Control of Cell Cultivation and Cell Sheet Detachment on the Surface of Polymer/Clay Nanocomposite Hydrogels

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Received June 8, 2006; Revised Manuscript Received August 10, 2006

Cell cultivation on the surface of a novel poly(*N*-isopropylacrylamide) (PNIPA) hydrogel (N-NC gel), consisting of a specific type of organic (PNIPA)/inorganic (clay) network, was studied using three cell types; HepG2 human hepatoma cells, human dermal fibroblasts, and human umbilical vein endothelial cells. For the first time, it was found that cells could be cultured to be confluent on the surfaces of PNIPA hydrogels using N-NC gels, regardless of gel thickness. Cell adhesion and proliferation on N-NC gels exhibit strong dependencies on clay concentration (C_{clay}), and the numbers of cultured cells are maximum at about $C_{clay} = 6 \times 10^{-2}$ mol (45.72 g)/1 L of H_2O. On the contrary, it was almost impossible to culture cells on conventional, chemically crosslinked PNIPA hydrogels, regardless of their cross-linker concentration. The reasons why cells cultured only on the surfaces of N-NC gels with their specific network structure and composition were discussed in terms of water content, protein adsorption, surface flatness, hydrophobicity of dehydrated PNIPA chains, and the anionic charge on exfoliated clay. Finally, it was found that cells cultured on the surfaces of N-NC gels could be detached in the forms of sheets of cells without trypsin treatment, but by just decreasing the temperature to 20 °C.

Introduction

Since polymer hydrogels with relatively high water content and a soft, rubbery nature generally have good prospects for biocompatibility and consistency with living tissue, they are expected to have important roles in many fields related to biomedical research and technology.[1,2] Among them, polymer hydrogels with stimuli-sensitivity (e.g., poly(*N*-isopropylacrylamide) (PNIPA) hydrogels in particular) have attracted considerable attention as smart gels for use in advanced devices in biomedical and tissue engineering, such as artificial insulin control systems,[3] efficient bioseparation devices,[4] carriers of immobilized enzyme,[5] drug delivery system,[6] and culture dishes for cell sheet engineering.[7] PNIPA, which exhibits a clear coil-to-globule transition at its lower-critical-solution temperature (LCST ≅ 32 °C),[8] could be readily produced in the form of covalently cross-linked hydrogels, either by using an organic cross-linker or by irradiation with γ-rays or an electron beam. However, these chemically cross-linked hydrogels have very limited applicability because of their poor mechanical properties.[2] Moreover, structural heterogeneity (e.g., lack of transparency) and restriction of the functions (e.g., slow and limited swelling and deswelling) are often the result of their large numbers of cross-links.

We recently succeeded in creating a new type of physically cross-linked hydrogel, a nanocomposite gel, with a specific organic (polymer)/inorganic (clay) network structure.[9−11] The novel nanocompsoite gels (abbreviated to NC gels), based on poly(alkyl acrylamides) and exfoliated inorganic clay, exhibit distinguished mechanical properties as well as excellent optical and swelling/deswelling properties, superior to the corresponding, conventional, chemically crosslinked hydrogels (abbreviated to OR gels). For example, NC gels exhibit ultrahigh elongation at break, near to or greater than 1000%, and their modulus and strength can be controlled over a wide range without sacrificing their high extensibility, whereas OR gels readily fractured at very low stress and strain. Also, NC gels are transparent, almost regardless of their clay content. Furthermore, due to a well-defined phase transition at their LCST, NC gels prepared from PNIPA exhibit clear reversible changes in properties such as hydrophilicity (hydrophilic/hydrophobic), transparency (transparent/opaque), and gel volume (swelling/de-swelling). In a previous paper,[12] we reported the effectiveness of NC gels as soft, transparent, and mechanically tough materials for biomedical applications. There, it was confirmed that NC gels, based on either thermo-sensitive PNIPA or thermo-stable poly(*N*,*N*-dimethyl acrylamide) (PDMAA), can absorb large amounts of water, saline solution, and blood plasma and can be purified sufficiently to pass the extraction test for contact-lens standards. Also NC gels can be sterilized using an autoclave or by γ-ray irradiation, and drying can be controlled by covering with a thin film for the purpose of wound dressings. Furthermore, some NC gels can exhibit good blood compatibility and do not cause inflammation or bleeding during short-term implantation tests, in addition to having the essential high mechanical toughness with the capability of being sewn.

In biomedical research, culturing cells on a substratum is one of the most important and indispensable experimental procedures and is widely utilized in medical, biological, pharmaceutical, and tissue engineering research and industry. For example, the cultivation of many kinds of cells were performed to study embryology, cytology, and tissue regeneration on scaffolds or to assess biocompatibility, safety, and in-vitro toxicity of newly developed medicines, medical devices, and materials.

In the present paper, the cell cultivation behavior on the surface of NC gels, based on PNIPA or PDMAA, with and without thermo-sensitivity, respectively, is demonstrated and compared with the corresponding, conventional OR gels. Then, the effects of network structure and gel composition on cell cultures, using three types of cells, are clarified. We reveal that

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10.1021/bm060549b CCC: $33.50
Published on Web 09/30/2006

thermo-sensitive PNIPA-NC gels with specific compositions can be used as a soft, wet substratum with the capability of thermally regulated cell adhesion and detachment without protease treatment.

Experimental Section

Materials. Two different types of hydrogel films, NC and OR gels, were prepared by free-radical polymerization using different cross-linkers, inorganic clay (hectorite) and *N*,*N*′-methylenebis(acrylamide) (BIS), respectively.

The sample code for NC gels defines the kind of monomer (N- and D- for NIPA and DMAA, respectively) and the concentrations of clay relative to water ($C_{clay} = n \times 10^{-2}$ mol of clay/1 L of H_2O). The monomer concentration was kept at 1 mol per liter of H_2O. For example, N-NC*n* gel indicates an NC gel prepared using $n \times 10^{-2}$ mol ($n \times 0.762$ g) of clay and 1 mol of NIPA in 1 L of water. The clay concentration, *n*, was varied from NC1 to NC15 in the present study, whereas, OR gels (e.g., N-OR*n*′ gels) were prepared using *n*′ mol % of organic cross-linker (BIS), instead of clay, relative to the monomer ($C_{BIS} = n' \times 10^{-2}$ mol of BIS/1 mol of monomer). The BIS concentration, *n*′, was varied from OR1 to OR7.

Raw Materials. N-Isopropylacrylamide (NIPA) monomer (Kohjin Co.) was purified by recrystallization from a toluene/*n*-hexane (10/90 w/w) mixture, followed by drying under vacuum. *N*,*N*-Dimethylacrylamide (DMAA) monomer (Kohjin Co.) was purified by filtering through activated alumina. As an inorganic clay, synthetic hectorite "Laponite XLG" (Rockwood Ltd.; $[Mg_{5.34}Li_{0.66}Si_8O_{20}(OH)_4]Na_{0.66}$) was used after washing and freeze-drying. Potassium persulfate (KPS) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were used as initiator and catalyst, respectively. The water used for all experiments was ultrapure water supplied by a PURIC-MX system (Organo Co.). Dissolved oxygen was removed by bubbling N_2 gas through the purified water for more than 3 h prior to use, and throughout all experiments, oxygen was excluded from the system. Other reagents were used without further purification.

Syntheses of Hydrogel Films. The synthetic procedure is almost the same as that reported previously.[11,13] For example, to prepare N-NC6 gel film (Figure 1a), a uniform, transparent reaction solution consisting of water (95 mL), Laponite XLG (4.572 g), NIPA (11.3 g), TEMED (80 μL), and KPS (0.1 g in 5 mL of H_2O) was prepared in an ice bath and poured into the laboratory-made molding apparatus made of poly(methyl methacrylate) substrates, with internal dimensions of 110 mm wide × 160 mm long × *t* mm thick. The thickness, *t*, was varied from 0.5 to 5 mm, with 2 mm used as a standard thickness. Then, free-radical polymerization was allowed to proceed in a water bath at 20 °C for 48 h.

Uniform, transparent NC and OR gels were obtained regardless of the cross-linker content, except for N-OR5 and N-OR7 gels (opaque). The polymerization yields were almost 100% for all hydrogels, evaluated from the weight of dried gels and thermogravimetric analysis. Thus, as-prepared hydrogels have the same compositions as the starting solution. The compositions (water, clay, and monomer) of the starting solutions for typical N-NC and N-OR gels (N-NC1, 3, 6, and 10 and N-OR1 and 3) and D-NC6 and D-OR3 gels are shown in Table 1. As reported previously, all NC gels exhibit large elongations at break (1000% or more), and their strength and modulus increased with increasing clay content, whereas all OR gels were weak and brittle regardless of the BIS content.

All hydrogels were purified prior to use for cell culture. In the case of PNIPA hydrogels, N-NC and N-OR gels were lightly washed with water at 20 °C and then immersed in an excess of pure water at 50 °C for total of 10 days, as shown in Figure 1b, during which period the gel contraction occurred progressively and the pure water was changed several times. At the end of the purification process, the contracted N-NC and N-OR gels were contracted and are henceforward referred to as contracted gels. The resulting contracted N-NC and N-OR gels were used as standard gels in cell culture procedures. The extent of contraction and, hence, the residual water content of each contracted gel differed, depending on the network composition. On the other hand, PDMAA-based hydrogels (D-NC and D-OR gels) were repeatedly washed using an excess of pure water at ambient temperature followed by drying slightly in air five times. The finally obtained D-NC and D-OR gels after the purification process, which are the standard PDMAA hydrogels for cell culture, contained almost same amount of water as in its as-prepared state. These variously defined standard gels were used as starting substrata for cell culture procedures, although their water contents were differed considerably, depending on the base polymer and the network composition. The water contents of typical standard gels are shown in a later section (2.4). In the present study, the hydrogels were not sterilized before using in cell culture procedures but were prepared and purified using pure water previously sterilized in an autoclave and handled under aseptic condition throughout the experiments.

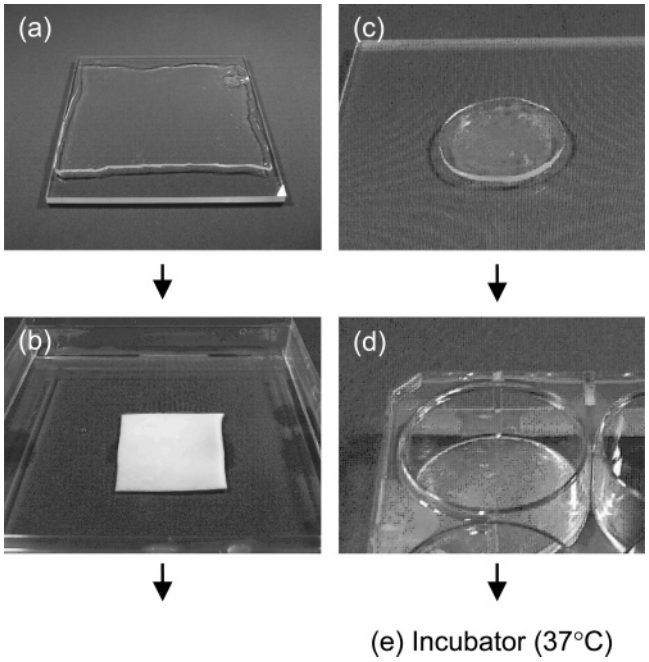

Figure 1. Preparation of N-NC6 gel film for cell cultivation. (a) As-prepared N-NC6 gel film. (b) Deswollen (contracted) N-NC6 gel film at 50 °C. (c) Cut disk N-NC6 gel film (observed at room temperature). (d) Film of N-NC6 gel disk in tissue culture polystyrene dish (at room temperature). (e) Moved into incubator (at 37 °C).

To study the effect of water content, some dried hydrogels (dried N-NC1 through N-NC10, dried N-OR1 through N-OR3, dried D-NC6, and dried D-OR3) were prepared by drying the corresponding purified standard hydrogels (initial gel thickness = 2 mm) placed in tissue-culture plates at 25 °C for 7 days under aseptic conditions. The water contents of dried gels were nearly zero in all cases and negligible when compared with the amount of medium absorbed during the early stages of cell cultivation.

Cell Culture. *Cell Types and Culture Media.* Three types of cells, human hepatoma cells (HepG2), normal human dermal fibroblast (both from Dainippon Sumitomo Pharma Co. Japan), and normal human umbilical vein endothelial cells (HUVEC; from Kurabo, Inc. Japan), were used to study cell cultivation on the surfaces of NC and OR gels. All cells, routinely subcultured in tissue culture polystyrene (TCPS) dishes at 37 °C under 5% CO_2 atmosphere, were harvested after treatment with 0.05% trypsin−EDTA solution (GIBCO; Invitrogen Co. U.S.A.). The culture medium for HepG2 was the minimum essential medium (MEM-EAGLE; Sigma Aldrich Co., U.S.A.) containing sodium pyruvate and nonessential amino acids (ICN Biomedicals, Inc., U.S.A.), supplemented with 15% heat-inactivated fetal bovine serum (biowest, France), penicillin−streptomycin (GIBCO), and sodium hydrogen carbonate (Wako Pure Chemical Industries Ltd., Japan). For

Table 1. Compositions (Water, Clay, and Monomer) of the Starting Solutions for Typical NC and OR Gels, and the Water (Medium) Contents Relative to Dry Gel for Various Hydrogels (and the Dry Gel) Used in Cell Cultivation Experiments

	$W_{liquid}/W_{dry} \times 100$				
	N-NC1	N-NC3	N-NC6	N-NC10	D-NC6
NC gel					
initial solution (g) (H_2O/Clay/monomer)	100/0.762/11.3	100/2.29/11.3	100/4.57/11.3	100/7.62/11/3	100/4.57/9.9
standard gel[a]	59	109	225	450	780
→5 days in the medium[b]	49 (57)	110 (100)	173 (152)	320 (360)	2090
dried NC gel					
→5 days in the medium[b]	40 (47)	49 (49)	67 (67)	185 (187)	1843

	$W_{liquid}/W_{dry} \times 100$		
	N-OR1	N-OR3	D-OR3
OR gel			
initial solution (g) (H_2O/BIS/monomer)	100/0.14/11.3	100/0.42/11.3	100/0.42/9.9
standard gel[a]	580	241	1082
→5 days in the medium[b]	465 (473)	86 (116)	1052
dried NC gel			
→5 days in the medium[b]	116 (92)	58 (63)	1024

[a] Water content. [b] Medium content: medium for HepG2 and (for fibroblast).

fibroblast and HUVEC, CS−C complete medium (Cell Systems Corp., U.S.A.) and HuMedia-EG2 (Kurabo) were used, respectively.

Cell Culture on Hydrogels. Four different types of hydrogel were used in cell culture experiments, namely N-NC gels (N-NC1 through N-NC15), N-OR gels (N-OR1 through N-OR7), D-NC gel (D-NC6), and D-OR gel (D-OR3). For N-NC and N-OR gels, standard gels cut into disks (3 cm diameter) at ambient temperature and placed in 6-well TCPS dishes (Falcon 3046, Becton Dickinson, U.S.A.), as shown in Figure 1c,d. For D-NC and D-OR gels, standard gels were also used in a similar manner. Then, HepG2, fibroblast and HUVEC in each medium were inoculated at a density of 6.3×10^4, 1.2×10^4, and 6.3×10^3 cell/cm^2, respectively, on the gel surface. All cells were incubated in 5% CO_2 − 95% air at 37 °C. Throughout the cell-culture process, for periods up to 1 week, the medium was replaced every 2 days. Cell adhesion and proliferation were documented by photomicrography using a phase-contrast optical microscope (OLYMPUS CKX41). The method employed for counting the number of cells that had grown onto the hydrogel surfaces was as follows. Cells that grew on the gel surfaces were dissociated by treating with 0.25% trypsin/EDTA solution at 37 °C for 5 min after culture times of 5 days. The number of cells was counted under the microscope after dying with trypan blue.

Cell Culture on Dried Hydrogels. Cells (HepG2, fibroblast) were cultured on four types of dried hydrogels, dried N-NC gels with different C_{clay} (dried N-NC1 through N-NC10), dried N-OR gels with different C_{BIS} (dried N-OR1 through N-OR3), dried D-NC6 gel, and dried D-OR3 gel, in a manner similar to that described above.

Cytotoxcity of the Constituents. The cytotoxicity of the main constituents of N-NC gels (PNIPA and hectorite) and of small residual amounts of other reactants (NIPA, TEMED, and KPS) were measured. HepG2 was cultured on TCPS dishes using a medium containing a certain amount of each component. Cytotoxcity was judged by whether HepG2 could be cultured satisfactorily using the medium.

Characterization of Hydrogels during Cell Culture. *Water Content of Hydrogels.* Typical examples of the water contents of standard gels and dried gels used for cell culture experiments are shown in Table 1. Here, the water content (C_{H2O}) was weight percentage of water relative to dried weight ($C_{H2O} = (W_{H2O}/W_{dry}) \times 100$). C_{H2O} changed considerably during cell-culture procedures, the extent depending on the gel sample and the medium used. In separate experiments, changes in C_{H2O} were measured under the same conditions of temperature (37 °C), time (∼7 days), and medium. Values of C_{H2O} (strictly speaking, this should be called the medium content) after 3 days in the medium are also listed in Table 1 for both standard and dried gels. In all media, C_{H2O} decreased when the starting gel was a standard gel, while it increased when the starting gel was a dried one.

Morphology of Hydrogels. To study the influence of hydrogel morphology on cell culture, the surface morphologies of starting, dried N-NC1, and dried N-NC6 gels were observed by scanning electron microscopy (SEM 5001, JEOL). Also, surface morphologies up to 5 days in the medium for HepG2 under cultivating conditions were observed by phase-contrast optical microscopy.

BSA Adsorption. The adsorption of bovine serum albumin (BSA) on hydrogel surfaces was examined by immersing standard gels into BSA solution, consisting of 0.14 mmol/mL of FITC (fluorescein isothiocyanate) − BSA/BSA solution (FITC: BIS = 10:90) and 50 mL of Tris-HCl buffer (pH 7.4), at 37 °C for 5 h. After rinsing lightly with water at 37 °C, the BSA adsorbed on the hydrogel was measured by using a fluoro image analyzer (FLA-3000, Fuji Photo Film Co. Japan) with excitation at 473 nm and emission at 510 nm.

Cell Sheet Detachment. After HepG2 and fibroblast were cultured almost to confluence on the surfaces of N-NC6 and dried N-NC6 gels on day 5 of culture, the medium temperature was decreased to about 10 °C, by exchanging with a cold medium, and then increased to 20 °C. The detachment of HepG2 cell sheets was observed by changing the temperature, followed by pumping the medium slightly on to the cell-gel interface with a pipet. In the case of fibroblast, it was observed that cell sheets became detached just by changing the temperature. The detachment behavior was monitored qualitatively under a phase-contrast microscope fitted with a video camera.

Results

Cytotoxicity of NC Gel Constituents. NC gels were prepared by in situ, free-radical polymerization of monomer (e.g., NIPA) in the presence of exfoliated clay (hectorite) and a small amount of initiator (KPS) and catalyst (TEMED). First, we checked the cytotoxicity of each of the main constituents of N-NC gels (i.e., PNIPA and hectorite) and of small amount of residues (i.e., NIPA, TEMED, and KPS) which might remain in NC gels even after purification.

It was found that HepG2 can be cultured satisfactorily for more than 5 days in a medium containing hectorite (0.10 wt %) or PNIPA (0.10 wt %), with no apparent difference in cell morphology from the control in either case. The effect of PNIPA addition was consistent with the result reported by Takizawa et

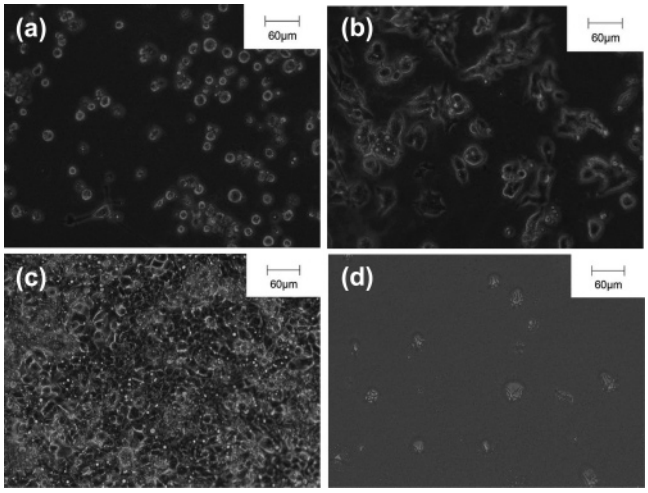

Figure 2. Phase-contrast photomicrographs of HepG2 cultures on the surfaces of (a−c) N-NC6 gels and (d) N-OR1 gel, after (a) 12 h, (b) 24 h, (c) and (d) 5 days.

al.[14] for human dermal fibroblasts. On the other hand, as was expected, all other reagents (NIPA, TEMED and KPS) showed cytotoxicity when the concentration was relatively high. However, at low concentrations, such as addition of 3.3×10^{-7} mol of NIPA, 5.0×10^{-7} mol of TEMED, and 3.3×10^{-8} mol of KPS, per gram of medium (although actual concentrations of the residues in purified gels were much lower than these concentrations), it was found that each component showed no cytotoxicity toward cultures of HepG2. Thus, it was concluded that residues of NIPA, TEMED, or KPS less than the above concentrations hardly affect cell cultures. The fact that the cell cultivation could be performed on N-NC gel films, as demonstrated in the present study, and the fact that TEMED, KPS, or NIPA was hardly detected on analyzing purified NC gels with high-performance liquid chromatography, indicates that the NC gels can be purified sufficiently to be safe for biomaterial uses.

Cell Cultivation (HepG2) on PNIPA and PDMAA Hydrogels. We investigated cell cultivation of HepG2 on the surface of two types of PNIPA hydrogels, N-NC6 and N-OR1 gels, both of which are transparent and contain almost the same amounts of water (90 wt %) and PNIPA (10 wt %). Although they only differ in the kind of cross-linker (i.e., the type of network structure), N-NC6 gel exhibited superior mechanical and swelling/de-swelling properties, as previously reported,[10] such as an extraordinarily large elongation at break (1000%), high strength (200 kPa), high fracture energy (0.7 J), high equilibrium absorption of water (about 3000 wt % relative to the dried gel at 20 °C), and rapid swelling and deswelling rates. On the contrary, N-OR1 gel was fragile and broke at very low elongation (40%) and had low strength (5 kPa).

Figure 2, panels a−c and d show phase-contrast micrographs of HepG2 after different culture times on the surfaces of N-NC6 and N-OR1 gels, respectively. It is clearly seen that HepG2 became attached to and began spreading on N-NC6 gel (Figure 2a, 24 h) and spreading continued to develop (Figure 2b, 48 h). Almost complete coverage by cells was observed within a 5 day culture period (Figure 2c, 5 days). In contrast, little adhesion and no proliferation of HepG2 was observed on the surface of N-OR1 gel throughout a 7 day culture period (Figure 2d, 5 days**)**. Taking the structural differences of both PNIPA hydrogels into consideration, the remarkable improvement for N-NC6 gel, compared with that for N-OR1 gel (conventional PNIPA hydrogel), was attributed to the particular network structure of the NC gel. We also examined the effect of gel thickness on cell cultures of HepG2 using N-NC6 gels 0.5∼5 mm thick in their as-prepared state. As a result, it was found that cell cultures always became confluent regardless of the gel thickness.

On the other hand, for two types of PDMAA hydrogels, D-NC6 and D-OR3 gels, it was found that little HepG2 adhered to or proliferated on their surfaces, regardless of the type of network structure (photos are not shown). This is probably because both D-NC6 and D-OR3 gels are totally hydrophilic under the cultivation conditions (at 37 °C). Actually, they swelled in the medium up to 2090 and 1050 wt % for D-NC6 and D-OR3 gels, respectively, on the third day.

Effects of the Cross-Linker Content of PNIPA Hydrogel on the Cultures of HepG2 Cells. HepG2 was cultured on the surface of various N-NC and N-OR gels with different cross-linker contents (C_{clay} and C_{BIS}). As previously reported,[10] all N-NC gels (NC1 through NC15) and N-OR gels with low C_{BIS} (OR1 through OR3) were optically transparent in the as-prepared state (at 20 °C), but N-OR gels with higher C_{BIS} (OR5 and OR7 gels) were opaque (white) due to the formation of heterogeneous networks. Therefore, the culturing of cells was examined using a normal phase-contrast microscope for the former and by counting the number of cells after trypsin treatment for the latter. It was found that cell cultures did not develop on the surface of any N-OR gels used here, regardless of C_{BIS} and the optical transparency. This result means that N-OR gels having conventional, chemical cross-links were intrinsically inert for cell culture.

On the other hand, HepG2 cultures on N-NC gels varied to a large extent depending on C_{clay}, as shown in Figure 3. It was found that N-NC gels with low C_{clay} (e.g., NC1 gel) or with high C_{clay} (e.g., NC15 gel) hardly supported cell cultivation. In contrast, spreading and proliferation of HepG2 was greatly enhanced to reach confluency on N-NC gels with an intermediate range of C_{clay} (e.g., NC6 gel). Quantification of the number of cells attached to the N-NC and N-OR gels is shown in Figure 4. The numbers of cells exhibit a maximum at around NC5 through NC7, whereas cells were hardly observed on any N-OR gels. Thus, it was concluded that the culturing of HepG2 cells on the surfaces of PNIPA hydrogels strongly depends not only on the network structure (i.e., the kind of cross-linker) but also the cross-link density (i.e., clay content) and that confluent cell layers are only achieved on specific N-NC gels with a intermediate range of C_{clay}. The reasons for the remarkable effects of cross-linker type (clay or BIS) of PNIPA hydrogels and their concentrations on cell cultures are discussed in a later section.

Cell Cultures of Fibroblast and HUVEC on PNIPA Hydrogels. To confirm the generality of the cell cultivation behavior on N-NC and N-OR gels, other types of cells, fibroblast and HUVEC, were also used on N-NC6 and N-OR1 gels. Consequently, it was observed that both fibroblast and HUVEC can adhere to and spread well on N-NC6 gel, in a manner similar to HepG2, and proliferate almost to confluence, as shown in Figure 5,panels a (fibroblast) and b (HUVEC). By contrast, both cell types hardly adhere to and did not spread at all on N-OR1 gel during 7 days in culture (Figure 5, panels c (fibroblast) and d (HUVEC)) analogous to HepG2. Thus, it was concluded that conventional PNIPA gel (N-OR1 gel) was generally nonsupportive for cell adhesion and spreading, but nanocomposite PNIPA gel (N-NC6 gel) was capable of culturing various types of cells.

The effects of cross-linker content on cell cultures of fibroblast and HUVEC were also investigated. As shown in Figure 6, taken after 5 days of culture, it was found that fibroblast adhered to, spread on, and proliferated differently on

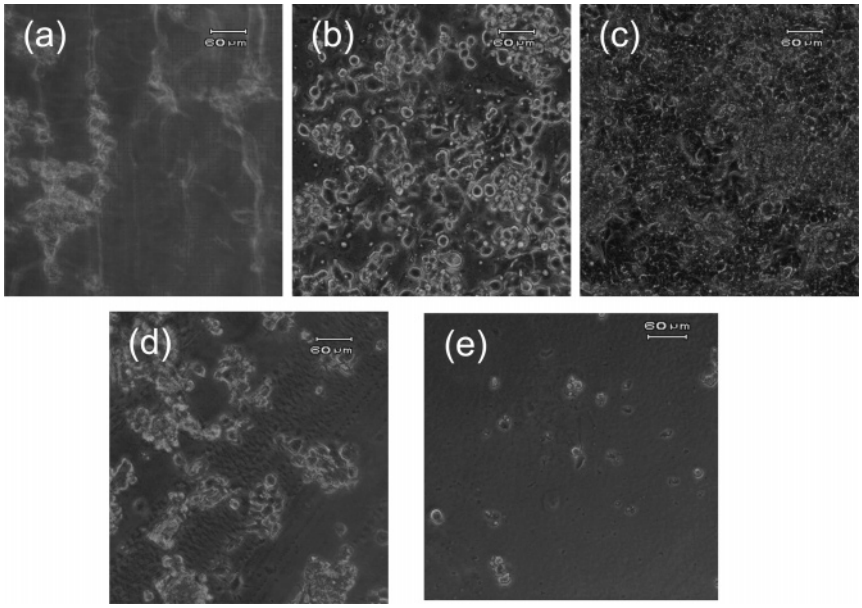

Figure 3. Phase-contrast photomicrographs of HepG2 cultured on the surface of N-NC gels with different clay contents (N-NC1 through N-NC15). (a) N-NC1 gel, (b) N-NC3 gel, (c) N-NC6 gel, (d) N-NC10 gel, and (e) N-NC15 gel.

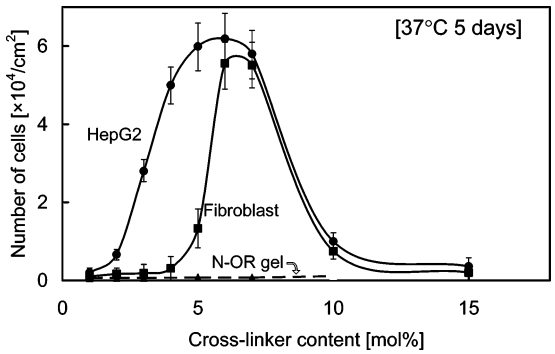

Figure 4. Densities of cells (HepG2 and fibroblast) cultured on the surfaces of PNIPA hydrogels (N-NC gels, N-OR gels) with different cross-linker contents after culturing for 5 days. ● HepG2 on N-NC gels, ■ fibroblast on N-NC gels, - - - HepG2 and fibroblast on N-NC gels.

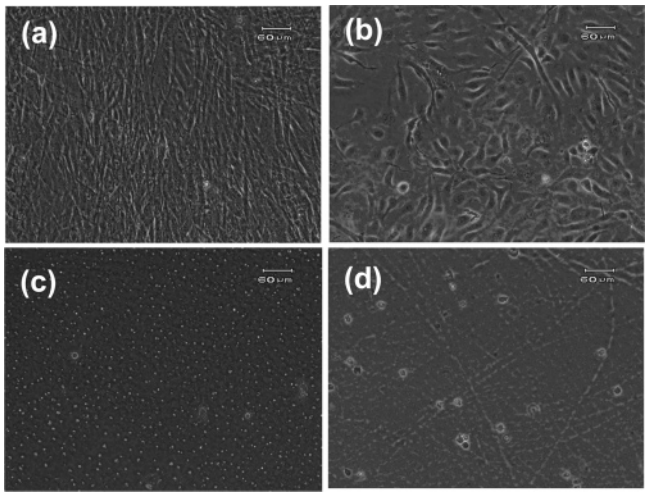

Figure 5. Phase-contrast photomicrographs of fibroblast and HUVEC proliferated on N-NC6 and N-OR1 gels after culturing for 5 days. (a) Fibroblast on N-NC6 gel, (b) HUVEC on N-NC6 gel, (c) fibroblast on N-OR1 gel, (d) HUVEC on N-OR1 gel.

the surfaces of N-NC gels depending on their C_{clay}. Here, fibroblast on an N-NC6 gel surface proliferated and eventually covered the gel surface, whereas it spread only slightly on N-NC3 and N-NC10 gel surfaces. Cell numbers were measured after culturing for 5 days and are shown in Figure 4 together with data for HepG2. Maximum numbers of cell were observed at around NC6 through NC7 gels, and the dependence on C_{clay} was sharper than that for HepG2. For HUVEC, a similar C_{clay} dependence on cell cultures was also observed for a consecutive series of N-NC gels. Thus, it was revealed that the most preferable clay concentration for cell culture was around NC6 for fibroblast and HUVEC, as well as for HepG2.

Influence of Water Content of PNIPA Hydrogels on Cell Cultures. In the former sections, it was revealed that cells can be cultured on the surfaces of N-NC gels with specific compositions. There, we used purified, contracted N-NC gels as the starting (standard) hydrogels. The water contents (C_{H2O}) of the standard N-NC gels are shown in Table 1. C_{H2O} varied depending on C_{clay}, and the best hydrogel (N-NC6 gel) on which to culture cells contained 225 wt % of water, relative to solids. However, it was also observed that the standard NC gel contracted further under the culturing conditions. From the time-dependence of C_{H2O} observed in separate experiments, it was found that the C_{H2O} decreased in the medium over several hours, after which the gel weight remained constant. In Table 1, C_{H2O}, which is correctly called the medium content, measured after 3 days of immersion in the media for HepG2 and fibroblast at 37 °C, are also shown. In general, C_{H2O} decreased considerably to 0.7∼0.8 times of that of the initial contracted (standard) gel. For example, the C_{H2O} of N-NC6 gel in the medium for HepG2 was 173 wt % vs 225 wt % for the standard gel. This is probably due to the effect of ionic components contained in the medium. Also, C_{H2O} increased with increasing C_{clay}, which is probably due to the increase of hydrophilicity introduced by the clay. Thus, it was found that N-NC6 gel, providing good conditions for cell culture, has quite a high C_{H2O} (173 wt %) under cultivation conditions, whereas N-NC1 gel, showing little cell culture, had a low C_{H2O} (49 wt %). However, the low C_{H2O} of N-NC1 gel does not seem to be the reason for poor cell culture, because an increase in hydrophobicity is normally considered suitable for the cell culture.

So, next, cell cultures of HepG2 and fibroblast on N-NC gels with lower water contents was undertaken, utilizing dried N-NC gels as starting materials. Consequently, it was found that dried

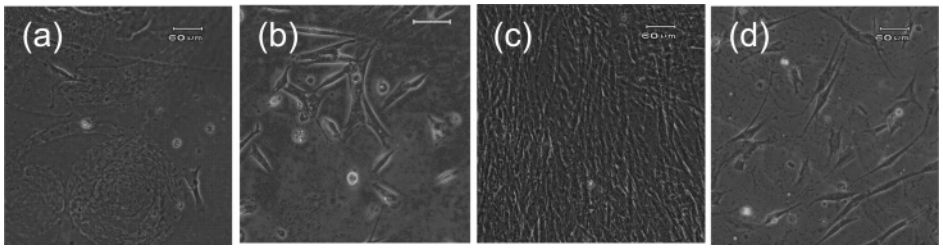

Figure 6. Phase-contrast photomicrographs of fibroblast cultured on the surfaces of N-NC gels with different clay contents (N-NC1 through N-NC10), taken after culturing for 5 days. (a) N-NC1 gel, (b) N-NC3 gel, (c) N-NC6 gel, (d) N-NC10 gel.

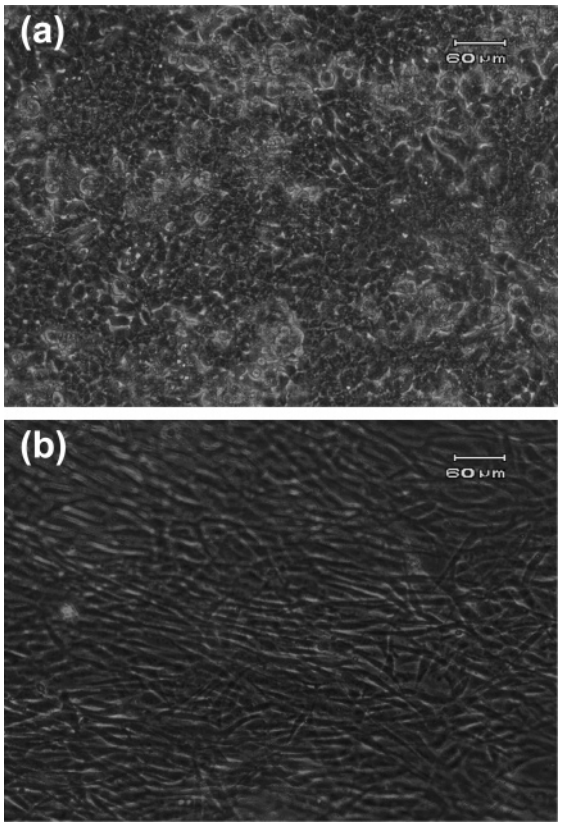

Figure 7. Phase-contrast photomicrographs of HepG2 and fibroblast cultured on the surface of dried N-NC6 gels, taken after culturing for 5 days. (a) HepG2, (b) fibroblast.

N-NC gels with different C_{clay} (NC1 through NC10) exhibited similar tendencies for cell cultivation to those of the contracted N-NC gels. That is, dried N-NC6 gel exhibited the best conditions for cell cultivation for both HepG2 and fibroblast (Figure 7). However, dried N-NC6 gel has a much lower C_{H2O} (67 wt %) than that of contracted N-NC6 gel (173 wt %). In the medium, it was observed that dried N-NC gels swelled rapidly and the weight increase almost reached equilibrium within a few hours. C_{H2O} for dried N-NC gels was in the range of 40∼82 wt % in the medium for HepG2, depending on C_{clay}. Thus, the results indicate that C_{H2O} is not the decisive factor to achieve good cell cultivation on N-NC gels, because cells did not culture well on dried N-NC10 gel (82 wt %) which contained intermediate C_{H2O} between N-NC6 gel (173 wt %) and dried N-NC6 gel (67 wt %). Furthermore, it was observed that dried N-OR gels containing 58∼116 wt % of medium did not cause cell adhesion and proliferation, regardless of their C_{H2O}. Thus, it was concluded that, despite the different water contents, both dried and standard N-NC gels with a specific clay concentration (around NC6) can act as effective substrata, whereas both N-OR gels and dried N-OR gels cannot be used to culture cells regardless of their C_{H2O}.

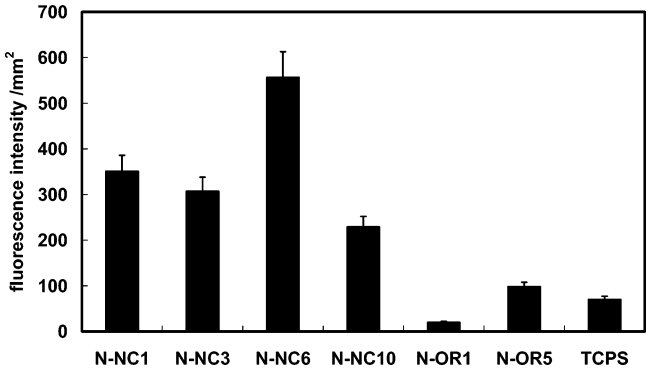

Figure 8. Adsorption of BSA on N-NC and N-OR gels with different cross-linker contents and TCPS.

Here, it should be noted that the utilization of dried NC gels has a large advantage as substrata in practical applications, particularly, in the process of sterilization. That is, in the case of dried N-NC gels, they can be readily sterilized by electron beam or γ-ray irradiation without sacrificing gel properties.

Factors Influencing Cell Cultivation on N-NC Gels. To clarify the reason for the totally different cell culture behaviors on the two types of PNIPA hydrogels, N-NC and N-OR gels, and the strong dependence on C_{clay} of N-NC gels, the adsorption of BSA on their surfaces was examined using FTIC−BSA/Tris-HCl aqueous solution (BSA: 0.1 mM, pH7.4) at 37 °C for 3 h. The results are summarized in Figure 8. It was found that, in general, NC gels can adsorb large quantities of BSA, and in contrast, OR gels adsorb only a little. This suggests that the clay−PNIPA network structure plays an important role in protein adsorption, thereby causing cells to adhere and proliferate. That is, NC gels can effectively adsorb some adhesive proteins or growth factors from the medium, resulting in improved cell adhesion. OR gels which cannot adsorb sufficient protein should result in the nonadherence of cells.

Despite the large protein adsorption of NC gels, regardless of C_{clay} (Figure 8), the fact that cell cultures on NC gels show strong dependencies on C_{clay} (Figures 3 and 4) indicates that some other factors, such as water content (hydrophilicity) and surface morphology (flatness), may affect cell culture behavior. Concerning the influence of C_{H2O}, as described in the preceding section, it is not necessary to maintain C_{H2O} within a small, strictly controlled range, because, as shown in Table 1, the C_{H2O} of N-OR gels (86∼465 wt %, standard N-OR gels; 58∼116 wt %, dried N-OR gels) and N-NC gels (49∼247 wt %, standard N-NC gels; 40∼82 wt %, dried N-NC gels) for the HepG2 medium were comparable but gave very different results for cell culture. Here, the rehydrated dried N-NC6 gel, with low C_{H2O} (67 wt %), can support cell culture similarly to the standard N-NC6 gel with high C_{H2O} (173 wt %). Thus, the C_{H2O} of a satisfactory substratum can be varied over quite a wide range, although an enormous water content in the medium, for example more than 300 wt %, may prevent cell culture due to the lack

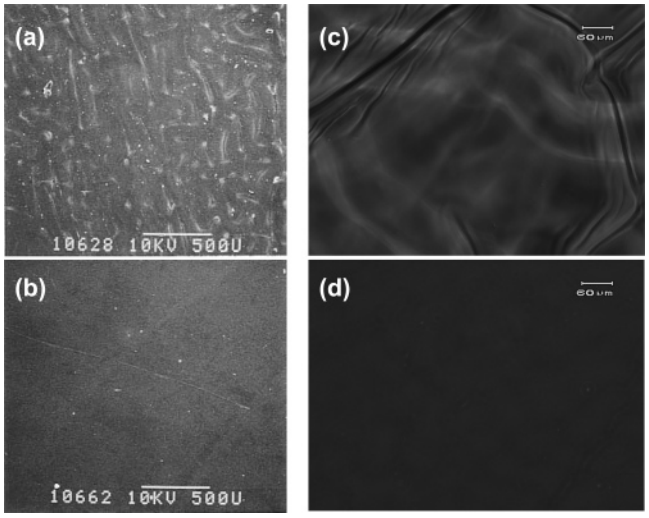

Figure 9. Different surface morphologies of N-NC1 and N-NC6 gels. (a and b): Scanning electron micrographs for dried N-NC1 and N-NC6 gels. (c and d): Photomicrographs of dried N-NC1 and N-NC6 gels after swelling for 12 h in the medium for HepG2.

of hydrophobicity. The reason for poor cell culture on N-NC gels with higher clay contents, NC10 or more, is attributed to the decrease in the hydrophobic nature at 37 °C. As reported previously,[15] the coil-to-globule transition was depressed by increasing C_{clay}, as evidenced by transmittance changes above LCST. This means that NC gels decrease in hydrophobicity with increasing C_{clay}. Thus, the ability to cultivate cells on the surface of N-NC gels gradually decreases at high C_{clay}.

Concerning the surface morphology, the flatness of the gel surface on the scale of cell sizes (a few tens of micrometers) may be important. Figure 9, panels a and b, shows scanning electron micrographs of dried N-NC1 gel and dried N-NC6 gel, respectively. The latter surface was flat, whereas the former surface was irregularly corrugated. This difference was also observed by phase-contrast optical microscopy for the surfaces of both rehydrated dried N-NC gels maintained in the medium for more than 12 h, as shown in Figure 9, panels c and d. Similar morphological differences with C_{clay} were also observed in the case of standard NC gels (NC1 and NC6 gels). Thus, both N-NC1 gels, starting from standard and dried N-NC1 gels, have irregular surfaces in the medium under cultivation conditions. In contrast, N-NC6 gels exhibit very smooth surfaces in the medium. This probably results in the different cell culture behaviors of N-NC gels. Hence, cell cultivation exhibits a maximum for N-NC gel with intermediate C_{clay}.

Detachment of Cell Sheets without Treatment by Trypsin. It was reported by Okano et al.[7,16−21] that cells, or cell sheets, cultivated on TCPS dishes modified by grafted PNIPA with temperature-sensitivity, can be detached just by decreasing the temperature below the PNIPA LCST. Since N-NC gels exhibit well-defined temperature sensitivity, due to the coil-to-globule transition at the PNIPA LCST, as previously reported,[9,10] it is expected that cells cultured on N-NC gels could be detached simply by decreasing the temperature from 37 °C (hydrophobic) to 10∼20 °C (hydrophilic). It was observed that HepG2 cell sheets cultured on N-NC6 gel separated from the surface by decreasing the temperature followed by slightly pumping the medium on to the cell−gel interface through a pipet. Figure 10a−c shows fibroblast cell sheets detached by decreasing the temperature to 10∼20 °C. With time, fibroblast cell sheets spontaneously detached from the substratum. In the present study, cell sheets were recovered from the gel surface within 20 min of starting the decrease in temperature. In contrast, in

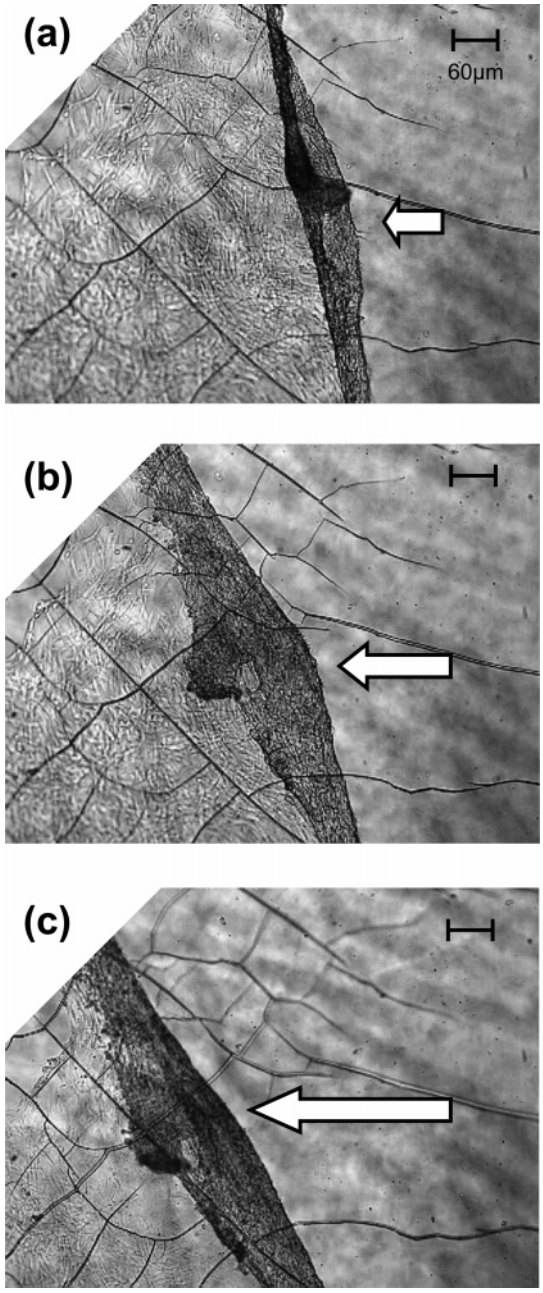

Figure 10. Cell sheet detachment of fibroblast by decreasing the temperature to 10 ∼ 20 °C. (a−c) Changes of cell sheet detachment from dried N-NC6 gels by time intervals of 1 min for (a−b) and 3 min for (b−c).

the case of conventional TCPS dishes, neither type of cell could be detached using the same treatment. Thus, the resulting cells and cell sheets detached from N-NC gel surfaces may contain extra-cellular matrix (ECM), since trypsin treatment is not necessary. So, this procedure could be useful in culturing cells for tissue engineering, such as transplantation or constructing two- or three-dimensional cell assemblies. Compared with TCPS dishes modified by grafted PNIPA, the key advantages of the present matrix are as follows: (1) transparent, soft, and deformable substratum (with high mechanical toughness); (2) available in various forms such as rod, hollow tube, thin film, sheet, etc. with widely different thicknesses; (3) The possibility of making uneven or patterned surfaces or chemically modified surfaces; (4) good temperature-sensitivity (controlled LCST behavior). Finally, we examined the reculture of cells obtained by temperature-regulated detachment. It was found that the cells

could be subcultured as usual, so cells obtained by temperature-regulated detachment are viable.

Discussion

The reason cells cannot be cultured on the dehydrated surfaces of conventional PNIPA hydrogels (N-OR gels), despite the resulting surface becoming hydrophobic, which is necessary to culture cells, is still not clearly understood. Takizawa et al.[14] reported that neither adhesion nor proliferation is observed on the surface of (linear) PNIPA coated on a PS dish. They concluded that, by conjugating PNIPA with collagen, it becomes possible for cells to adhere to and proliferate on the surfaces, due to the increase in surface wettability and cell adhesion. Also, Okano et al.[16] reported that PNIPA layers, which may be cross-linked by γ-ray irradiation, grafted onto TCPS dishes can support cell cultures only when the thickness of the PNIPA layer is less than 30 nm. Thus, the results of the present study, using conventional, chemically crosslinked N-OR gels with large thicknesses exceeding the micrometer scale, are consistent with their results.

The most important factor in realizing the successful culturing of cells on PNIPA hydrogel (N-NC gel) surfaces may be the incorporation of molecularly dispersed inorganic clay and the formation of a specific PNIPA−clay network. The incorporation of inorganic clay (hectorite) changes the property of PNIPA network above the PNIPA LCST in the following ways. One is to increase the hydrophilicity of the contracted PNIPA network. Then, a suitable balance of hydrophobicity (due to contracted PNIPA chains) and hydrophilicity (due to hydrophilic hectorite) may be attained. This is consistent with the fact that many hydrophobic polymers (solids) were modified, by for example plasma discharge, to achieve the adequate balance between hydrophilicty and hydrophobicity required to sustain adhesion and proliferation of cells.[22]

Another aspect is the increase of anionic charges. Since exfoliated hectorite sheets have anionic surface charges, the resulting PNIPA−clay network is negatively charged. In the present study, it is considered that the anionic charges associated with the surfaces of the exfoliated clay sheets may act as a promoter for cell adhesion on dehydrated PNIPA gels. Then, from the dependence of cell cultivation on C_{clay} (Figures 3, 4, and 6), it seems likely that a limited range of clay content is optimum for culturing cells. There have been many reports relating to the effects of surface charges on solid substrates and hydrogels on cell cultivation, such as the incorporation of polar groups on PS,[23−25] the formation of gradients of functional groups on polyethylene (PE) surfaces,[26] the grafting of ionic monomer on PE,[27] and copolymerization of various amounts of positively or negatively charged monomers with poly(2-hydroxy ethyl methacrylate) (PHEMA) hydrogel[28] or poly-(ethylene glycol),[29] and the treatment of PHEMA hydrogel with concentrated sulfuric acid.[30] In these studies, although the positive charges can be predominantly used to improve culturing of cells, the detailed effects of the natures and densities of the charges differ markedly with the nature of the base polymer or hydrogel and cell types. In the present study, it was concluded that the incorporation of a certain amount of negatively charged clay sheets into hydrophobic dehydrated PNIPA chains can improve the adhesion and proliferation of various cell types.

Unlike N-NC gels, D-NC gels, consisting of permanently hydrophilic PDMAA and clay, did not allow cells to adhere and proliferate at all, as described in section 3.2. This result indicates that those hydrogels, consisting of a three-dimensional network of hydrophilic polymer and negatively charged hydrophilic clay, are intrinsically cell nonadhesive. Instead, the combination of hydrophobic polymer (contracted PNIPA chains above their LCST) and hydrophilic, anionic clay on a nanometer scale provides a feasible basis for cell culture.

Conclusions

As for the cytotoxicities of each component of PNIPA hydrogels, it was found that their main components (PNIPA, clay) and very small amounts of residual NIPA, TEMED, and KPS did not disturb cell cultures of HepG2. As a result of investigations on the culturing of cells on the surfaces of two different types of thermo-sensitive PNIPA hydrogels, it was concluded that cells could be cultured only on the surfaces of dehydrated PNIPA hydrogels, regardless of the gel thickness, by using N-NC gels with certain specific clay concentrations; this is the first observation of successfully culturing cells on a PNIPA hydrogel. The adhesion and proliferation of HepG2 were found to depend strongly on the clay content (C_{clay}) of N-NC gels and to be maximal at $C_{clay} \approx 6 \times 10^{-2}$ mol/1 L of H_2O. Different types of cells, fibroblast and HUVEC, were found to be confluent on the surface of N-NC6 gel, and to exhibit similar C_{clay} dependencies on the numbers of cultured cells. It was concluded that the development of cell cultures had little dependence on either the water contents or the thicknesses of N-NC gel sheets. On the other hand, it was established that cell cultures hardly developed on the surfaces of conventional N-OR gels, regardless of their cross-linker content, although their compositions were almost the same as those of N-NC gels, except for the nature of the cross-linker. The failure of cell cultures to develop on either type of thermo-stable, hydrophilic NC gels, D-NC and D-OR gels, was attributed to their lack of hydrophobicity. The reason cells adhered and proliferated only on the surfaces of N-NC gels with their specific network structure and compositions, was discussed in terms of protein absorption on the surface, surface flatness, the hydrophobicity of dehydrated PNIPA chain and the surface anionic charges of the incorporated exfoliated clay. Finally, it was concluded that a decrease in temperature from that of the cultivation temperature (37 °C) to below that of the PNIPA LCST (e.g., 10∼20 °C) caused cell cultures to detach from the surfaces of N-NC gels without treatment by trypsin. Confluent cell layers of HepG2 and fibroblast could be spontaneously separated as cell sheets. The ability to culture cells on N-NC gels, found in the present study, together with the distinguished optical and mechanical properties of the gels, may be useful for their development in various biomedical applications.

Acknowledgment. The authors thank Ms. M. Hasegawa for technical assistance on the cell culture.

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BM060549B