Novel Method of Forming Human Embryoid Bodies in a Polystyrene Dish Surface-Coated with a Temperature-Responsive Methylcellulose Hydrogel

Mei-Ju Yang,[†,‡,§] Chun-Hung Chen,[‡,§] Pei-Ju Lin,[†] Chih-Hao Huang,[‡] Wannhsin Chen,[*,†] and Hsing-Wen Sung[*,‡]

Biomedical Engineering Research Laboratories, Industrial Technology Research Institute, Hsinchu, Taiwan, Republic of China, and Department of Chemical Engineering, National Tsing Hua University, Hsinchu, Taiwan, Republic of China

Received April 17, 2007; Revised Manuscript Received May 28, 2007

A temperature-responsive hydrogel composed of aqueous methylcellulose (MC) blended with distinct concentrations of PBS was prepared and characterized. The developed MC hydrogel underwent a sol−gel reversible transition upon heating or cooling at ∼32 °C. This temperature-responsive hydrogel was employed to coat the surface of a polystyrene dish and used to cultivate human embryonic stem (hES) cell clumps for the formation of embryoid bodies (EBs) in liquid suspension culture (LSC-MC/PS). The conventional hanging drop culture (HDC) and LSC in the uncoated polystyrene dish (LSC-PS) or in the Corning Ultralow-Attachment plate (LSC-ULAP) were used as controls. The results indicated that LSC-PS failed to generate EBs in an efficient manner, whereas the efficiencies of EB formation observed in LSC-ULAP and LSC-MC/PS were significantly greater than in HDC. The hES cells within the EBs were shown to express molecular markers specific for representative cells from the three embryonic germ layers. These results indicated that the MC-coated dish can be used to produce a large scale of hES cell derivatives through the formation of EBs.

1. Introduction

Human embryonic stem (hES) cells are of significant interest as a renewable source of therapeutically useful cells. ES cell aggregation is important for the formation of embryoid bodies (EBs) and the subsequent generation of ES cell derivatives.[1,2] Several culture methods such as forced aggregation, spinner flask, and reactor-based cultures[1,3−5] have been developed to produce a large scale of ES cell derivatives through the formation of EBs for further applications. These methods, however, may have the disadvantage of generating shear forces, and although manageable, these forces still damage the cells.[6−8]

Other methods including the conventional hanging drop culture (HDC) and the liquid suspension culture (LSC) in bacterial-grade polystyrene dishes have been reported in the literature.[8,9] To reduce the adhesion of cells onto substrate during LSC, a Corning Ultralow-Attachment plate (ULAP) was recently used to form aggregates in the ES cell differentiation culture.[10,11] It has been shown to successfully inhibit the attachment of anchorage dependent cells.[12] However, each of the above-mentioned methods is suffered by at least one of the drawbacks, such as time-consuming, labor intensive, complicated production process, unable to scale up, low efficiency of EB formation, or economical impracticality.[1,2,8,9,13,14]

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.[15] Owing to this feature, MC has been added in media (i.e., the conventional semisolid MC culture medium) for the culture of mouse ES cells. With MC in media, the viscous liquid reduced ES cell movement and aggregation and resulted in a higher efficiency of EB formation.[8] Nevertheless, the replacement of semisolid MC media during cell culture is rather difficult due to their high viscosity.

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. Aqueous MC undergoes a sol−gel reversible transition upon heating or cooling.[16,17] This unique temperature-responsive behavior of MC has made it a promising functional hydrogel for various biomedical applications.[18,19]

In the study, a temperature-responsive hydrogel composed of aqueous MC blended with phosphate buffered saline (PBS) was prepared and characterized and subsequently was used to coat the surface of a polystyrene dish. The coated dish was prepared by pouring an aqueous MC blended with PBS in the dish at room temperature (∼20 °C). After being gelled at 37 °C (i.e., in an incubator), a thin layer of MC hydrogel was evenly coated on the surface of the dish. The MC-coated dish was then used to cultivate hES cell clumps for the formation of EBs in LSC (LSC-MC/PS, Figure 1a). Differentiation of hES cells into EBs comprising the three embryonic germ layers was investigated. The conventional HDC and LSC in the bacterial-grade polystyrene dish (LSC-PS) or in the ULAP (LSC-ULAP) were used as controls. Additionally, using this novel LSC method, the differentiation of cells with features of hepatocytes from hES cells was reported.

* Authors to whom correspondence should be addressed. Fax: +886-3-572-6832 (H.-W. S.). E-mail: WhsinChen@itri.org.tw; hwsung@che.nthu.edu.tw.

† Industrial Technology Research Institute.

‡ National Tsing Hua University.

§ Contributed equally to this work.

10.1021/bm0704166 CCC: $37.00
Published on Web 08/03/2007

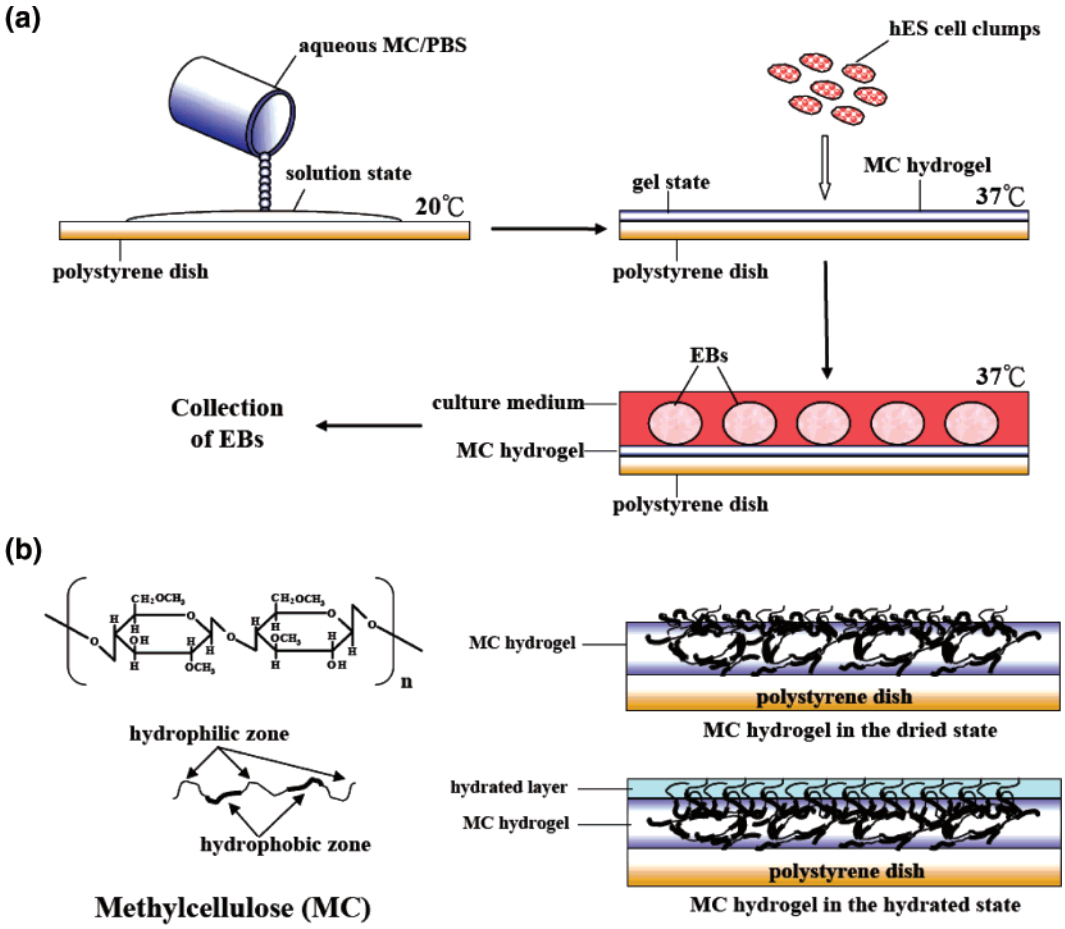

Figure 1. Schematic illustrations of (a) the procedure used to prepare for the MC-coated polystyrene dish for the hES cell differentiation culture and (b) the physical structures of the MC hydrogel coated in the polystyrene dish in the dried or hydrated state. MC: methylcellulose; hES cell: human embryonic stem cell; EBs: embryoid bodies; PBS: phosphate buffered saline.

2. Materials and Methods

2.1. Preparation of Aqueous MC. MC (cell culture grade, viscosity 15 cP for a 2% aqueous solution at 20 °C, Mn 14 000, methoxy substitution 27.5−31.5%, degree of substitution 1.5−1.9) was purchased from Sigma-Aldrich (St. Louis, MO). Aqueous MC solutions in different concentrations (6%, 9%, 12%, and 15% by w/v) were prepared by dispensing the weighed MC powders in heated water with the addition of PBS (cell culture grade) at varying known concentrations (2.5, 5.0, and 10.0 g/L) at 50 °C. After being thoroughly shaken, they were kept in a refrigerator at 4 °C for 24 h until homogeneous MC solutions were obtained. The osmolality of the prepared MC solutions was measured using an osmometer (model 3300, Advanced Instruments, Inc., Norwood, MA). For the cell-culture experiments, the prepared MC solutions were autoclaved for 30 min at 121 °C, 100 kPa.

2.2. Gelation Temperatures of Aqueous MC. The physical gelation phenomena of aqueous MC with temperature were visually observed as per an inversion method described in the literature.[18] Briefly, aqueous MC solutions blended with distinct concentrations of PBS (2 mL samples) were exposed to elevating temperatures via a standard hot-water bath. 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.[18]

2.3. Preparation of the MC-coated Polystyrene Dish. A total of 1 mL of test MC solutions was poured into a commercially available polystyrene dish (Falcon 351008, diameter 35 mm, Becton Dickinson Labware, Franklin Lakes, NJ) at room temperature (~20 °C). A thin transparent layer of the poured solution was evenly distributed on the surface of the dish. Subsequently, the dish was preincubated at 37 °C for 1 h and a gelled opaque layer (the MC hydrogel, with a thickness of 1.5−2.0 mm) was formed on the dish. To evaluate whether the salts blended in the MC hydrogel would leach out with time, the coated dish was loaded with a pre-warmed PBS at 37 °C (2 mL, with an osmolality of 280 ± 5 mOsm/kg). The osmolality of the loaded PBS solution was monitored with time. An uncoated polystyrene dish loaded with the same PBS was used as a control. Additionally, the surface hydrophilicity of the MC-coated dish in the dried or hydrated state (at 37 °C) was examined by measuring its water contact angle using deionized water. The measurements were performed on five different spots on test samples.

2.4. Culture of hES Cells. The derivation, routine culture, and characterization of the hES cell line H9 were previously described[20,21] and approved by the institutional review board (IRB). hES cells were maintained on feeders (mitomycin-C inactivated mouse embryonic fibroblasts, MEF) in ES media, which contained 85% DMEM/Ham's F12 medium supplemented with 15% knockout serum replacement, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, and 4 ng/mL human basic fibroblast growth factor (Gibco Invitrogen Corporation, Grand Island, NY). Cultures were passaged as cells became subconfluent (i.e., about once a week) by incubation in 200 units/mL collagenase IV (Gibco) for 5−10 min at 37 °C, dissociated, and then seeded on a fresh MEF feeder layer.

2.5. EB Formation. To induce EB formation, undifferentiated hES cell colonies were split into small clumps using Dispase and mechanical dissecting. The obtained hES cell clumps were grown in HDC or LSC. For HDC, cell clumps were plated as hanging drops as described previously.[8] For LSC, cell clumps (approximately 50 pieces per dish, corresponding to a total cell concentration of 1.5−2.0 × 10^5 cells per

Table 1

gene	primer-probe sequence 5′-3′	product size	annealing temp (°C)	cycle no.
β-actin	F: TGGCACCACACCTTCTACAATGAGC R: GCACAGCTTCTCCTTAATGTCACGC	387 bp	60	25
Oct-4	F: AAGAACATGTGTAAGCTGCGGCCC R: GGAAAGGCTTCCCCCTCAGGGAAAGG	454 bp	60	25
nanog	F: ATGAGTGTGGATCCAGCTTGTCC R: TCACACGTCTTCAGGTTGCATGTT	918 bp	60	25
α-fetoprotein (α-FP)	F: AGAACCTGTCACAAGCTGTG R: GACAGCAAGCTGAGGATGTC	675 bp	56	25
cardiac actin	F: TCTATGAGGGCTACGCTTTG R: CCTGACTGGAAGGTAGATGG	668 bp	60	25
keratin	F: AGGAAATCATCTCAGGAGGAAGGGC R: AAAGCACAGATCTTCGGGAGCTACC	782 bp	68	25
albumin	F: CCTTTGGCACAATGAAGTGGGTAACC R: CAGCAGTCAGCCATTTCACCATAGG	350 bp	68	25
tyrosine aminotransferase (TAT)	F: CTAGAAGCTAAGGACGTCAT R: GAGGAAGCTCAGAGTGTTGT	642 bp	60	30

dish) were cultivated in suspension in the Falcon 35-mm polystyrene dish (LSC-PS), the Corning ULAP (LSC-ULAP, 6 well plate, Acton, MA),[22] or the above-mentioned MC-coated polystyrene dish (LSC-MC/PS). EBs were grown in media consisting of 80% DMEM, supplemented with 20% fetal bovine serum (HyClone, Utah), 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, 1 mM L-glutamine, and 1% insulin-transferrin-selenium-G supplement (Gibco). The number of EBs formed per dish, cultivated by different methods, was counted using a light microscope.

Photomicrographs of representative EBs were taken for diameter measurements, using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) at a $\times$40 magnification and converted to mm. To dissociate EBs for single cell analysis, EBs were subjected to 0.125% trypsin/0.5 mM EDTA digestion for 10−20 min. The resulting single cell suspension was subjected to trypan blue dye exclusion to determine total viable cell concentrations of each culture at a given time point 10 days after differentiation.

2.6. Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from undifferentiated hES cells and the EBs after distinct culture periods in HDC, LSC-PS, LSC-ULAP, and LSC-MC/PS using RNeasy Mini Kit (Qiagen, Valencia, CA). Complementary DNA was synthesized from 50 ng of total RNA using One-Step RT-PCR Kit (Qiagen). PCR conditions included an initial denaturation step at 95 °C for 15 min, followed by repeated cycles at 95 °C for 30 s, a 30 s annealing step, and a final step of 1 min extension at 72 °C. Details of PCR primer sequences, sizes of final products, annealing temperatures and cycle numbers are described below. Products were analyzed on 1.5% agarose gel and visualized with ethidium bromide staining (see Table 1).[13]

2.7. Hepatocyte Differentiation. Day-7 EBs maintained in LSC-MC/PS were allowed to attach to a collagen I-coated dish (5 μg/cm^2) in an SF medium supplemented with hepatocyte growth factor (HGF, 50 ng/mL, R&D systems, Minneapolis, MN) and fibroblast growth factor (FGF4, 10 ng/mL, R&D systems) as previously described.[11] The medium was changed every 2 days. Total RNA extracted from undifferentiated and differentiated hES cells were analyzed by RT-PCR. The mRNA from the C3A hepatoma cells (Cat. #CRL-10741, ATCC, Manassas, VA) was used as a control.

2.8. Immunostaining. The differentiated hES cells were fixed in 4% paraformaldehyde for 2 h and then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Either goat anti-human α-FP (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-human albumin (Biogenesis, NH) was used as a primary antibody and revealed by a peroxidase-antiperoxidase technique.[23] Isotype matched IgG (Jackson Immunoresearch Laboratories, West Grove, PA) was used as a negative control. Immunostained cells were observed under an inverted microscope (ECLIPSE-TE2000-U, Nikon, Tokyo, Japan).

2.9. 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 $\pm$ SD). Differences were considered to be statistically significant when the p values were less than 0.05.

3. Results and Discussion

Commercial MC is a heterogeneous polymer consisting of highly methoxy-substituted zones (hydrophobic zones) and less substituted ones (hydrophilic zones, Figure 1b).[24] Aqueous MC gels upon heating, due to hydrophobic interactions.[25,26] It was found that the gelation temperature of a 12% (by w/v) aqueous MC was approximately 41 $\pm$ 1.5 °C (n = 5). This temperature is apparently too high for the specific application proposed in the study. It is known that blending of salts in an aqueous MC lowers its gelation temperature.[16,18,19] The salts blended usually have a greater affinity for water molecules than polymers, resulting in the removal of water of hydration from polymers and thus dehydrating or "salting out" the polymeric molecules. This can further increase the hydrophobic interaction among MC molecules and lead to a decrease in their gelation temperature.[19,26]

3.1. Gelation Temperatures of Aqueous MC. In the study, PBS was blended in aqueous MC to further reduce its gelation temperature. PBS is a salty solution containing sodium chloride, sodium phosphate, and potassium phosphate. It is a buffer solution commonly used in biochemistry. PBS has many uses because it is isotonic and nontoxic to cells. It can be used to dilute substances and to disengage the attached and clumped cells.[27]

As shown in Figure 2a, the gelation temperature of aqueous MC blended with PBS decreased significantly the MC concentration was increased. At the same MC concentration, the gelation temperature of aqueous MC decreased when the concentration of PBS used was increased. As described before, for the specific application proposed in the study, the prepared aqueous MC must have a gelation temperature significantly

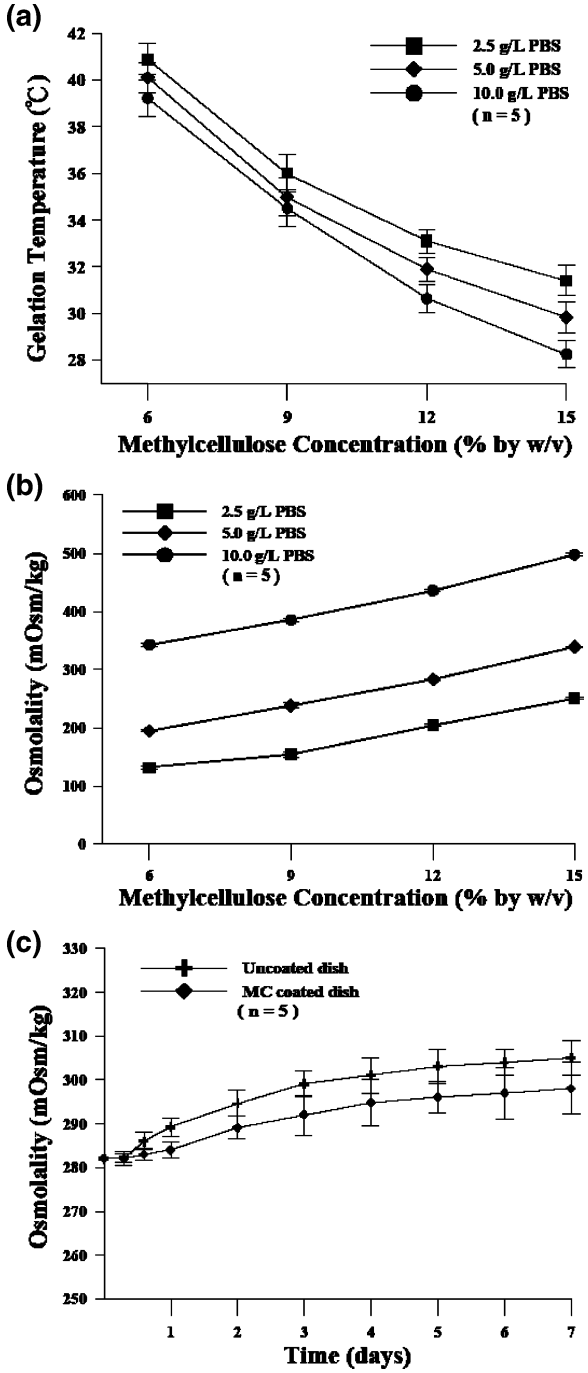

Figure 2. (a) Gelation temperatures and (b) osmolality values of aqueous MC blended with distinct concentrations of PBS; (c) Changes in osmolality of the PBS loaded in the MC-coated polystyrene dish with time. An uncoated polystyrene dish was used as a control. MC: methylcellulose; PBS: phosphate buffered saline.

below 37 °C. It was found that aqueous MC was too viscous to be manipulated when its concentration was close to 15% (by w/v). Therefore, the MC concentration used in the study for coating polystyrene dishes was 12%.

The osmolality values of aqueous MC solutions blended with distinct concentrations of PBS are shown in Figure 2b. As shown, the aqueous MC (12% by w/v) blended with 5 g/L PBS had an osmolality value of 283 ± 2 mOsm/kg, which is comparable to that of a culture medium (290 ± 30 mOsm/kg) normally maintained for cultivating cells.[28] Therefore, the composition of aqueous MC chosen in the study to coat polystyrene dishes was 12% aqueous MC (by w/v) blended with

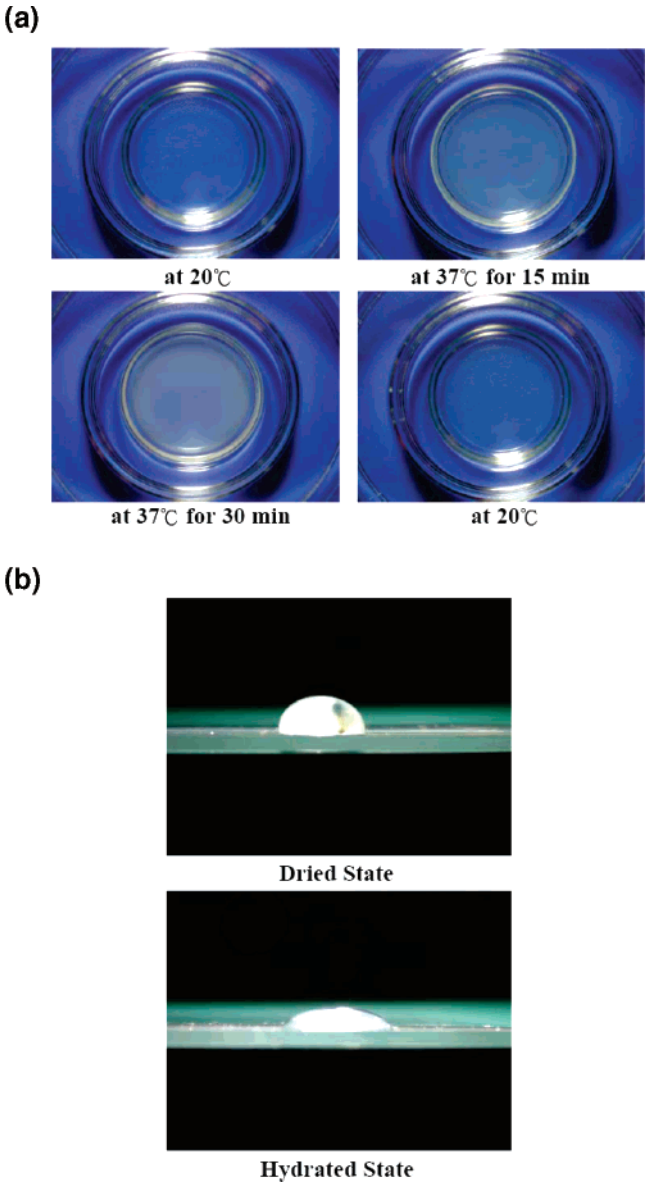

Figure 3. (a) Photographs of the MC hydrogel coated in a polystyrene dish at distinct temperatures; (b) Photographs of a water drop on the surface of the MC hydrogel coated in a polystyrene dish in the dried or hydrated state. MC: methylcellulose.

5 g/L PBS. We found that this specific aqueous MC underwent a sol−gel reversible transition upon heating or cooling at approximately 32 ± 1 °C (n = 5, Figure 2a). It was reported that a large hysteresis loop was observed in the heating/cooling circle of aqueous MC due to different kinetics of association and dissociation of MC in water.[29]

To evaluate the stability of the MC hydrogel coated on polystyrene dishes, a PBS solution (10 g/L) with an osmolality of 280 ± 5 mOsm/kg at 37 °C, simulating that of a cell culture medium, was loaded in the coated dish. An uncoated 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 shown in Figure 2c, the osmolality of the PBS loaded in the MC-coated dish was comparable to that of the control one. Additionally, the MC hydrogel coated on the polystyrene dish stayed intact throughout the entire course of the study.

3.2. Characterization of the MC-coated Polystyrene Dish. As shown in Figure 3a, the MC hydrogel in a polystyrene dish at room temperature (~20 °C) was a clear viscous solution. At

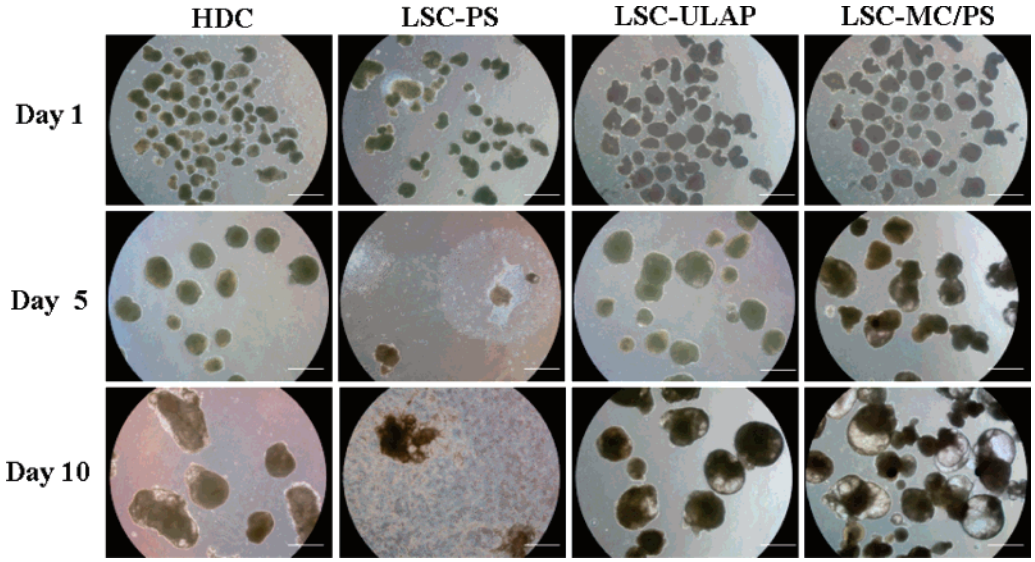

Figure 4. Photomicrographs of the hES cell clumps cultivated by different methods for distinct periods (original magnification 40x). HDC: hanging drop culture; LSC-PS: liquid suspension culture in the polystyrene dish; LSC-ULAP: liquid suspension culture in the Corning Ultralow Attachment plate; LSC-MC/PS: liquid suspension culture in the MC-coated polystyrene dish. hES cell: human embryonic stem cell; MC: methylcellulose. Scale bars, 1.0 mm.

Table 2. Numbers of Total and Cystic EB and Their Formation Efficiencies (Relative to the Original hES Cell Clumps) Cultivated by Different Methods for Distinct Periods[a]

	total EB number and their formation efficiency				cystic EB number and their formation efficiency			
	HDC	LSC-PS	LSC-ULAP	LSC-MC/PS	HDC	LSC-PS	LSC-ULAP	LSC-MC/PS
day 5								
total number	125	15	142	141	27	0	26	36
efficiency (%)	86.2	10.3	97.9	97.2	18.6	0	17.9	24.8
day 10								
total number	108	7	133	132	64	1	83	98
efficiency (%)	74.4	4.8	91.7	91.0	44.1	0.7	57.2	67.5

[a] HDC: hanging drop culture; LSC-PS: liquid suspension culture in the polystyrene dish; LSC-ULAP: liquid suspension culture in the Corning Ultralow Attachment plate; LSC-MC/PS: liquid suspension culture in the MC-coated polystyrene dish. There were 145 hES cell clumps in each studied group in the beginning. MC: methylcellulose; EB: embryoid body.

37 °C, the clear solution started to become opaque. The transition of sol−gel was continuous with time. At about 30 min later, a gel-network structure began to form. It was found that this hydrogel was thermoreversible. Back at room temperature, the opaque gel gradually became a clear viscous solution again.

The surface hydrophilicity of the MC hydrogel coated on polystyrene dishes in the dried or hydrated state was examined by measuring its water contact angle. As shown in Figure 3b, the water contact angle of the MC hydrogel in the hydrated state ($42.4 \pm 2.2°$, $n = 5$) was significantly lower than that in the dried state ($75.2 \pm 3.5°$, $n = 5$), indicating that the surface of the MC hydrogel in the hydrated state is considerably hydrophilic than in the dried state. This is because the hydrophilic segments of MC molecules are inclined to be exposed to an aqueous environment (i.e., in the hydrated layer, Figure 1b).

3.3. EB Formation. To evaluate the potential application of the MC-coated polystyrene dish to the liquid suspension culture (LSC-MC/PS) of hES cell-derived EBs, we compared the formation of EBs in LSC-MC/PS to those maintained in HDC, LSC-PS, and LSC-ULAP. Figure 4 shows photomicrographs of the hES cell clumps cultivated by different methods for distinct periods. As shown, cultivation of clumps of hES cells in LSC-PS resulted in clump dissociation and cell adhesion onto the substrate over the course of the culture period. The HDC method was developed to reduce cell adhesion.[3] However, this method consists of some troublesome processes and the efficiency of EB formation significantly depends on the skill of technicians.[30] Of note is that clumps of hES cells did not adhere onto the substrate in LSC-ULAP and LSC-MC/PS and generated EBs with time. As shown in Figure 4, dissected hES cell clumps formed rounded discrete structures and acquired an EB appearance within 24 h. The external morphology of EBs was highly variable, even between EBs growing under the same conditions.

To reduce the adhesion of cells onto the substrate during LSC, a Corning ULAP was recently used to inhibit the attachment of anchorage dependent cells.[12] The surface of the ULAP is a covalently bound hydrogel layer that is hydrophilic and neutrally charged. It was reported that it can effectively inhibit the protein adsorption and cell adhesion.[12] Although the ULAP is already commercially available, it is rather expensive for routine hES cell differentiation cultures. Similar to the ULAP, the hydrated surface of our MC-coated dish is hydrophilic as discussed above and MC molecules are neutrally charged. As illustrated in Figure 1a, the procedure used to prepare the MC-coated dish is simple and can be readily done in the lab.

The efficiency of total EB formation was significantly affected by the culture method used. As shown in Table 2, the efficiencies of total EB formation observed in LSC-ULAP and LSC-MC/PS were significantly greater than that seen in HDC

Table 3. Average Diameter of EB, Average Cell Number in Each EB, and Cell Number in Total EB Formed by Different Culture Methods for 10 Days[a]

	HDC	LSC-PS	LSC-ULAP	LSC-MC/PS
EB diameter (mm)	1.23 ± 0.29	N/A[b]	1.22 ± 0.37	1.23 ± 0.10
cell number in each EB ($\times 10^4$)	6.81 ± 2.79	N/A[b]	5.54 ± 2.13	5.72 ± 1.43
cell number in total EB ($\times 10^6$)	1.53	N/A[b]	1.51	1.64

[a] HDC: hanging drop culture; LSC-PS: liquid suspension culture in the polystyrene dish; LSC-ULAP: liquid suspension culture in the Corning Ultralow Attachment plate; LSC-MC/PS: liquid suspension culture in the MC-coated polystyrene dish. MC: methylcellulose; EB: embryoid body. [b] N/A: data are not available because cultivation of clumps of hES cells in LSC-PS resulted in clump dissociation and cell adhesion onto substrate over the course of the culture period.

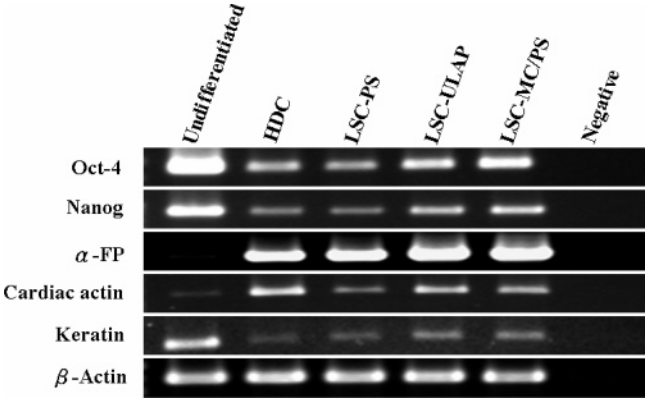

Figure 5. RT-PCR analysis for the expression of Oct-4, nanog, and representative genes of the three embryonic germ layers of hES cells cultivated by different culture methods for 10 days. HDC: hanging drop culture; LSC-PS: liquid suspension culture in the polystyrene dish; LSC-ULAP: liquid suspension culture in the Corning Ultralow Attachment plate; LSC-MC/PS: liquid suspension culture in the MC-coated polystyrene dish. hES cell: human embryonic stem cell; MC: methylcellulose.

over the course of the culture periods. This result clearly demonstrated that the majority of hES cell clumps had the capacity to form EBs in LSC-ULAP and LSC-MC/PS. Also, it was found that the efficiency of total EB formation observed in HDC decreased significantly with time, whereas the efficiencies of total EB formation cultivated in LSC-ULAP and LSC-MC/PS remained relatively stable.

The average diameters of EBs formed by different culture methods were determined by analyzing their photomicrographs. As shown in Table 3, the average diameters of the EBs formed in LSC-ULAP and LSC-MC/PS were comparable to that cultivated in HDC. The number of cells per EB observed in HDC was significantly greater than those seen in LSC-ULAP and LSC-MC/PS. However, the total cell number in LSC-MC/PS was greater than in HDC.

Continuous growth of EBs in HDC, LSC-ULAP, and LSC-MC/PS resulted in the acquisition of a cystic appearance and increased diameter. The center of the bodies became cavitated and began to accumulate fluid and turned into cystic EBs. Ten days after the initiation of cellular aggregation, the efficiencies of cystic EB formation observed in HDC, LSC-ULAP, and LSC-MC/PS were 44.1%, 57.2%, and 67.5%, respectively (Table 2).

The EBs matured by the process of spontaneous differentiation and cavitations. The hES cells within the EBs were shown to express molecular markers specific for representative cells from the three embryonic germ layers. The pluripotency and germ-lineage marker expression in developing EBs were analyzed by RT-PCR. Markers of embryonic transcription factors (Oct-4 and nanog) and the germ lineages, endoderm (α-FP), mesoderm (cardiac actin), and ectoderm (keratin), were included in the analysis. The housekeeping β-actin was used as an internal control.[31−33] As shown in Figure 5, low level

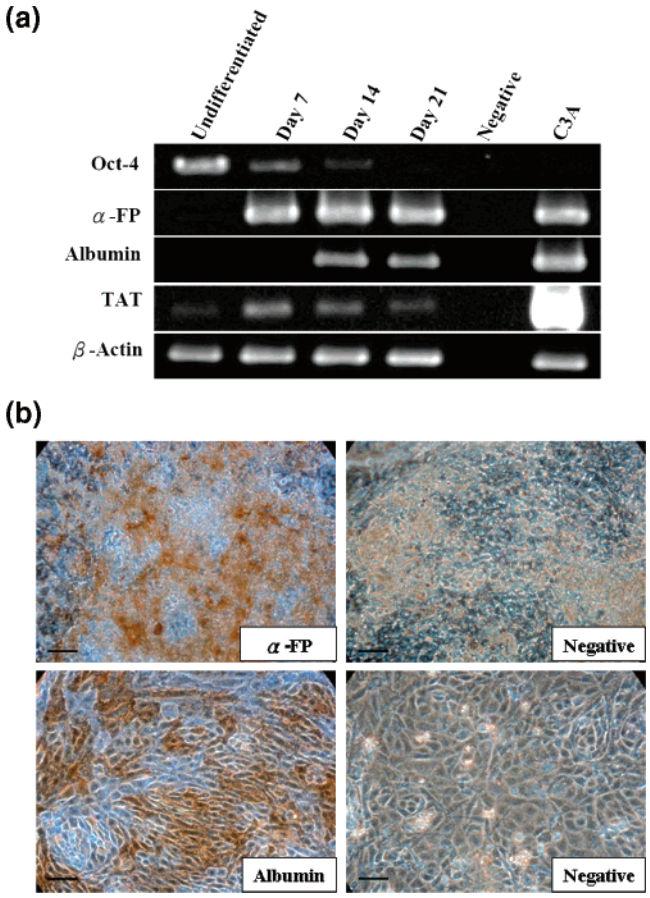

Figure 6. (a) RT-PCR analysis for the expression of hepatic-lineage cell-related genes. (b) Immunostaining with antibodies against albumin and α-FP for the differentiated hES cells (21 days after initiating the differentiation culture, original magnification 200x). Scale bars, 70 μm. Negative: negative control.

expressions of Oct-4 and nanog were observed for the EBs formed by different culture methods. In contrast, expression of cardiac actin and, in particular, α-FP increased. Keratin was observed in undifferentiated hES cells and the formed EBs. Generally, the expressions of lineage markers for the EBs formed in LSC-ULAP and LSC-MC/PS were similar. The LSC-ULAP has recently been used to form EB aggregates in the hES cell differentiation culture.[11,13] These results suggested that LSC-MC/PS did not affect the gene expression of the formed EBs.

3.4. Differentiation of hES Cells into Hepatocyte-Lineage Cells. The significant increase in the expression of α-FP (a clue of endodermal differentiation) prompted us to analyze the potential of hES cells to differentiate into hepatic lineage cells. To determine if the EBs formed in LSC-MC/PS could differentiate into hepatic lineage cells, they were subsequently cultured under conditions known to support the development of this lineage.[34−36]

As shown in Figure 6a, RT-PCR analysis of hepatocyte-associated markers revealed that the hepatocyte-like cells derived from hES cells had an expression profile similar to that of the C3A hepatoma cells. Gene expression of Oct-4, a marker of the pluripotent state, was detected in undifferentiated hES cells but decreased progressively during the subsequent differentiation. In contrast, the expression of three hepatic markers including α-FP, albumin, and TAT increased upon induction with growth factors when compared with the undifferentiated hES cells. It was reported that EBs cultivated under the optimal hepatocyte differentiation conditions express significant levels of hepatocyte-specific markers, such as α-FP, albumin, and TAT.[11,35,37,38] The results of immunostaining showed that the differentiated hES cells were positive for α-FP and albumin (Figure 6b). These results demonstrated that the hES cells cultivated in LSC-MC/PS can generate cells with hepatic characteristics.

4. Conclusions

In order to use hES cells as a source of cells for clinical applications, methods for generating a large scale of the desired cell types must be developed. In the study, we demonstrated that the hES cells cultivated in LSC-MC/PS can efficiently form EBs and may be differentiated into hepatic lineage cells. These results provided the basis for initiating further studies to establish human hepatic lineage cells that may have utility in cell-based therapeutics and in toxicology, pharmacology, and physiology experiments.

Acknowledgment. This work was supported by grants from the Ministry of Economic Affair (5301XS5410 and 94A0317P2) and the National Health Research Institute (94A0050J2), Taiwan, Republic of China.

References and Notes

(1) Dang, S. M.; Gerecht-Nir, S.; Chen, J.; Itskovitz-Eldor, J.; Zandstra, P. W. *Stem Cells* **2004**, *22*, 275−282.
(2) Yim, E. K.; Leong, K. W. *J. Biomater. Sci.-Polym. Ed.* **2005**, *16*, 1193−1217.
(3) Zandstra, P. W.; Bauwens, C., Yin, T.; Liu, Q.; Schiller, H.; Zweigerdt, R.; Pasumarthi, K. B.; Field, L. J. *Tissue Eng.* **2003**, *9*, 767−778.
(4) Burridge, P. W.; Anderson, D.; Priddle, H.; Barbadillo Munoz, M. D.; Chamberlain, S.; Allegrucci, C.; Young, L. E.; Denning, C. *Stem Cells* **2007**, *25*, 929−938.
(5) Ulloa-Montoya, F.; Verfaillie, C. M.; Hu, W. S. *J. Biosci. Bioeng.* **2005**, *100*, 12−27.
(6) Chisti, Y. *Crit. Rev. Biotechnol.* **2001**, *21*, 67−110.
(7) Hammond, T.; Hammond, J. M. *Am. J. Physiol. Renal Physiol.* **2001**, *281*, F12−F25.
(8) Gerecht-Nir, S.; Cohen, S.; Itskovitz-Eldor, J. *Biotechnol. Bioeng.* **2004**, *86*, 493−502.
(9) Dang, S. M.; Kyba, M.; Perlingeiro, R.; Daley, G. Q.; Zandstra, P. W. *Biotechnol. Bioeng.* **2002**, *78*, 442−453.
(10) Paek, H. J.; Morgan, J. R.; Lysaght, M. J. *Stem Cells* **2005**, *23*, 862−867.
(11) Schwartz, R. E.; Linehan, J. L.; Painschab, M. S.; Hu, W. S.; Verfaillie, C. M.; Kaufman, D. S. *Stem Cells Dev.* **2005**, *14*, 643−655.
(12) Shen, M. C.; Horbett, T. A. *J. Biomed. Mater. Res.* **2001**, *57*, 336−345.
(13) Cameron, C. M.; Hu, W. S.; Kaufman, D. S. *Biotechnol. Bioeng.* **2006**, *94*, 938−948.
(14) Harrison, J.; Pattanawong, S.; Forsythe, J. S.; Gross, K. A.; Nisbet, D. R.; Beh, H.; Scott, T. F.; Trounson, A. O.; Mollard, R. *Biomaterials* **2005**, *25*, 4963−4970.
(15) Ott, E.; Spurlin, H. M.; Grafflin, M. W. *Cellulose and Cellulose Derivatives*; Interscience Publishers Inc: New York, 1963; pp 930−938.
(16) Lee, J. W.; Hua, F. J.; Lee, D. S. *J. Controlled Release* **2001**, *73*, 315−327.
(17) Jeong, B.; Bae, Y. H.; Lee, D. S.; Kim, S. W. *Nature* **1997**, *388*, 860−862.
(18) Tate, M. C.; Shear, D. A.; Hoffman, S. W; Stein, D. G.; LaPlaca, M. C. *Biomaterials* **2001**, *22*, 1113−1123.
(19) Liang, H. F.; Hong, M. H.; Ho, R. M.; Chung, C. K.; Lin, Y. H.; Chen, C. H.; Sung, H. W. *Biomacromolecules* **2004**, *5*, 1917−1925.
(20) Amit, M.; Carpenter, M. K.; Inokuma, M. S.; Chiu, C. P.; Harris, C. P.; Waknitz, M. A.; Itskovitz-Eldor, J.; Thomson, J. A. *Dev. Biol.* **2000**, *227*, 271−278.
(21) Thomson, J. A.; Itskovitz-Eldor, J.; Shapiro, S. S.; Waknitz, M. A.; Swiergiel, J. J.; Marshall, V. S.; Jones, J. M. *Science* **1998**, *282*, 1145−1147.
(22) Itskovitz-Eldor, J.; Schuldiner, M.; Karsenti, D.; Eden, A.; Yanuka, O.; Amit, M.; Soreq, H.; Benvenisty, N. *Mol. Med.* **2000**, *6*, 88−95.
(23) Allaire, E.; Guettier, C.; Bruneval, P.; Plissonnier, D.; Michel, J. B. *J. Vasc. Surg.* **1994**, *19*, 446−456.
(24) Kundu, P. P.; Kundu, M. *Polymer* **2001**, *42*, 2015−2020.
(25) Chen, C. H.; Tsai, C. C.; Chen, W.; Mi, F. L.; Liang, H. F.; Chen, S. C.; Sung, H. W. *Biomacromolecules* **2006**, *7*, 736−743.
(26) Li, L.; Shan, H.; Yue, C. Y.; Lam, Y. C.; Tam, K. C.; Hu, X. *Langmuir* **2002**, *18*, 7291−7298.
(27) Dulbecco, R.; Vogt, M. *J. Exp. Med.* **1954**, *99*, 167−182.
(28) Freshney, R. I. *Culture of Animal Cells: a Manual of Basic Technique*; Wiley-Liss: New York, 1994; pp 83−84.
(29) Zheng, P. J.; Li, L.; Hu, X.; Zhao, X. Y. *J. Polym. Sci. Part B-Polym. Phys.* **2004**, *42*, 1849−1860.
(30) Konno, T.; Akita, K.; Kurita, K.; Ito, Y. *J. Biosci. Bioeng.* **2005**, *100*, 88−93.
(31) Khoo, M. L.; McQuade, L. R.; Smith, M. S.; Lees, J. G.; Sidhu, K. S.; Tuch, B. E. *Biol. Reprod.* **2005**, *73*, 1147−1156.
(32) Amit, M.; Shariki, C.; Margulets, V.; Itskovitz-Eldor, J. *Biol. Reprod.* **2004**, *70*, 837−845.
(33) Melville, A. J.; Harrison, J.; Gross, K. A.; Forsythe, J. S.; Trounson, A. O.; Mollard R. *Biomaterials* **2006**, *27*, 615−622.
(34) Thomson, J. A.; Marshall, V. S. *Curr. Top. Dev. Biol.* **1998**, *38*, 133−165.
(35) Shirahashi, H.; Wu, J.; Yamamoto, N.; Catana, A.; Wege, H.; Wager, B.; Okita, K.; Zern, M. A. *Cell Transplant* **2004**, *13*, 197−211.
(36) Lavon, N.; Benvenisty, N. *J. Cell. Biochem.* **2005**, *96*, 1193−1202.
(37) Walkup, M. H.; Gerber, D. A. *Stem Cells* **2006**, *24*, 1833−1840.
(38) Lavon, N.; Yanuka, O.; Benvenisty, N. *Differentiation* **2004**, *72*, 230−238.

BM0704166