Novel Living Cell Sheet Harvest System Composed of Thermoreversible Methylcellulose Hydrogels

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In this study, a novel yet simple method, using a thermoreversible hydrogel system coated on tissue culture polystyrene (TCPS) dishes, was developed for harvesting living cell sheets. The hydrogel system was prepared by simply pouring aqueous methylcellulose (MC) solutions blended with distinct salts on TCPS dishes at 20 °C. For the applications to cell culture, only those aqueous MC compositions that may form a gel at 37 °C were chosen for the study. It was found that the hydrogel coating composed of 8% MC blended with 10 g/L PBS (phosphate buffered saline) (the MC/PBS hydrogel, with a gelation temperature of ∼25 °C) stayed intact throughout the entire course of cell culture. To improve cell attachments, the MC/PBS hydrogel at 37 °C was evenly spread with a neutral aqueous collagen at 4 °C. The spread aqueous collagen gradually reconstituted with time and thus formed a thin layer of collagen (the MC/PBS/collagen hydrogel). After cells reached confluence, a continuous monolayer cell sheet formed on the surface of the MC/PBS/collagen hydrogel. When the grown cell sheet was placed outside of the incubator at 20 °C, it detached gradually from the surface of the thermoreversible hydrogel spontaneously, without treating with any enzymes. The results obtained in the MTT assay demonstrated that the cells cultured on the surface of the MC/PBS/collagen hydrogel had an even better activity than those cultured on an uncoated TCPS dish. After harvesting the detached cell sheet, the remaining viscous hydrogel system is reusable. Additionally, the developed hydrogel system can be used for culturing a multilayer cell sheet. The obtained living cell sheets may be used for tissue reconstructions.

1. Introduction

Methylcellulose (MC) is a water-soluble polymer derived from cellulose, the most abundant polymer in nature. As a viscosity-enhancing polymer, it thickens solutions without precipitation over a wide pH range.[1] This feature makes it widely used as a thickener in the food and paint industries.[2] It is recognized as an acceptable food additive by the U.S. Food and Drug Administration.[3,4] Additionally, the physiological inertness and the storage stability of MC permit its use in cosmetics and pharmaceuticals products.[1,2]

Recently, investigations of hydrogels have been focused on functional hydrogels. These functional hydrogels may change their structures as per the environments they are exposed to such as temperature or pH.[5−14] MC gels from aqueous solutions upon heating or salt addition.[15,16] This unique phase-transition behavior of MC has made it as a promising functional hydrogel for various biomedical applications.[17,18]

Using a thermoreversible polymer, poly(*N*-isopropylacrylamide) (PNIPAAm), chemically grafted on TCPS dishes, Okano's group developed a novel technique of cell-sheet engineering for tissue reconstructions.[19−21] PNIPAAm is hydrophobic at 37 °C and hydrophilic at 20 °C, thus the cultured cells can be harvested as a continuous cell sheet after incubation at 20 °C.[18] The harvested cell sheets have been used for various tissue reconstructions, including ocular surfaces, periodontal ligaments, cardiac patches, and bladder augmentations.[22] In their method, PNIPPAm is polymerized and concurrently grafted to TCPS dishes by means of irradiation with an electron beam.[19] The whole grafting process is relatively complicated and time-consuming.[23]

In this study, a simple and inexpensive method was proposed by simply pouring aqueous MC solutions blended with distinct salts on TCPS dishes at room temperature (∼20 °C), which subsequently gelled at 37 °C (the MC hydrogel). The gelled coating at 37 °C was then evenly spread with a neutral aqueous collagen at 4 °C. The physical behaviors of the prepared MC hydrogels were reported as a function of temperature as the polymer systems transitioned from the solution to gel states. Additionally, cell cultures and their harvests on the prepared hydrogel systems were investigated.

2. Experimental Section

Preparation of Aqueous MC Solutions. MC (with a viscosity of 3000−5500 cps for a 2% by w/v aqueous solution at 20 °C) was obtained from Fluka (64630 Methocel MC, Buchs, Switzerland). Aqueous MC solutions in different concentrations (1%, 2%, 3%, or 4% by w/v) were prepared by dispersing the weighed MC powders in heated water with the addition of distinct salts (NaCl, Na_2SO_4, Na_3PO_4) or in phosphate buffered saline (PBS) in varying concentrations at 50 °C. The osmolalities of the prepared aqueous MC solutions were then measured using an osmometer (Model 3300, Advanced Instru-

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ments, Inc., Norwood, MA). For the cell-culture experiments, the prepared MC solutions were autoclaved for 30 min at 121 °C and 100 kPa.

Gelation Temperatures of Aqueous MC Solutions. The physical gelation phenomena of aqueous MC solutions with temperature were visually observed per an inversion method described in the literature[17] and measured by a differential scanning calorimeter (DSC, Pyris Diamond, Perkin-Elmer, Shelton, CT). For the former method, aqueous MC solutions blended with distinct salts (2 mL samples) were exposed to elevating temperatures via a standard hot-water bath. Their behavior was recorded at intervals of approximately 0.5 °C over the range of 20−70 °C. The heating rate between measurements was approximately 0.5 °C/min. At each temperature interval, the solutions/gels were allowed to equilibrate for 30 min. A "gel" criterion was defined as the temperature at which the solution did not flow upon inversion of the container.[17] For the latter method, a DSC was used to determine the transition temperatures of the prepared aqueous MC solutions heating from 20 to 90 °C. A heating rate of 10 °C/min was used for all test samples.

Preparation of the MC−Hydrogel Coated TCPS Dish. The prepared aqueous MC solutions that had a gelation temperature below 37 °C were used to coat TCPS dishes (Falcon 3653, diameter 35 mm, Becton Dickinson Labware, Franklin Lakes, NJ). An amount of 450 μL of test MC solutions was poured into the center of each TCPS dish at room temperature (~20 °C). A thin transparent layer of the poured solution was evenly distributed on the TCPS dish. Subsequently, the TCPS dish was preincubated at 37 °C for 1 h, and a gelled opaque layer (the MC hydrogel) was formed on the dish. To evaluate whether the salts blended in the MC hydrogel would leach out with time, the coated TCPS dish was loaded with a pre-warmed PBS at 37 °C (2 mL, with an osmolality of 280 ± 10 mOsm/kg). The osmolality of the loaded PBS solution was monitored with time. An uncoated TCPS dish loaded with the same PBS was used as a control.

For that further coated with collagen, 100 μL of aqueous type I collagen (0.5 mg/mL, bovine dermis collagen, Sigma Chemical Co., St. Louis, MO), adjusted to pH 7.4 by dialysis against PBS at 4 °C, was evenly spread onto the aforementioned TCPS dish coated with the MC hydrogel at 37 °C.

Cell Culture. Human foreskin fibroblasts (HFF) were cultured in Dulbecco's modified Eagle's minimal essential medium (12800 Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (JRH, Brooklyn, Australia) and 0.25% penicillin−streptomycin (15070 Gibco, Grand Island, NY). The cells were maintained at 37 °C with 5% CO_2, and the cultured media were changed three times a week until ready for use. After reaching confluence, cells were isolated from culture dishes with a 0.05% trypsin and then seeded uniformly on the coated TCPS dishes at a density of 4×10^4 cells/cm^2 at 37 °C. Cell attachment and growth were observed daily using a microscope. An uncoated TCPS dish and a collagen-coated TCPS dish were used as the controls. Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide, Sigma] assay. Details of the methodology used in the MTT assay were previously described.[24]

Detachment of Cell Sheets. Cells grown on the coated TCPS dishes for 1 or 2 weeks (with media changes three times per week) were taken out from the incubator with media present. The dishes were then allowed to cool at approximately 20 °C. Changes in the morphology of the cell sheets on the dishes with time were photographed every 5 s for up to 15 min.

Immunofluorescence Staining. Monoclonal mouse anticollagen types I (1:150, ICN Biomedicals, Inc., Aurora, OH) and III (1:200, Chemicon International Inc., Temecula, CA) antibodies were used for localizing type I and type III collagen secreted by HFF, respectively. A Cy5-conjugated affinity-purified goat antimouse IgG + IgM (H + L) (1.5 mg/mL, Jackson ImmunoResearch Laboratories, Inc., PA) was used as the secondary antibody for labeling the monoclonal antibody. Cell sheets grown on the coated TCPS dishes were fixed in 4% phosphate buffered formaldehyde at 37 °C for 10 min and then permeabilized with 0.1% Triton X-100 in PBS containing 1% bovine serum albumin (PBS-BSA) and RNase 100 μg/mL. After washing three times with PBS-BSA, the cell sheets were exposed to the primary antibody for 60 min at 37 °C. The cell sheets were then incubated for another 60 min with the secondary antibody (1:400) at room temperature. Additionally, the cell sheets were costained to visualize F-actins and nuclei acids by phalloidin (Oregon Green 514 phalloidin, Molecular Probes, Inc., Eugene, OR) and propidium iodide (PI, P4864, Sigma), respectively.

Subsequently, the stained cell sheets were evenly mounted on the slides and examined with excitations at 488, 543, and 633 nm, respectively, using an inversed confocal laser scanning microscope (TCS SL, Leica, Germany). Superimposed images were performed with an LCS Lite software (version 2.0).

Statistical Analysis. Statistical analysis for the determination of differences in the measured properties between groups was accomplished using one-way analysis of variance and determination of confidence intervals, performed with a computer statistical program (Statistical Analysis System, Version 6.08, SAS Institute Inc., Cary, NC). All data are presented as a mean value with its standard deviation indicated (mean ± SD).

3. Results and Discussion

Gelation of Aqueous MC Solutions. Commercial MC is a heterogeneous polymer consisting of highly substituted zones (hydrophobic zones) and less substituted ones (hydrophilic zones).[25] Aqueous MC solutions undergo a sol−gel reversible transition upon heating or cooling.[15,16,26,27] In the solution state at lower temperatures, MC molecules are hydrated and there is little polymer−polymer interaction other than simple entanglements.[28] As temperature is increased, aqueous MC solutions absorb energy (the endothermic peaks observed in the DSC thermograms discussed later) and gradually lose their water of hydration.[15] Eventually, a polymer−polymer association takes place, due to hydrophobic interactions, causing cloudiness in solution and subsequently forming an infinite gel−network structure.[28]

The temperature in forming this gel−network structure, at which the aqueous MC solution did not flow upon inversion of its container, was defined as the gelation temperature in the study. Therefore, the gelation temperature of the aqueous MC solution determined by inverting its container should be slightly greater than the onset temperature of the endothermic peak observed in its corresponding DSC thermogram.

It was reported that the addition of salts lowers the gelation temperature of the aqueous MC solution.[15,17,18] Upon the addition of salts, water molecules are placed themselves around the salts, thus reducing the intermolecular hydrogen-bond formations between water molecules and the hydroxyl groups of MC. This can increase the hydrophobic interaction between MC molecules and lead to a decrease in their gelation temperature.[18]

The salts blended in aqueous MC solutions played an important role in their physical sol−gel behavior. Examples of the DSC thermograms of aqueous MC solutions (2% by w/v) blended with distinct concentrations of NaCl are shown in Figure 1a. As shown, an endothermic peak was observed for each test sample in the heating process. The endothermic peak shifted to the left ($p < 0.05$) with increasing NaCl concentration. This indicated that the addition of NaCl in the aqueous MC solution may slow down its sol−gel transition temperature. Additionally, if a higher concentration of NaCl is used, then a lower temperature in its sol−gel transition was observed. This fact was also observed in the determination of the gelation temper-

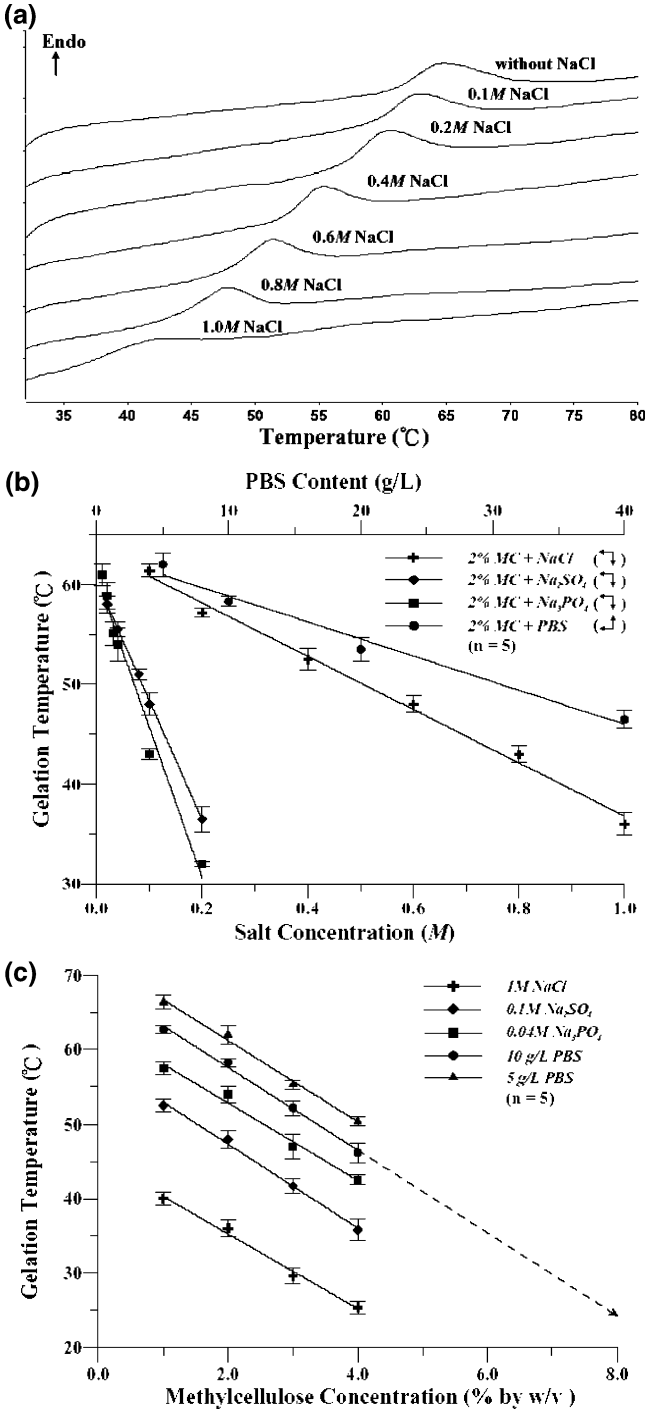

Figure 1. (a) Examples of the DSC thermograms of aqueous methylcellulose solutions (2% by w/v) blended with distinct concentrations of NaCl. Gelation temperatures of aqueous methylcellulose solutions blended with distinct salts: (b) effect of the concentration of salt and (c) effect of the concentration of methylcellulose.

ature of each test sample by inverting its container (Figure 1b). As expected, the onset temperatures of the endothermic peaks of aqueous MC solutions observed in the DSC thermograms were lower than their corresponding gelation temperatures obtained by the inversion method, ranging approximately from 1 to 3 °C (Table 1).

Similar phenomena were observed when Na_2SO_4, Na_3PO_4, or PBS was blended into aqueous MC solutions (Figure 1b and Table 1). Normally, an electrolyte (the salt blended) has a greater affinity for water than polymers resulting in the removal of water of hydration from the polymer and thus dehydrating or "salting out" the polymer. The ability of an electrolyte to salt out a polymer from its solution generally follows the salts order in the lyotropic series.[29] The cations follow the order $Li^+ > Na^+ > K^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$, and more common anions follow the order $PO_4^{3-} > SO_4^{2-} >$ tartrate $> Cl^- > NO_3^- > Br^- > I^- > SCN^-$.[29] Accordingly, more water molecules were removed from aqueous MC solutions when Na_2SO_4 or Na_3PO_4 was added, resulting in a lower gelation temperature. As shown in Figure 1b and Table 1, at the same concentration of the salt blended, generally, the gelation temperatures of aqueous MC solutions followed the order $Na_3PO_4 < Na_2SO_4 <$ NaCl ($p < 0.05$).

The effects of the addition of PBS in aqueous MC solutions on the onset temperatures of the endothermic peaks observed in the DSC thermograms and their gelation temperatures were similar to those blended with NaCl, Na_2SO_4, or Na_3PO_4 (Figure 1b and Table 1). It was reported that the effect of cations on salting-out polymers in solution is less significant than that of anions.[2,30] Therefore, salting-out MC polymers from aqueous solutions blended with PBS was mainly caused by its constituent anions such as Cl^-, HPO_4^{2-}, or $H_2PO_4^-$.[2]

It was found that the concentration of MC in aqueous solution also played a significant role in its physical sol−gel behavior. As shown in Figure 1c, the gelation temperatures of aqueous MC solutions blended with distinct salts decreased approximately linearly with increasing MC concentration. In the preparation of the aqueous MC solution, it was found that the solution was too viscous to be manipulated when the MC concentration was greater than 4% (by w/v). Therefore, no data were available when the concentration of MC was greater than this limit.

For the applications to cell culture, only those aqueous MC compositions that may form a gel (the MC hydrogel) at 37 °C were used to coat the TCPS dishes: 2% MC + 1 M NaCl, 2% MC + 0.2 M Na_2SO_4, 2% MC + 0.2 M Na_3PO_4 (Figure 1b), and 8% MC + 10 g/L PBS. For the last case, a 4% aqueous MC solution blended with 5 g/L PBS was used to coat the TCPS dish and subsequently dried in a laminar flow hood to remove 50% of its moisture content. Thus obtained MC hydrogel had a gelation temperature of about 25 °C (extrapolated from Figure 1c). As shown in Figure 1c, the gelation temperature of a 4% MC solution blended with PBS was significantly greater than 37 °C. Additionally, as mentioned above, the aqueous MC solution was too viscous to be manipulated when its concentration was greater than 4%. It was observed in the study that this specific aqueous MC solution (8% MC + 10 g/L PBS) underwent a sol−gel reversible transition upon heating or cooling at approximately 25 °C.

Stability of the Coated MC Hydrogel. It is speculated that the MC hydrogels coated on TCPS dishes may become swelled and gradually disintegrate when loaded with the cell culture media due to the differences in osmotic pressure between the two.[31,32] It was found that the osmolalities of aqueous MC solutions, used to prepare the MC hydrogels, increased nearly linearly with increasing the concentrations of the salt blended and MC (Figure 2).

To evaluate the stability of the coated MC hydrogels, a PBS solution (10 g/L) with an osmolality of 280 ± 10 mOsm/kg at 37 °C, in simulating that of the cell culture media, was loaded onto the coated TCPS dishes. The osmolality of the cell culture media is normally maintained at 290 ± 30 mOsm/kg.[33] An uncoated TCPS dish loaded with the same PBS solution was used as a control. Changes in osmolality of the loaded PBS solution with time were monitored by an osmometer. As

Table 1. Onset Temperatures (T_{onset}) of the Endothermic Peaks of Aqueous Methylcellulose Solutions (2% by w/v) Blended with Distinct Salts in Varying Concentrations Observed in the DSC Thermograms and Their Gelation Temperatures ($T_{gelation}$) Measured by an Inversion Method ($n = 5$)

NaCl						
concentration (M)	0.1	0.2	0.4	0.6	0.8	1.0
T_{onset}	59.0 ± 0.8	55.6 ± 0.3	52.0 ± 0.1	47.4 ± 0.3	42.3 ± 0.4	35.2 ± 0.3
$T_{gelation}$	61.4 ± 0.6	57.2 ± 0.4	52.5 ± 1.1	48.0 ± 0.8	43.0 ± 0.9	36.0 ± 1.1

Na_2SO_4					
concentration (M)	0.02	0.04	0.08	0.10	0.20
T_{onset}	57.3 ± 0.2	54.8 ± 0.3	50.4 ± 0.4	47.4 ± 0.3	35.1 ± 0.3
$T_{gelation}$	58.0 ± 0.8	55.5 ± 0.7	51.0 ± 0.5	48.0 ± 1.1	36.5 ± 1.3

Na_3PO_4						
concentration (M)	0.01	0.02	0.03	0.04	0.10	0.20
T_{onset}	60.1 ± 0.5	58.4 ± 0.5	54.6 ± 0.5	53.4 ± 0.3	42 ± 0.4	30 ± 0.2
$T_{gelation}$	61.0 ± 1.1	58.9 ± 1.3	55.1 ± 1.1	54.0 ± 1.7	43 ± 1.1	32 ± 1.3

PBS				
concentration (g/L)	5	10	20	30
T_{onset}	57.5 ± 0.2	55.1 ± 0.5	52.4 ± 0.3	44.1 ± 0.2
$T_{gelation}$	62.0 ± 1.2	58.3 ± 0.5	53.5 ± 1.1	46.5 ± 0.9

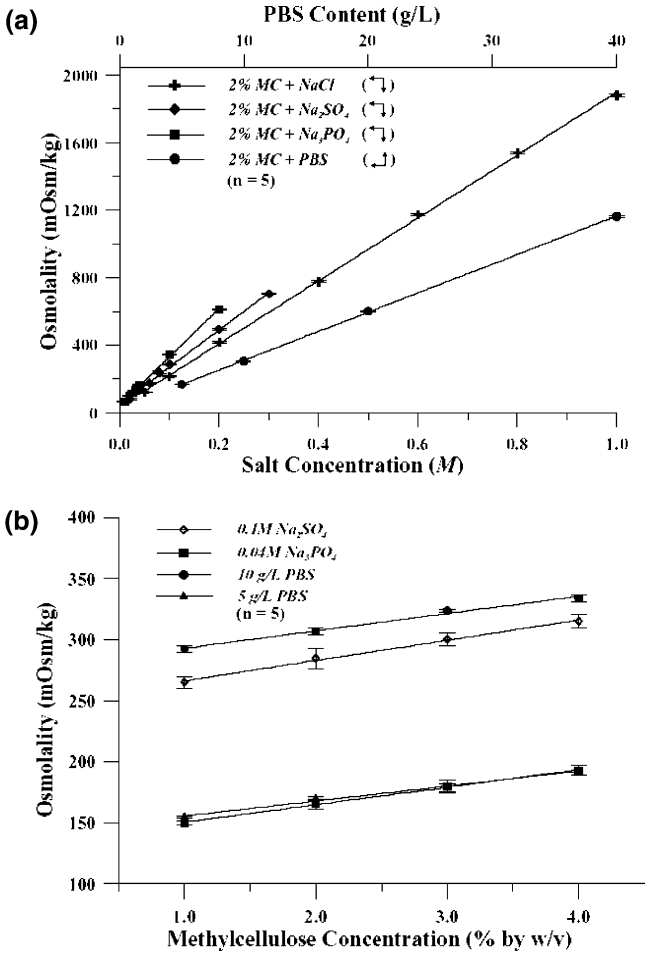

Figure 2. Osmolalities of aqueous methylcellulose solutions blended with distinct salts: (a) effect of the concentration of salt and (b) effect of the concentration of methylcellulose.

compared to the uncoated control group, the osmolalities of the loaded PBS solutions increased significantly within 1 day (>325 mOsm/kg) for the MC hydrogels blended with NaCl, Na_2SO_4, or Na_3PO_4 ($p < 0.05$, Figure 3). This observation may be attributed to the differences in osmolality between these MC hydrogels (>500 mOsm/kg, Figure 2a) and the originally loaded

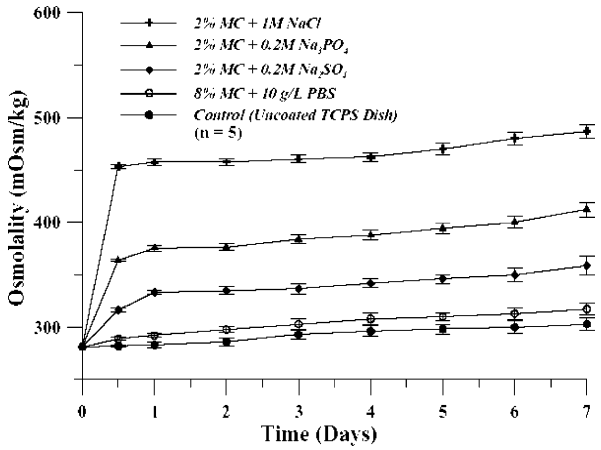

Figure 3. Changes in osmolality of the PBS solution loaded onto each studied TCPS dish with time.

PBS solutions (~280 mOsm/kg) and thus caused a significant amount of water from the loaded PBS solutions to diffuse into the MC hydrogels. This led to a significant increase in osmolality for the loaded PBS solutions together with a noticeable swelling and gradual disintegration of the MC hydrogels.

In contrast, the osmolality of the PBS solution (10 g/L) loaded on the MC hydrogel blended with PBS (10 g/L) only increased slightly as compared to the uncoated control group (Figure 3). Additionally, the MC hydrogel coated on the TCPS dish stayed intact throughout the entire course of the study. The aforementioned results indicated that the MC hydrogel blended with PBS (8% by w/v MC + 10 g/L PBS) was more suitable for cell cultures than those blended with NaCl, Na_2SO_4, or Na_3PO_4 and thus was chosen for the study (the MC/PBS hydrogel).

As shown in Figure 4a, the MC/PBS hydrogel at 20 °C was a clear, viscous solution. At 37 °C, the clear solution started to become opaque (Figure 4b). The transition of sol−gel was continuous with time. About 30 min later, a gel−network structure began to form (Figure 4c). It was found that this hydrogel was thermoreversible. Back at 20 °C, the opaque gel gradually became a clear, viscous solution again (parts d and e of Figure 4).

Cell Culture on the Surface of the MC Hydrogel. Figure 5 shows photomicrographs of cells cultured on the surface of

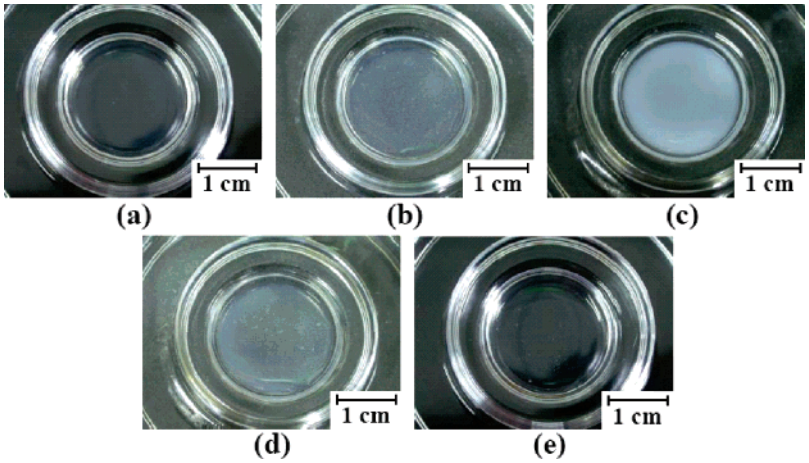

Figure 4. Photographs of the TCPS dish coated with the MC/PBS hydrogel: (a) at 20 °C, (b) at 37 °C for 5 min, (c) at 37 °C for 30 min, (d) back at 20 °C for 2 min, and (e) back at 20 °C for 20 min.

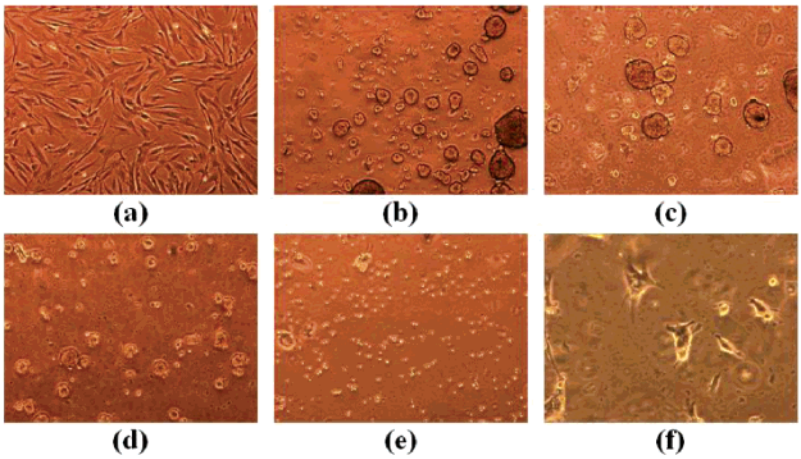

Figure 5. Photomicrographs of cells cultured on (a) an uncoated TCPS dish, 40x; (b) the TCPS dish coated with the 2% MC + 1 M NaCl hydrogl, 40x; (c) the TCPS dish coated with the 2% MC + 0.2 M Na_2SO_4 hydrogel, 40x; (d) the TCPS dish coated with the 2% MC + 0.2 M Na_3PO_4 hydrogel, 40x; (e) the TCPS dish coated with the MC/PBS (8% MC + 10 g/L PBS) hydrogel, 40x and (f) 100x.

an uncoated TCPS dish (the control group) and those coated with the MC hydrogels blended with distinct salts for 1 day, respectively. As shown, the seeded cells attached very well on the surface of the uncoated TCPS dish (Figure 5a). However, cells did not attach at all on the surfaces of the MC hydrogels blended with NaCl, Na_2SO_4, or Na_3PO_4 and mainly suspended in the culture media in the form of aggregates (parts b−d of Figure 5). In contrast, a few cells were found to attach on the surface of the MC/PBS hydrogel, and others remained suspended in the culture media (parts e and f of Figure 5).

To improve cell attachments, a neutral aqueous bovine type I collagen at 4 °C was evenly spread on the TCPS dish coated with the MC/PBS hydrogel at 37 °C (Figure 6). It was reported that under the influence of increasing temperature, collagen molecules self-assemble into a gel network.[34] Thermal triggering of collagen gelation was demonstrated at temperatures as low as 20 °C and at concentrations as low as 0.1 mg/mL.[35] Thus, a thin layer of bovine type I collagen was formed on the surface of the MC/PBS hydrogel gradually (the MC/PBS/collagen hydrogel, Figure 6).

Figure 7 presents photomicrographs of cells cultured on an uncoated TCPS dish and those coated with the MC/PBS/collagen hydrogel or collagen for 1, 3, and 7 days, respectively. The results of their relative cell activities of test-to-control evaluated by the MTT assay are shown in Table 2. As shown, after coating with the bovine type I collagen, cell attachments and proliferations were significantly improved as compared to those observed on the surface of the MC/PBS hydrogel (parts e and f of Figure 5). The results obtained in the MTT assay demonstrated that the cells cultured on the surfaces of the MC/PBS/collagen hydrogel or collagen had a better activity than those cultured on the uncoated TCPS dish ($p < 0.05$). Collagen is known to have the capacity to regulate cell behaviors such as adhesion, spreading, proliferation, and migration and thus has been used extensively to enhance cell−material interactions for both in vivo and in vitro applications.[36]

Detachment of Cell Sheets. After cells reach confluence, a continuous monolayer cell sheet formed on the surface of the MC/PBS/collagen hydrogel (Figures6 and 8a). When the grown cell sheet was placed outside of the incubator at 20 °C, it detached gradually from the surface of the thermoreversible hydrogel spontaneously, without treating with any enzymes (e.g., trypsin/EDTA, Figure 6 and parts b−j of Figure 8). It was observed that the grown cell sheet started to detach from its edge at about 2 min after cooling at 20 °C. Detachment of the entire cell sheet was completed within 20 min (or within 10 min by shaking the TCPS dish gently by hand). With the same method, a large size of living cell sheet, cultured on a coated 100-mm Petri dish, can be readily obtained in our lab and may be utilized in applications to tissue reconstructions.

For most types of cells, and especially for a connective-tissue cell, the opportunities for anchorage and attachment depend on the surrounding matrix, which is usually made by the cell itself.[37] It is known that fibroblasts are dispersed in connective tissue

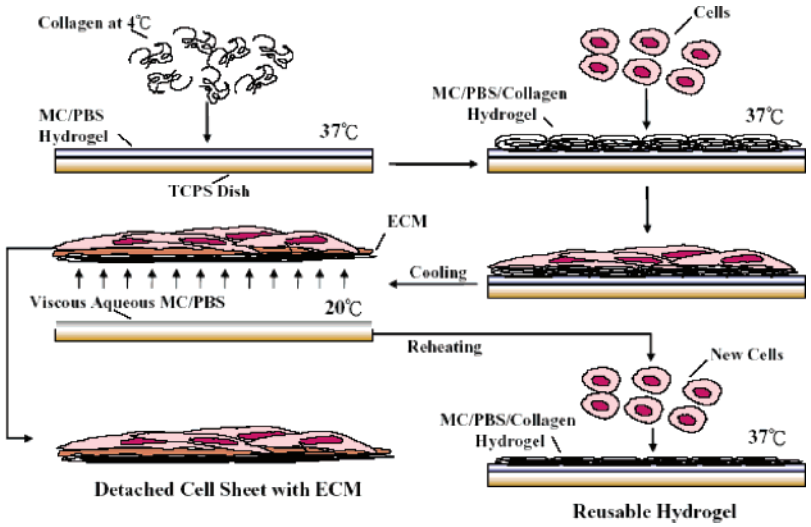

Figure 6. Schematic illustrations of cells cultured on the TCPS dish coated with the MC/PBS/collagen hydrogel and detachment of its grown cell sheet.

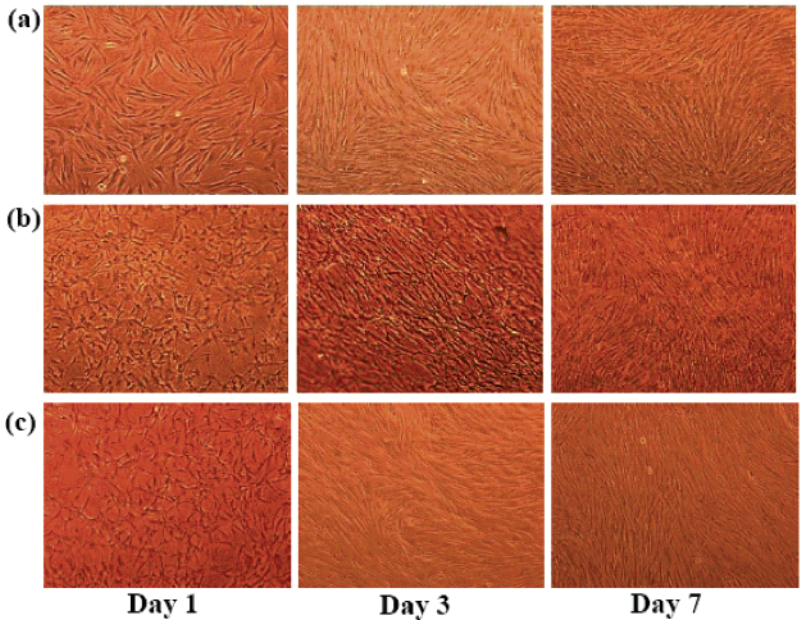

Figure 7. Photomicrographs of cells cultured on (a) an uncoated TCPS dish, 40x; (b) on the TCPS dish coated with the MC/PBS/collagen hydrogel, 40x; (c) the collagen-coated TCPS dish, 40x for 1, 3, and 7 days, respectively.

Table 2. Results of the Relative Cell Activities of Test-to-Control Obtained in the MTT Assay for the Cells Cultured on an Uncoated TCPS Dish (uncoated dish), the TCPS Dish Coated with the MC/PBS/collagen Hydrogel (coated dish), and the Collagen-Coated TCPS Dish (collagen-coated dish) for 1, 3, and 7 days, Respectively ($n = 5$)

relative cell activity[a]	day 1	day 3	day 7
uncoated dish	100.0 ± 2.3%	161.9 ± 9.4%	203.0 ± 12.3%
coated dish	159.1 ± 7.7%	286.3 ± 13.5%	339.9 ± 18.7%
collagen-coated dish	223.1 ± 3.5%	384.4 ± 7.3%	465.3 ± 14.7%

[a] The cell activity of the cells cultured on the uncoated TCPS dish for 1 day was used as a control.

throughout the body, where they secrete an extracellular matrix (ECM) that is rich in type I and/or type III collagen.[37] The detached cell sheet was fixed and immunostained with antitype I or type III collagen and subsequently costained with phalloidin for F-actins and propidium iodide for nuclei acids.

Results of the immunofluorescence images of the cell sheets grown on the MC/PBS/collagen hydrogel for 1 and 2 weeks are shown in Figure 9. As shown, the F-actins (in green) and cell nuclei (in red) of the cultured cells (HFF) together with the secreted type III collagen (in blue) were clearly identified. Type I collagen was also found in the study (data not shown). However, the labeled type I collagen may be from the originally coated bovine collagen or that secreted by the cultured cells. These results indicated that the cultured cells can secrete their own ECM during culture. On the contrary, the originally coated bovine type I collagen may be degraded gradually. It was reported that human skin fibroblasts can secrete collagenase as two proenzyme forms. These enzymes play an essential role in the maintenance of the ECM during tissue development and remodeling.[38]

Applications of the Developed Technique. After harvesting the detached cell sheet, the remaining viscous MC/PBS hydrogel can be reused after recoating a thin layer of type I collagen on its surface as described before (Figure 6). Additionally, a

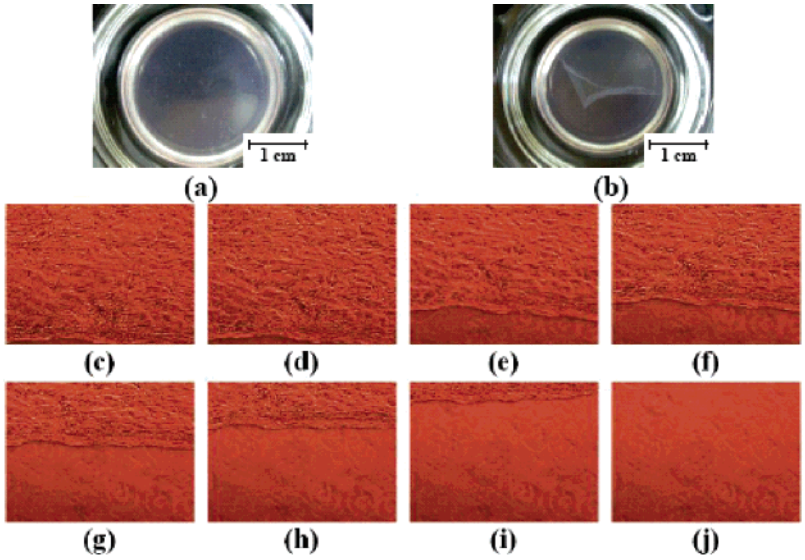

Figure 8. Photographs of (a) a grown cell sheet on the TCPS dish coated with the MC/PBS/collagen hydrogel and (b) its detaching cell sheet. Photomicrographs of the detaching cell sheet with time (c−j), 40x.

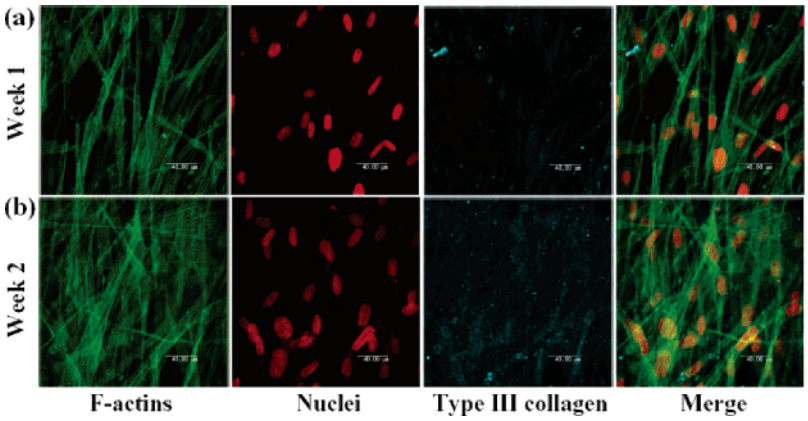

Figure 9. Immunofluorescence images of the cell sheets grown on the TCPS dish coated with the MC/PBS/collagen hydrogel for (a) 1 week and (b) 2 weeks.

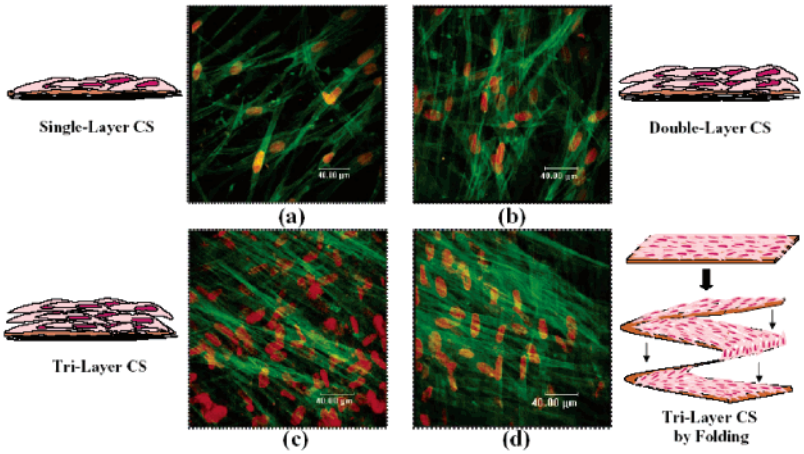

Figure 10. Immunofluorescence images of (a) a single-layer cell sheet (CS), (b) a double-layer cell sheet, and (c) a trilayer cell sheet obtained from the TCPS dish coated with the MC/PBS/collagen hydrogel and (d) a trilayer cell sheet obtained by folding a single-layer cell sheet.

multilayer cell sheet can be obtained with one of the following two methods. For the first method, a double-layer cell sheet can be obtained by seeding new cells directly on top of the first grown cell sheet (without detaching it from the surface of the MC/PBS/collagen hydrogel) and then culturing until confluence (Figure 10b). The same procedure can be repeated again to obtain a trilayer cell sheet (Figure 10c). The other method is to fold the detached cell sheet into multilayers and reculture it. The folded multilayer cell sheet would then stick together between layers within 2 days and form an integrated multilayer cell sheet (Figure 10d).

The aforementioned single-layer or multilayer cell sheets may be used in the applications to tissue reconstructions. Cell sheet engineering is being developed as an alternative approach for

tissue engineering. It may have the advantages of eliminating the use of biodegradable scaffolds and maintaining the cultured cell−cell and cell−ECM interactions.[21,39,40]

4. Conclusions

In the study, a novel method, using a thermoreversible MC/PBS/collagen hydrogel coated on the TCPS dish, was developed for harvesting a living cell sheet with ECM. The coated hydrogel system is reusable and can be used for culturing a multilayer cell sheet. The obtained living cell sheets may be used for tissue reconstructions.

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