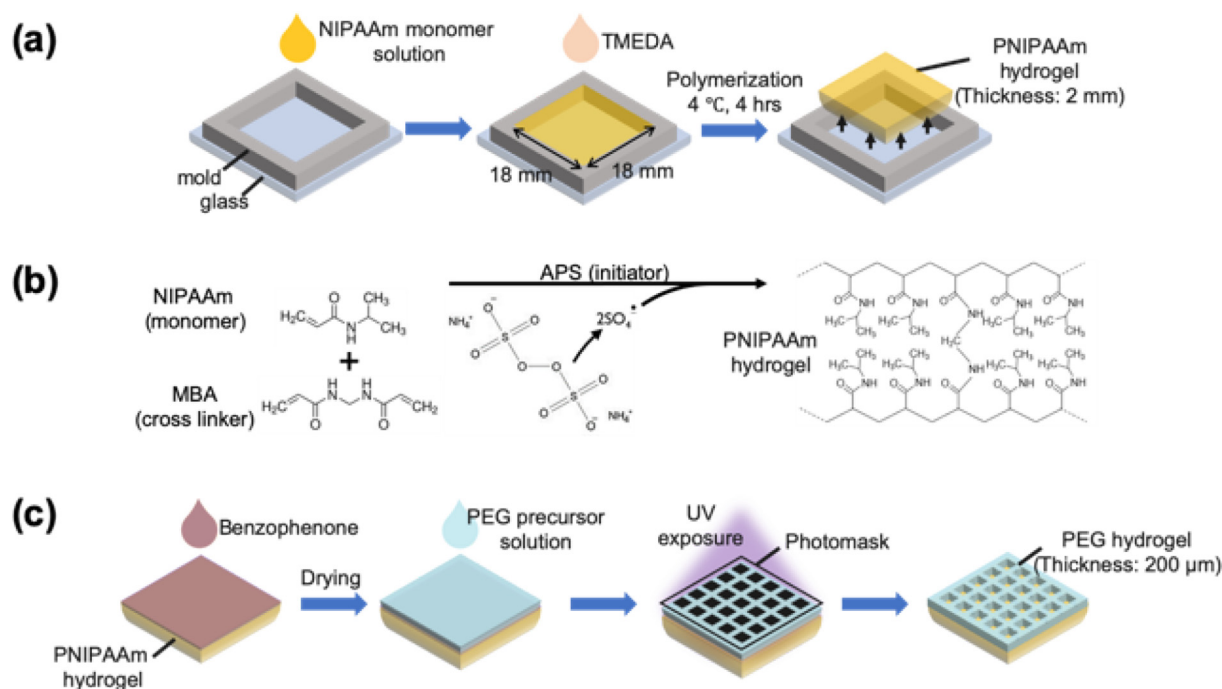


Fig. 1. Schematic illustrations of micropatterned thermoresponsive hydrogel fabrication. (a) PNIPAAm hydrogel substrate (PHS) synthesis. (b) Chemical structure scheme of PNIPAAm hydrogel formation. (c) PEG microwell patterning and grafting PHS.

2.4. Adipose-derived stem cell (ASC) culture and spheroid formation

Human ASCs were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The ASCs, cryopreserved in the second passage, were cultured in culture medium (DMEM/low glucose, 10 v/v% FBS, and 1 v/v% penicillin/streptomycin) and used at passage 5 for all experiments.

Before cell seeding, microwell-patterned PHS was cut into circles 15 mm in diameter, placed in 24-well plates, and sterilized under UV light for 30 min. The PHS in the well plate was incubated at 37 °C until they turned opaque and adhered to the bottom of the plate, and 1 mL of culture medium per well was added.

ASCs were trypsinized by treating with trypsin-EDTA for 3 min and centrifuged at 1200 rpm for 5 min. After the supernatant was removed, ASCs were resuspended in the culture medium. Approximately 2.0×10^5 cells/cm² (about 3.5×10^5 cells/well of 24-well plate) were seeded onto the PHS. ASCs were cultured for 5 days after seeding, and the culture medium was changed every 2 days.

On days 1, 3, and 5, ASC spheroids formed in the microwells were stained with the LIVE/DEAD viability/cytotoxicity assay kit. The culture medium was removed and rinsed with PBS. The spheroids were incubated in the assay solution, which was prepared by adding 10 μ L of Calcein-AM and 10 μ L of EthD-1 in 10 mL of PBS, for 1 h at 37 °C. The prepared samples were observed using a fluorescence microscope. To analyze the spheroid size distribution, ASC spheroids formed in the microwells on days 1, 3, and 5 were photographed. One hundred ASC spheroids were selected from five photos each day, and their sizes were measured using ImageJ software. The average size and standard deviation of spheroid sizes were calculated, and the size distributions on each day were presented in distribution graphs.

2.5. ASC spheroid immunostaining

For immunofluorescence (IF) staining, ASC spheroids were fixed in 4 v/v% paraformaldehyde for 15 min, and the fixed spheroids were washed with PBS several times. Triton X-100 (0.2 v/v%) was applied to the spheroids to increase permeability for 20 min. The resulting

spheroids were blocked in blocking buffer (1 w/v% BSA in PBS) for 1 h and incubated with Alexa Fluor 488-conjugated antibody solution (fibronectin and laminin diluted 1:100 in blocking buffer) for 1 h at room temperature. After washing with PBS, IF-stained spheroids were mounted in DAPI solution (1:1000 dilution in blocking buffer). Subsequently, the stained spheroids were washed with PBS and observed by using a fluorescence microscope. The fluorescence image intensity was measured using ImageJ software.

2.6. ASC spheroid retrieval

To measure the percentage of ASC spheroids retrieved from microwell-patterned PHS by decreasing the temperature below the LCST on days 1, 3, and 5, the culture medium was removed, and ASCs were subjected to LIVE/DEAD viability/cytotoxicity assay as described above. The total number of spheroids in each sample was counted before decreasing the temperature. After incubating the sample containing spheroids at 4 °C for 10 min, the number of spheroids remaining in the microwells was counted. The percentage of cell spheroids retrieved from microwell-patterned PHS was calculated as follows:

(Retrieved cell spheroids (%))

$$= \left\{ 1 - \frac{(\text{number of spheroids on PHS after incubation})}{(\text{number of spheroids on PHS before incubation})} \right\} \times 100$$

To count the number of cells per spheroids on 200- and 300-PHS, the retrieved ASC spheroids were collected, and the numbers of spheroids were counted firstly. The spheroids were trypsinized to isolate each cell, and the total cell number was counted. Then, the total cell number was divided by the spheroids number, and it was repeated three times.

2.7. Statistical analysis

All data are expressed as the mean $\pm$ standard deviation (SD). Each experiment was repeated at least 3 times unless otherwise indicated. Statistical evaluation was performed using one-way ANOVA. A value of $p < 0.05$ was considered statistically significant. Statistical analysis

was performed using the statistical software GraphPad Prism 7.

3. Results

3.1. Micropatterned thermoresponsive hydrogel fabrication

We designed a platform that consisted of a thermoresponsive substrate and microwell patterns to form and retrieve cell spheroids. The thermoresponsive hydrogel substrate was fabricated using redox initiation polymerization of *N*-isopropylacrylamide (NIPAAm) (Fig. 1a and b). NIPAAm monomer solution containing *N,N'*-methylenebis(acrylamide) (MBA) as a crosslinker and ammonium persulfate (APS) as an initiator were added to a silicon mold, and polymerization was initiated by adding *N,N,N',N'*-tetramethylethylenediamine (TMEDA) to the PNIPAAm hydrogel precursor solution. The resulting polymer precipitated when the temperature was higher than the lower critical solution temperature (LCST) of PNIPAAm; therefore, the polymerization was performed at 4 °C for 24 h. The fully crosslinked PNIPAAm hydrogel was removed from the glass substrate and used as a thermoresponsive PNIPAAm hydrogel substrate (PHS).

The mechanical property of PHS was evaluated using a compressive test. The compressive modulus of PHS was 153 ± 11 kPa from the linear region of the compressive stress curve (Table 1). Our increased monomer concentration and crosslinker ratio led to higher stiffness compared to that with the same free radical polymerization method [28]. Fei et al. applied a higher concentration of monomer and crosslinker with UV photocuring polymerization, but the compressive modulus was lower than that of our PHS [29].

The poly(ethylene glycol) (PEG) hydrogel was micropatterned on the PNIPAAm hydrogel with photoinduced grafting to anchor the PEG hydrogel on the substrate (Fig. 1c). Benzophenone was used as a surface grafting initiator, and the PEG diacrylate (PEG-DA) molecules linked onto the surface [30]. Benzophenone bounds and generates surface initiator by UV irradiation, and it was adsorbed onto the PNIPAAm surface followed by applying PEG precursor solution to enhance the efficiency of links between PNIPAAm and PEG hydrogel. The residual acrylate group participated in the crosslinking reaction with other acrylate groups of PEG-DA, and the photomask photolithography generated the microwell pattern. PEG hydrogel microwell size was controlled using the photomask dimensions, and 200, 300, and 500 μm well patterns were fabricated on the PHS.

PEG hydrogel microwell-patterned PHS was named 200-PHS, 300-PHS, and 500-PHS depending on the microwell pattern size. The surface morphologies of PNIPAAm substrates and PHS were observed using microscopy (Fig. 2a). PEG hydrogel patterns were fabricated and firmly grafted to PHS, and there was no tangible border between PNIPAAm and PEG hydrogels. PHS heights were about 200 μm , and previous studies showed that it was a proper height to form cell spheroids [31,32].

Quantum dots (QDs) were added to the PEG hydrogel precursor solution, and they were entrapped in the hydrogel matrix after UV exposure. The residual PEG hydrogel precursor solution blocked from UV by the photomask was entirely washed out; therefore, the QD fluorescence was not detectable at the PNIPAAm substrate-exposed area (Fig. 2b). The atomic composition of PNIPAAm was quantified before and after PEG hydrogel patterning to chemically confirm that unreacted

PEG hydrogel precursor solution was removed (Fig. 3 and Table 2). The N1s peak was found from the PHS sample, which was derived from the amide group, and the N1s peak was maintained after the patterning process at the PNIPAAm substrate-exposed region. The atomic composition of the PNIPAAm surface did not change markedly compared to the PEG hydrogel region composition. It was confirmed from fluorescence images of patterned substrate and surface atomic composition that the thermoresponsive substrate was exposed to the surface at the desired parts after PEG hydrogel patterning.

3.2. Thermoresponsive property of PHS

The thermoresponsive property of the substrate is required to detach following the formation of cell spheroids. The thermoresponsive property of PHS was evaluated by the transparency and volumetric changes (Fig. 4).

The molecular structure folding of PNIPAAm hydrogel affects hydrophobic/hydrophilic states depending on the temperature, and the interaction between water molecules and PNIPAAm hydrogel is changed by hydrogen bonding strength between PNIPAAm hydrogel amide groups and water molecules [33,34]. The patterned PNIPAAm hydrogel (200-PHS) showed transparencies to discriminate against the logo under the PHS below LCST, but 200-PHS became opaque when the temperature was higher than LCST (Fig. 4a). The transparency of patterned PHS was changed from 31 to 34 °C, and it corresponds with the PNIPAAm LCST. The volume of 200-PHS was calculated after measuring length, width, and thickness, and the volume at 4 °C was set to 100%. From 4 to 31 °C, the volume was maintained between 99 and 100% without a significant decline. Like with transparency, there was a significant volumetric change between 31 and 34 °C from 99.4 ± 0.7 to $93.2 \pm 1.2\%$ (Fig. 4b). The volumetric changes of 200-PHS had the same tendency as transparency, showing that physical property changes were maintained at the LCST boundary.

The contact angle was measured to evaluate the thermoresponsive surface property (Fig. 4c). The contact angle of water on PHS was measured immediately after incubating at 37 °C for 30 min, and it was 89.00°. The incubated substrate was placed at 20 °C. The water contact angle was measured when the surface temperature was 34 °C, and the contact angle was 84.46°. After reaching the temperature to 20 °C, the contact angle changed to 73.18°. At 4 °C, the water contact angle was 72.95°. The contact angle was reduced when the temperature changed from 20 to 4 °C, but there was no drastic decrease. The PHS showed physical property changes with temperature, and the hydrophilicity was increased when the temperature was decreased below LCST.

3.3. Adipose-derived stem cell (ASC) culture and spheroid formation

Adipose-derived stem cells (ASCs) were cultured to form cell spheroids on the PHS, and the cell viability was evaluated (Fig. 5a). ASCs aggregated on the PHS without PEG hydrogel microwell-patterns and tended to increase in size, but the size distribution ranged from a few microns to a few hundred microns. On day 5, the cells in the center of some spheroids with a diameter > 300 μm died. The control of spheroid size was impossible without the PEG hydrogel pattern. When the 200 and 300 μm PEG hydrogel well patterns were introduced on the PHS, single spheroids were formed in each microwell pattern. Dead

Table 1
Mechanical properties of PNIPAAm hydrogel at room temperature.

Monomer concentration (M)	Crosslinker ratio ^a	Polymerization method	Compressive modulus (kPa)	Reference
0.87	1.47E−02	Free radical (4 °C, 24 h)	153 ± 11	PHS in this study
0.21	1.20E−02	Free radical (20 °C, 24 h)	12.2	Puleo et al. ²⁷
1.07	2.97E−02	Photocuring (UV, 30 min)	81 ± 14	Fei et al. ²⁸

^a Crosslinker ratio = mol of crosslinker/mol of monomer.

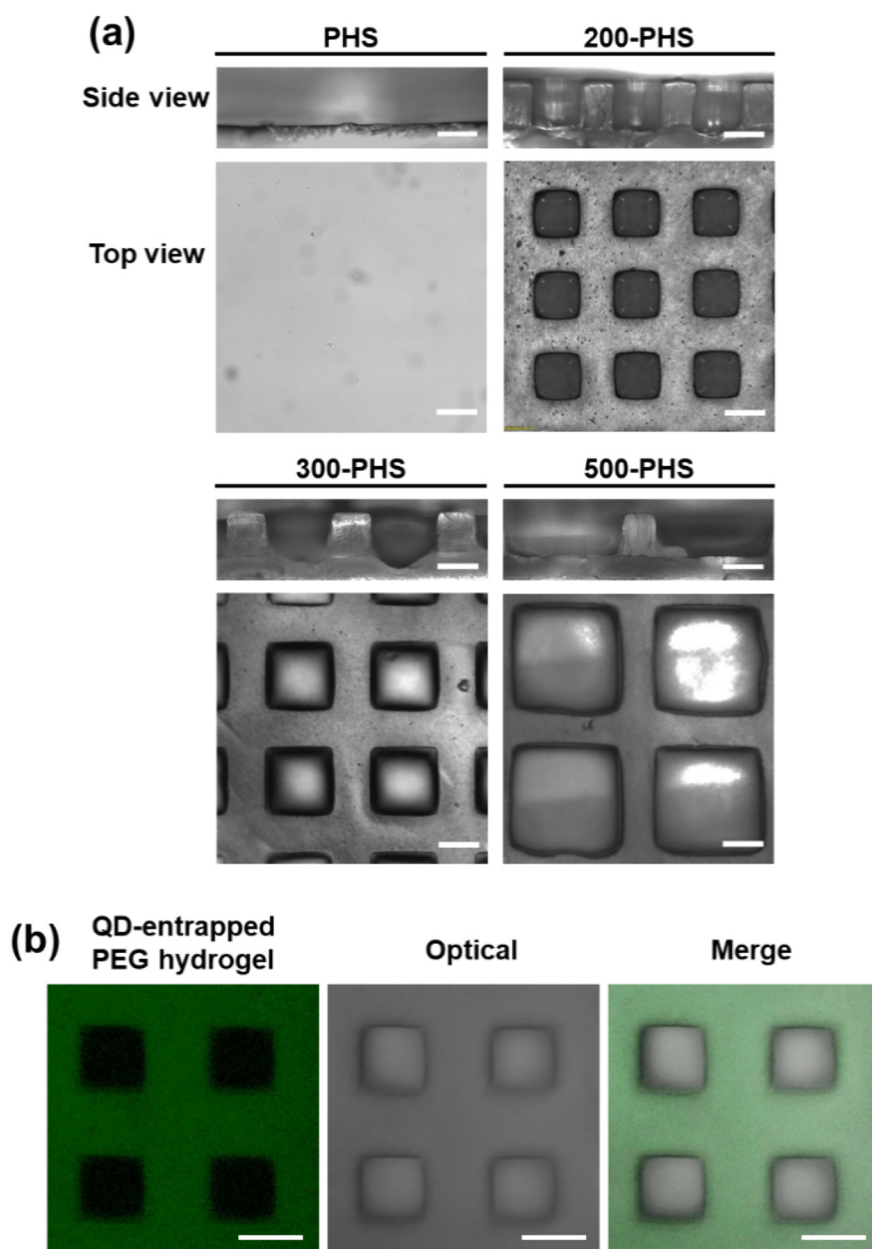


Fig. 2. Morphologies of PEG hydrogel microwell-patterned PHS. (a) Optical images of PHS and microwell-patterned PHS with different pattern sizes. The upper and lower images represent side views and top views, respectively. (b) Fluorescence and optical images of QD-entrapped PEG hydrogel grafted on PHS. Scale bars: 200 μm .

cells were not observed on the 200- and 300-PHS. Additionally, no dead cells were found on the 500-PHS, but several spheroids formed in one well instead of a single spheroid.

The sizes of the ASC spheroids were 133 ± 24 and 160 ± 30 μm on 200- and 300-PHS on day 1, respectively, and they gradually decreased until day 5. The spheroids on 300-PHS were approximately 30 μm larger than those on 200-PHS, and the size differences were maintained during the culture period. On 500-PHS, the size of the ASC spheroids was 115 ± 38 μm on day 1, and it was smaller than the spheroids on 200-PHS. The size of spheroids was slightly increased on day 3 but decreased again on day 5; 200- and 300-PHS were suitable for controlling the size of the spheroids.

The numbers of cells per spheroids on PHS were counted, and they were 142 ± 52 and 222 ± 10 for 200- and 300-PHS, respectively. The number of cells per spheroids on 500-PHS was not evaluated since more than two spheroids were formed in a well with a wide size

distribution of spheroids.

3.4. Fibronectin and laminin expression by ASC spheroids

To evaluate whether the spheroids produce extracellular matrix (ECM) proteins, ASCs were cultured on 200- and 300-PHS to obtain spheroids of uniform size, and immunostaining was performed on day 5 (Fig. 6). When ASCs were cultured on tissue culture plate (TCP), the cells grew by spreading into a monolayer without forming spheroids, and fibronectin and laminin were rarely expressed. On the other hand, the two ECM proteins were highly expressed from ASC spheroids formed on 200- and 300-PHS. The intensity of the green fluorescence region, except for the background region, was measured to quantify ECM protein expression (Fig. 6c and d). Relatively high intensities were observed for both fibronectin and laminin expression when ASCs formed spheroids. The spheroids on 200-PHS showed statistical

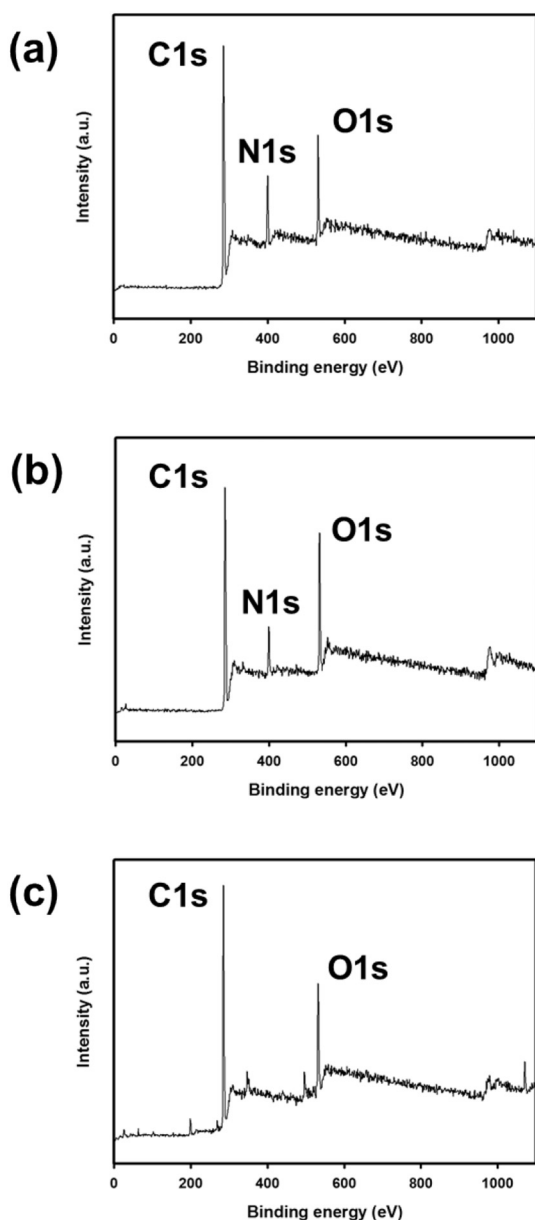


Fig. 3. XPS spectra of PNIPAAm substrate (a) before and (b) after patterning and (c) PEG region after patterning.

Table 2

The relative atomic composition of PHS before and after PEG hydrogel micro-pattern.

	Relative atomic composition (%)		
	C1s	N1s	O1s
PNIPAAm substrate before patterning	77.07	11.55	11.38
PNIPAAm region after patterning	74.25	7.86	17.89
PEG region after patterning	81.69	0.00	18.31

difference compare to the cells on TCP for fibronectin expression, but there was no statistical difference between the spheroids on 300-PHS and cells on TCP. Also, there were no statistical differences in the expression level of fibronectin and laminin between 200- and 300-PHS (Fig. 6c and d).

3.5. ASC spheroid retrieval

The retrievability of spheroids of the appropriate size and function after formation is necessary to utilize them. The PHS was incubated at 20 or 4 °C for 10 min to detach spheroids from the substrate using physical property changes of the surface, and the spheroids were disassembled from substrates by gentle pipetting without additional mechanical forces. The shape of spheroids detached by temperature change was maintained without superficial damage (Fig. 7a). The number of undetached spheroids from the substrate was counted, and the retrieval ratio was calculated (Fig. 7b). The percentage of retrieved cell spheroids was about 45 ± 12 and $74 \pm 19\%$ on day 5 at 20 and 4 °C, respectively. The lower temperature was effective for detaching ASC spheroids. Viability was assessed using LIVE/DEAD staining to investigate damage to the cells during the detachment process. It was confirmed that the number of dead cells increased compared to spheroids before reducing the temperature (Fig. 7c). It was difficult to count the live and dead cells because the aggregated cells were very dense, and therefore, the green and red fluorescence areas were measured (Fig. 7d). The average ratio of red area/green area was $2.4 \pm 1.2\%$ for the spheroids from 200-PHS and suggesting that approximately 97.5% of cells remained viable after the retrieval procedure.

4. Discussion

Adipose-derived stem cells (ASCs) are mesenchymal stem cells (MSCs) derived from adipose tissue [35]. Bone marrow-derived MSCs (BM-MSCs) have been widely used in MSC studies. However, adipose tissue is an abundant MSC source because 1% of adipose cells are estimated to be MSCs, which is 500 to 1000 times higher value than bone marrow cells [36]. ASCs can be promising MSCs for a wide range of stem cell applications; thus, they were used in this study as a cell material for spheroid formation.

Here, thermoresponsive poly(*N*-isopropylacrylamide) (PNIPAAm) was utilized to form and detach ASC spheroids, and a poly(ethylene glycol) (PEG) hydrogel microwell pattern was introduced for spheroid size uniformity. The fabrication process of the microwell patterned thermoresponsive substrate consisted of two steps (Fig. 1). The first step was a PNIPAAm hydrogel substrate (PHS) synthesis, and the second step was PEG hydrogel patterning on the PHS. In the first step, the thermoresponsive substrate was synthesized by redox initiation polymerization. *N,N'*-methylenebis(acrylamide) (MBA)-crosslinked PNIPAAm hydrogel was manufactured. Moreover, the thermoresponsive property of the resulting substrate could be controlled via the composition of MBA/PNIPAAm monomers, incubation temperature, and temperature [37–39].

We found that ASCs could not fully adhere to PHS that we fabricated (Fig. 5a), but the cells fully adhere and spread on the general PNIPAAm surface [22,26,27]. It is known that the cells could not adhere stably on a bulk PHS [40]. Our bulk PHS was fabricated with higher compositions of crosslinker and initiator than in other studies, and the high stiffness increased cell adhesion stability, as observed in another study [41]. The compressive modulus of PHS was 153 ± 11 kPa, and this high modulus compared to the general PNIPAAm hydrogel allowed the cells to adhere over LCST on the bulk PHS. In contrast, the cells do not adhere to the surface of the general bulk PNIPAAm [40]. Thus, cell spheroids can be formed while attached to the semi-adhesive surface.

The second step was introduced for spheroid size control. PEG microwell pattern was fabricated on PHS, and the cells were separated by the cell non-adhesive properties of PEG to form cell spheroids of a uniform size. PEG hydrogel has no thermoresponsive feature, while PNIPAAm hydrogel changed volume depending on the temperature. The grafting of the two hydrogels was performed by benzophenone to avoid disassembly of hydrogels.

The thermoresponsive surface should be exposed after PEG

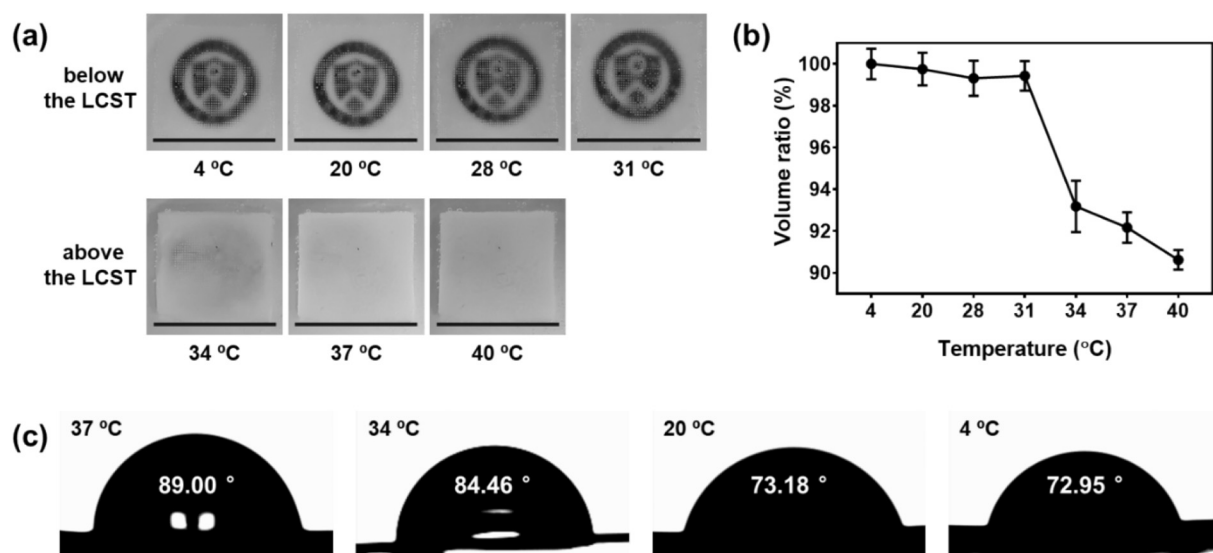


Fig. 4. Thermoresponsive behavior of PNIPAAm hydrogels. (a) Images of 200-PHS depending on temperature. Scale bars: 2 cm. (b) Volumetric changes depending on temperatures of 200-PHS ($n = 5$). (c) Contact angles of water on the PHS at 37, 34, 20, and 4 °C.

microwell patterning to retrieve cell spheroids, and thus it was evaluated using fluorescence (Fig. 2b) and XPS (Fig. 3). When we assessed the atomic composition of the PNIPAAm region after patterning, the oxygen composition was slightly increased compared to the composition before patterning. However, the atomic compositions of the PNIPAAm region before and after patterning did not deviate significantly

from PNIPAAm's theoretical atomic composition (75% C, 12.5% N, 12.5% O) [27].

The physical property changes with temperature were transparency and hydrogel substrate volume change, which are intuitive observations to detect the transition of the thermoresponsive property of the substrate (Fig. 4a and b). The transparent substrate below 31 °C became

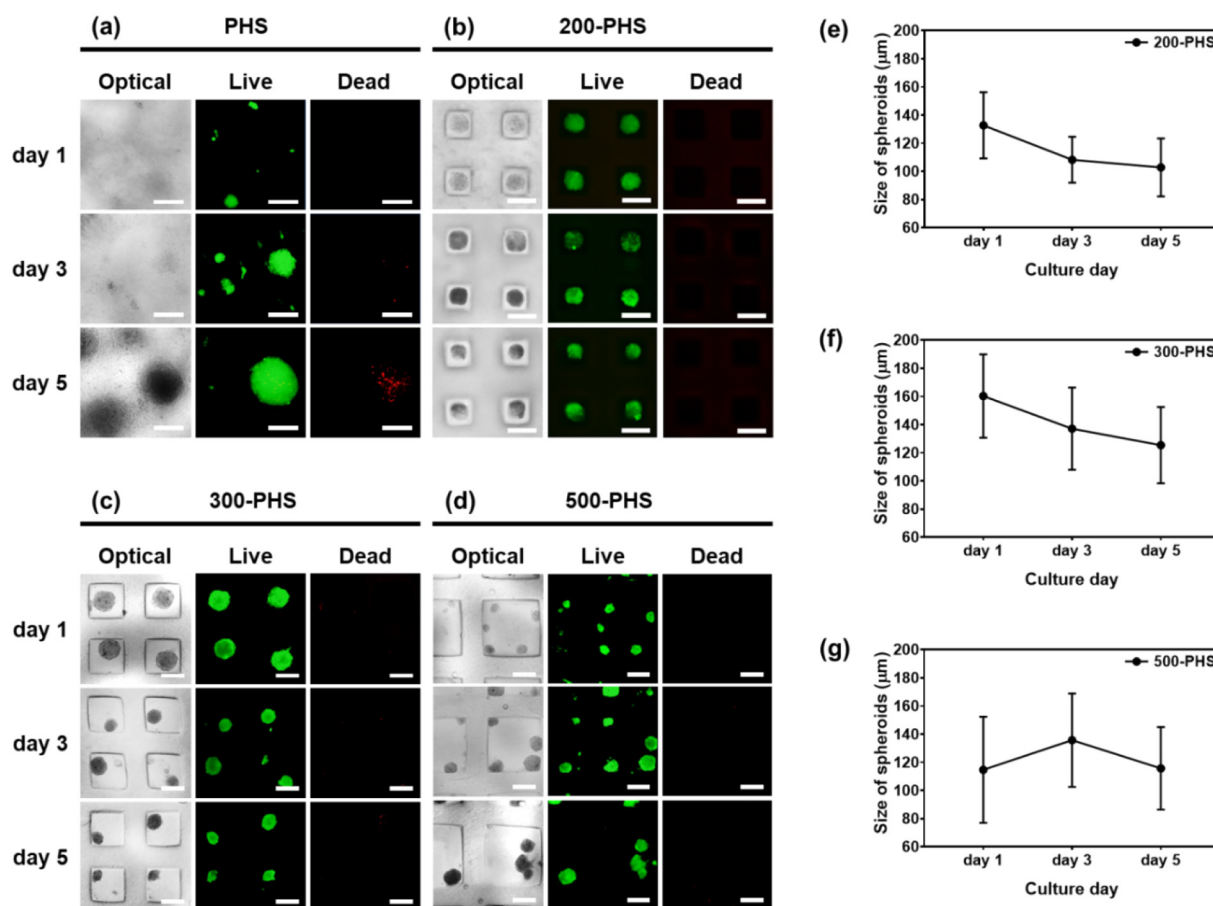


Fig. 5. Morphology and viability of ASCs cultured on the (a) PHS, (b) 200-PHS, (c) 300-PHS, and (d) 500-PHS. Scale bars: 200 μm. Spheroid sizes of ASCs on the (e) 200-PHS, (f) 300-PHS, and (g) 500-PHS ($n = 100$).

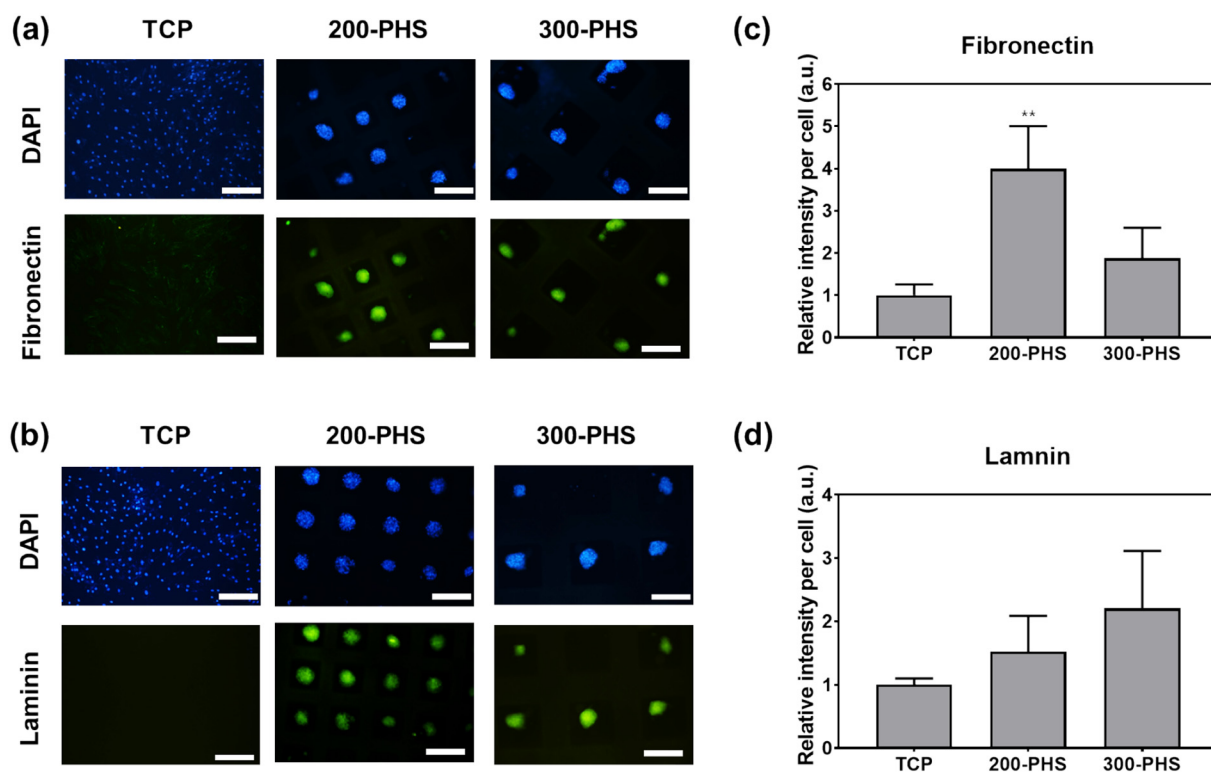


Fig. 6. Immunostaining images of (a) fibronectin and (b) laminin expressed from ASCs cultured on tissue culture plate (TCP), 200-PHS, and 300-PHS. Scale bars: 200 μ m. Relative fluorescence intensity per cell of (c) fibronectin and (d) laminin expressed from ASCs cultured on TCP, 200-PHS, and 300-PHS. Statistical analysis was performed with the TCP intensity. Significant differences are represented by $p < 0.01$ (**).

opaque between 31 and 34 $^{\circ}$ C, and it indicates the phase separation by the hydrophilic/hydrophobic transition of the PNIPAAm hydrogel matrix above LCST. Water was gradually absorbed into the PNIPAAm hydrogel matrix when the temperature was below 31 $^{\circ}$ C, reducing the light scattering and making the substrate transparent. The hydrophilicity of the PNIPAAm matrix was attenuated with a temperature increase over 34 $^{\circ}$ C, and the water molecules got out from the PNIPAAm network. Thus, the substrate became opaque due to the loss of water that could reduce light scattering, which is directly related to PHS hydrophilicity. The volume changes also confirmed the decrease in the capacity of the substrate to absorb water as the temperature increased. PHS volume was maintained between 4 and 31 $^{\circ}$ C but decreased to about 93% when the temperature passed the LCST. Generally, the volume of PNIPAAm hydrogels decreased by 60% when the temperature rose above the LCST [42].

The surface property based on the contact angle showed the thermoresponsive property (Fig. 4c). The contact angle of water was 89.00 $^{\circ}$ at 37 $^{\circ}$ C, and it decreased to 73.18 $^{\circ}$ at 20 $^{\circ}$ C. The contact angle of water was 84.46 $^{\circ}$ at 34 $^{\circ}$ C, and the hydrophilic/hydrophobic transition was progressing at the temperature. At the below LCST, there was no significant difference in water contact angle when the temperature changed from 20 to 4 $^{\circ}$ C, which is consistent with the results of transparency and volume change. Also, The contact angle of water on a typical PNIPAAm hydrogel was about 90 and 50 $^{\circ}$ above and below LCST, respectively [43]. The contact angle above LCST was similar, but the difference in the contact angle below LCST was about 20 $^{\circ}$ on our surface. It represents the degree of conversion from hydrophobic to hydrophilic was relatively small when the crosslinking density was high. As we mentioned, our PNIPAAm hydrogel was fabricated using a high composition of crosslinker and initiator, and therefore, the degree of hydrophilic/hydrophobic transition was lesser than that of general PNIPAAm molecules. However, we confirmed that the LCST was maintained regardless of the crosslinking density and degree of hydrophilic/hydrophobic transition.

The effect on cell behaviors of the fabrication process that contained high compositions of crosslinker and initiator was observed when ASCs were cultured on PHS without patterns (Fig. 5a). The cells formed aggregates like cultured cells on a nonadhesive surface, but the cell aggregates adhered to the substrate and did not float. It demonstrated that our fabricated substrate provided a semi-adhesive surface, which is an appropriate device to form cell spheroids in general cell culture conditions. However, it was difficult to control the spheroid size uniformly like other nonadhesive surface cell culture methods, and some cell aggregates over 300 μ m showed dead cells at the spheroid center due to limited transfer of nutrients, oxygen, or wastes.

The size of the ASC spheroids became uniform when PEG hydrogel micropatterns were introduced to PHS (Fig. 5b–g). In particular, one spheroid was formed in each well in 200-PHS and 300-PHS, while there were several spheroids in one well in 500-PHS. In 200-PHS and 300-PHS, the ASC spheroids sizes slightly decreased until day 5 after spheroid formation on day 1. Generally, the spheroids in the microwell tend to be dense due to size maintenance rather than growth when the PEG microwell is applied [44]. The uniform sizes of spheroids, under 200 μ m, showed high cell viability for 5 days, and dead cells were rarely observed in PHS. Based on the morphology and viability of ASC spheroids on PHS, it was shown that the PEG hydrogel microwell-patterned PHS was a simple platform to generate uniform ASC spheroids under 200 μ m.

The proper action of the 3D cellular environmental effect is important after spheroid formation; therefore, the cell-cell interactions in the cell spheroids were evaluated using fibronectin and laminin expression. The extracellular matrix (ECM) proteins, including fibronectin and laminin, have critical roles in connecting to surrounding tissues when ASCs are transplanted, and this is related to enhancing the viability of the transplanted cells with microenvironment construction [45]. ASC spheroids formed in 200-PHS and 300-PHS highly expressed fibronectin and laminin compared to conventional monolayer cultured ASCs (Fig. 6). The high expression of ECM proteins suggests that

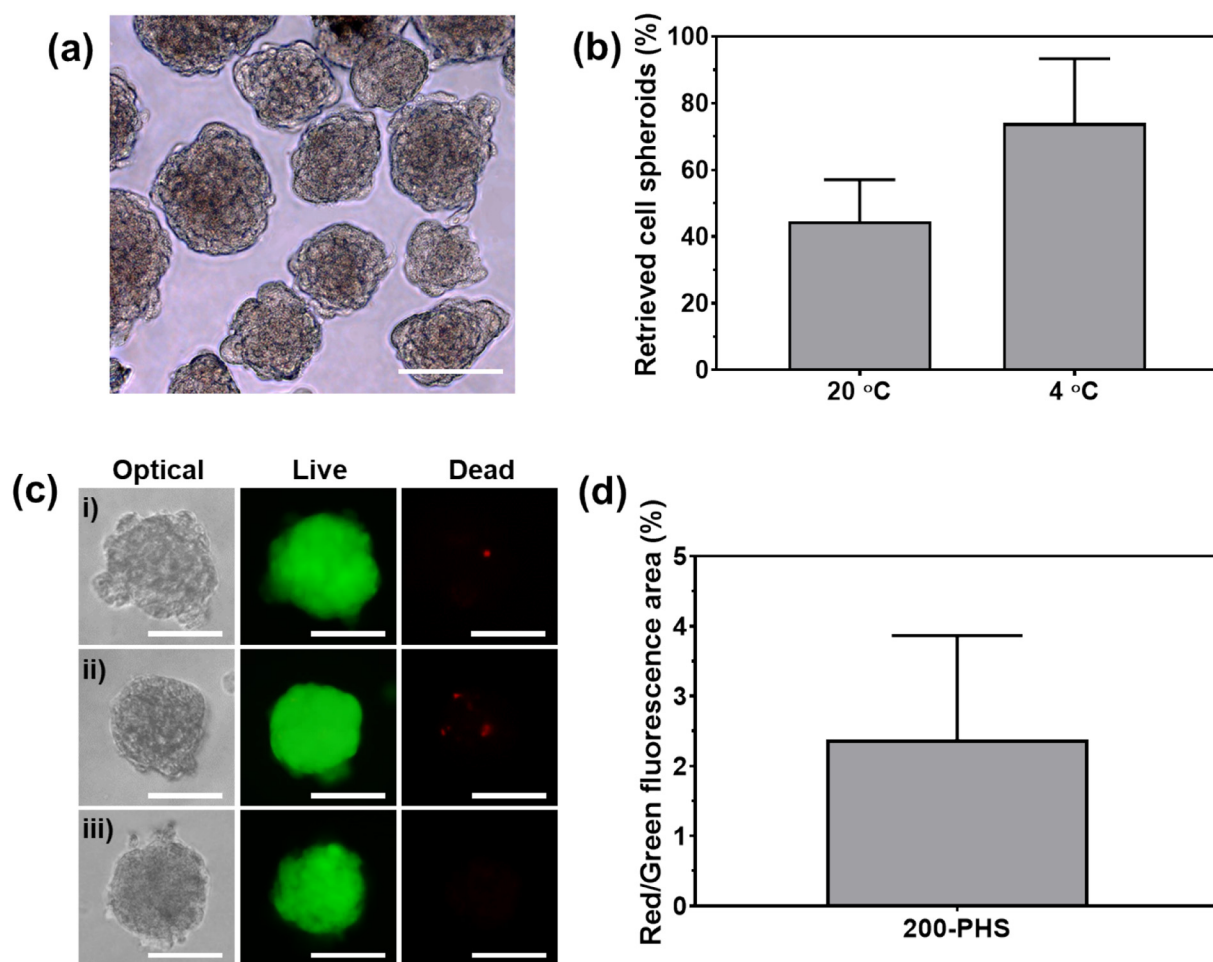


Fig. 7. ASC spheroid retrieval. (a) Image of retrieved ASC spheroids. (b) ASC spheroid retrieval efficiency from 200-PHS at 20 and 4 °C on day 5. (c) Optical and fluorescence images of retrieved ASC spheroids from 200-PHS at 4 °C on day 5. Scale bars: 100 μ m. (d) The red/green fluorescence area ratio of retrieved ASC spheroid LIVE/DEAD staining images. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

spheroid formation enhances cell-cell and cell-matrix interactions. The spheroid diameter would be a parameter to control the cell-cell interaction, but the effect of spheroid size depending on the microwell pattern size was not tested in this study. There were no significant size differences of ASC spheroids on 200- and 300-PHS; the diameters of resulting spheroids were 102 ± 21 and 125 ± 27 μ m on 200- and 300-PHS on day 5, respectively. The fibronectin expression result represented that the spheroid on 200-PHS showed higher expression level than spheroid on 300-PHS, but there were no statistical differences in the expression level of fibronectin and laminin for 200- and 300-PHS (Fig. 6c and d). The spheroid size in the 100 to 150 μ m region did not affect protein expression. Since ASC fibronectin- and laminin-expressing spheroids promoted therapeutic effects for ischemic kidney [46] and angiogenesis [47], our uniform spheroids would be a promising tool for therapeutic ASC applications.

Spheroid retrieval is necessary for the application and formation of uniform spheroids. The thermoresponsive semi-adhesive surface allowed spheroids to detach from the substrate when the temperature decreased below LCST, and the sphere morphologies were maintained after separation from PHS (Fig. 7a). We expected that ASCs formed spheroids on the semi-adhesive surface above 34 °C, and they were readily detached from PHS if the temperature decreased below 31 °C. In previous studies that used physical changes of PNIPAAm according to temperature, 20 °C or room temperature was applied as the temperature below LCST for the hydrophobic/hydrophilic transition [21,22,26,38,39]. We applied 20 °C as the temperature to detach ASC spheroids, but the number of detached spheroids from 200-PHS was less

than half (Fig. 7b). In another study, cells were maintained at 4 °C for detachment from the substrate using geometric expansion [23]; therefore, we also applied the low temperature to detach ASC spheroids by the surface physical property change. About three-quarters of ASC spheroids were dissembled from the substrate after incubation for 10 min at 4 °C.

Our substrate was the weak hydrophobic/hydrophilic transition compared to conventional PNIPAAm hydrogel and required a much lower temperature than LCST for enough surface property change to remove the spheroids. Our PHS was designed to have a semi-adhesive property, and the extent of physical property change was smaller than general PNIPAAm hydrogel based on the volume change results according to temperature. Thus, the incubation at 20 °C for 10 min achieved about 50% retrieval efficiency, and it was improved to about 75% at 4 °C. Although there was no significant volume difference between 4 and 20 °C in Fig. 4b, the rapid temperature descent would be beneficial for retrieval during the same period. The hydrophilic transition was smaller than the general PNIPAAm hydrogel, but high-efficiency spheroid retrieval was achieved at 4 °C. There was an additional effect of bulk PNIPAAm hydrogel property to detach the ASC spheroids from the surface. The surface roughness of the bulk PNIPAAm hydrogel surface varies in the micrometer range, while the roughness change of the grafting surface is only in the nanometer range [41]. The increase of surface roughness of bulk hydrogel supported ASC spheroid detachment from the surface along with the hydrophilic transition of the surface.

The cell viability after retrieval was evaluated using LIVE/DEAD staining, and there were few dead cells after the detaching process

(Fig. 7c). Counting the number of live cells from the cell spheroids was not possible, and thus, the green and red fluorescence areas were measured. The ratio of red/green fluorescence area was evaluated, which indirectly represents cell viability. The percentage of dead cells was estimated to be approximately 2.5%, and this is higher viability than about 8 to 9% from another study that used the same 4 °C and 10 min incubation conditions [23]. The diameter of spheroids commonly determines cell viability because nutrients, wastes, and gas transfer are limited, and the favorable size is 50 to 200 µm [15,48,49]. Some dead cells distributed in the outer shell, but most of the dead cells were generally observed at the center of the sphere because of the limited transfer. The outer cells could be exposed to cold damage by instantaneous low temperature or physical damage like pipetting during the detaching process.

Here, we designed a simple platform device that can form ASC spheroids of uniform size and retrieve the spheroids using a temperature stimulus. The modified composition of PHS with high compositions of crosslinker and initiator provided a semi-adhesive surface to form ASC spheroids, and PEG hydrogel microwell-patterns contributed to the uniform spheroid size. The resulting spheroids had an optimal diameter of about 100–150 µm, and the availability of ASC spheroids was demonstrated by cell viability and ECM protein expression. The mass-produced uniform spheroids were retrieved by incubating at 4 °C for 10 min for the next utilization. This substrate is a device that can assist the formation and retrieval of uniform spheroids depending on the temperature, and it will be beneficial for creating spheroids for future therapeutic applications.

5. Conclusion

Although the thermoresponsive PNIPAAm surface was utilized for many applications in the cell sheet engineering field, there was no direct spheroid formation on the PNIPAAm surface. Here, ASC spheroids of uniform size were cultured directly on the PEG hydrogel microwell-patterned PNIPAAm surface, and the resulting spheroids were retrieved while maintaining cell viability for the first time. The bulk PNIPAAm hydrogel suggested a different application direction for the thermoresponsive PNIPAAm material, and the morphological change could be combined with a hydrophobic/hydrophilic transition. We believe that further studies of bulk PNIPAAm hydrogel with adjusted compositions and mechanical properties will expand the utilization for many cell types. Our proposed PEG hydrogel patterned bulk PHS provides a new cell spheroid formation system that is convenient to retrieve. It will be a promising spheroid fabrication method for the clinical and biomedical fields.

CRedit authorship contribution statement

Gyeongju Kim:Methodology, Formal analysis, Investigation, Writing - original draft.**Yuna Jung:**Formal analysis, Investigation.**Kanghee Cho:**Formal analysis, Investigation.**Hyun Jong Lee:**Writing - review & editing, Supervision.**Won-Gun Koh:**Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the National Research Foundation (NRF) grant funded by the Ministry of Science and ICT (MSIT) (NRF-2017M3D1A1039289) and the Korea Health Technology R&D Project

through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI15C1744).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.msec.2020.111128>.

References

- [1] X. Yin, B.E. Mead, H. Safaee, R. Langer, J.M. Karp, O. Levy, *Cell Stem Cell* 18 (2016) 25–38.
- [2] D. Antoni, H. Burckel, E. Josset, G. Noel, *Int. J. Mol. Sci.* 16 (2015) 5517–5527.
- [3] S. Nath, G.R. Devi, *Pharmacol. Ther.* 163 (2016) 94–108.
- [4] E. Fennema, N. Rivron, J. Rouwkema, C. van Blitterswijk, J. de Boer, *Trends Biotechnol.* 31 (2013) 108–115.
- [5] P.R. Baraniak, T.C. McDevitt, *Cell Tissue Res.* 347 (2012) 701–711.
- [6] W. Wang, K. Itaka, S. Ohba, N. Nishiyama, U.I. Chung, Y. Yamasaki, K. Kataoka, *Biomaterials* 30 (2009) 2705–2715.
- [7] N.C. Cheng, S. Wang, T.H. Young, *Biomaterials* 33 (2012) 1748–1758.
- [8] S. Sart, A.C. Tsai, Y. Li, T. Ma, *Tissue Eng Part B Rev* 20 (2014) 365–380.
- [9] M. Schroeder, S. Niebruegge, A. Werner, E. Willbold, M. Burg, M. Ruediger, L.J. Field, J. Lehmann, R. Zweigert, *Biotechnol. Bioeng.* 92 (2005) 920–933.
- [10] S. Agastin, U.B. Giang, Y. Geng, L.A. Delouise, M.R. King, *Biomicrofluidics* 5 (2011) 24110.
- [11] S.I. Montanez-Sauri, K.E. Sung, J.P. Puccinelli, C. Pehlke, D.J. Beebe, *J Lab Autom* 16 (2011) 171–185.
- [12] X. Qiu, Y. Zhang, X. Zhao, S. Zhang, J. Wu, H. Guo, Y. Hu, *Biomaterials* 53 (2015) 600–608.
- [13] M. Bauer, L.F. Kang, Y.L. Qiu, J.H. Wu, M. Peng, H.H. Chen, G. Camci-Unal, A.F. Bayomy, D.E. Sosnovik, A. Khademhosseini, R. Liao, *PLoS One* 7 (2012) e50491.
- [14] H. Tekin, M. Anaya, M.D. Brigham, C. Nauman, R. Langer, A. Khademhosseini, *Lab Chip* 10 (2010) 2411–2418.
- [15] O. Jeon, R. Marks, D. Wolfson, E. Alsberg, J. Mater. Chem. B 4 (2016) 3526–3533.
- [16] H. Otsuka, A. Hirano, Y. Nagasaki, T. Okano, Y. Horiike, K. Kataoka, *Chembiochem* 5 (2004) 850–855.
- [17] H.S. Shin, Y.M. Kook, H.J. Hong, Y.M. Kim, W.G. Koh, J.Y. Lim, *Acta Biomater.* 45 (2016) 121–132.
- [18] C.S. Ong, X. Zhou, J. Han, C.Y. Huang, A. Nashed, S. Khatir, G. Mattson, T. Fukunishi, H. Zhang, N. Hibino, *Biotechnol. Adv.* 36 (2018) 494–505.
- [19] S. Lanzalaco, E. Armelin, *Gels* 3 (2017) 36.
- [20] M.J. Taylor, P. Tomlins, T.S. Sahota, *Gels* 3 (2017) 4.
- [21] H. Tekin, M. Anaya, M.D. Brigham, C. Nauman, R. Langer, A. Khademhosseini, *Lab Chip* 10 (2010) 2411–2418.
- [22] R. Yoshida, T. Okano, Stimuli-responsive hydrogels and their application to functional materials, in: R.M. Ottenbrite, K. Park, T. Okano (Eds.), *Biomedical Applications of Hydrogels Handbook*, Springer New York, New York, NY, 2010, pp. 19–43.
- [23] Y.B. Lee, E.M. Kim, H. Byun, H.-k. Chang, K. Jeong, Z.M. Aman, Y.S. Choi, J. Park, H. Shin, *Biomaterials* 165 (2018) 105–120.
- [24] T. Takezawa, Y. Mori, T. Yonaha, K. Yoshizato, *Exp. Cell Res.* 208 (1993) 430–441.
- [25] S. Jiang, B. Lyu, M. Müller, D. Wesner, H. Schönherr, *Langmuir* 34 (2018) 14670–14677.
- [26] N. Matsuda, T. Shimizu, M. Yamato, T. Okano, *Adv. Mater.* 19 (2007) 3089–3099.
- [27] M.A. Cooperstein, H.E. Canavan, *Biointerphases* 8 (2013) 19.
- [28] G.L. Puleo, F. Zulli, M. Piovanelli, M. Giordano, B. Mazzolai, L. Beccai, L. Andreozzi, *React. Funct. Polym.* 73 (2013) 1306–1318.
- [29] R. Fei, J.T. George, J. Park, A.K. Means, M.A. Grunlan, *Soft Matter* 9 (2013) 2912–2919.
- [30] H. Ma, R.H. Davis, C.N. Bowman, *Macromolecules* 33 (2000) 331–335.
- [31] H.W. Lee, Y.-M. Kook, H.J. Lee, H. Park, W.-G. Koh, *RSC Adv.* 4 (2014) 61005–61011.
- [32] H.J. Lee, H.-S. Kim, H.O. Kim, W.-G. Koh, *Lab Chip* 11 (2011) 2849–2857.
- [33] K. Otake, H. Inomata, M. Konno, S. Saito, *Macromolecules* 23 (1990) 283–289.
- [34] X.S. Wu, A.S. Hoffman, P. Yager, *J. Polym. Sci. A Polym. Chem.* 30 (1992) 2121–2129.
- [35] B.A. Bunnell, M. Flaatt, C. Gagliardi, B. Patel, C. Ripoll, *Methods* 45 (2008) 115–120.
- [36] J.K. Fraser, I. Wulur, Z. Alfonso, M.H. Hedrick, *Trends Biotechnol.* 24 (2006) 150–154.
- [37] R. Luo, C.-H. Chen, *Chem. Commun.* 51 (2015) 6617–6620.
- [38] Y.M. Mohan, T. Premkumar, D.K. Joseph, K.E. Geckeler, *React. Funct. Polym.* 67 (2007) 844–858.
- [39] M. Lehmann, P. Krause, V. Miruchna, R. von Klitzing, *Colloid Polym. Sci.* 297 (2019) 633–640.
- [40] R.M.P. da Silva, J.F. Mano, R.L. Reis, *Trends Biotechnol.* 25 (2007) 577–583.
- [41] A. Choi, K.D. Seo, H. Yoon, S.J. Han, D.S. Kim, *Biomaterials Science* 7 (2019) 2277–2287.
- [42] A. Burmistrova, M. Richter, M. Eisele, C. Üzüim, R. Von Klitzing, *Polymers* 3 (2011) 1575–1590.
- [43] J. Zhang, R. Pelton, Y. Deng, *Langmuir* 11 (1995) 2301–2302.

- [44] J.M. Karp, J. Yeh, G. Eng, J. Fukuda, J. Blumling, K.-Y. Suh, J. Cheng, A. Mahdavi, J. Borenstein, R. Langer, A. Khademhosseini, *Lab Chip* 7 (2007) 786–794.
- [45] S. Lee, E. Choi, M.-J. Cha, K.-C. Hwang, *Oxidative Med. Cell. Longev.* (2015) 9.
- [46] Y. Xu, T. Shi, A. Xu, L. Zhang, *J. Cell. Mol. Med.* 20 (2016) 1203–1213.
- [47] J.H. Lee, Y.-S. Han, S.H. Lee, *Biomol Ther (Seoul)* 24 (2016) 260–267.
- [48] B.-H. Liu, H.-Y. Yeh, Y.-C. Lin, M.-H. Wang, D.C. Chen, B.-H. Lee, S.-h. Hsu, *BioResearch Open Access* 2 (2013) 28–39.
- [49] X. Qiu, Y. Zhang, X. Zhao, S. Zhang, J. Wu, H. Guo, Y. Hu, *Biomaterials* 53 (2015) 600–608.