Journal of Stem Cells & Regenerative Medicine
ISSN 0973 - 7154

REVIEW ARTICLE

JSRM Code: 008010200002

Development of Soft Nanocomposite Materials and Their Applications in Cell Culture and Tissue Engineering

Kazutoshi Haraguchi

Novel soft nanocomposite materials with unique organic/inorganic network structures have been developed by extending the strategy of "organic/inorganic nanocomposites" to the field of soft materials. The structures described here were synthesized by *in-situ* free-radical polymerization of various monomers in the presence of exfoliated clay (hectorite) in aqueous media. The nanocomposite hydrogels (NC gels) and soft nanocomposites (M-NCs) obtained were flexible and transparent soft materials, regardless of the clay content, that could be prepared in various shapes and surface forms, each consisting of individually different polymer/clay network structures. Owing to these unique network structures, both NC gels and M-NCs showed extraordinary mechanical properties such as ultrahigh elongation at break and widely controlled modulus and strength, which could overcome the problems (e.g., mechanical fragility, optical turbidity, poor processing ability) associated with conventional chemically crosslinked materials. In addition, the NC gels and M-NCs exhibited a number of new characteristics related to optical anisotropy, morphology, biocompatibility, stimulus sensitivity and cell culture. In the present review, we outline the novel features of these soft nanocomposites, and demonstrate their potential as soft culture substrates useful for tissue engineering as well as soft, transparent, absorbing, and mechanically tough biomaterials for many bio-applications.

1. Introduction

Although the study of hydrogels is one of the oldest areas of research in polymer science, hydrogels still have many exciting potential applications in the fields of biology, biochemistry, medicine, pharmacy, food chemistry, analytical chemistry, electrochemistry, and photochemistry.[1-7] Polymer hydrogels, which consist of a three-dimensional polymer network and a large amount of water, have long been believed to be interesting but mechanically fragile materials because of their randomly arranged chemically crosslinked network.[8]In addition, polymer hydrogels have serious problems such as optical opaqueness at high crosslink density due to the structural heterogeneity, low degree of swelling due to a large number of cross links, and low deswelling rates in the case of stimuli-responsive hydrogels.[9]In order to overcome these limitations, we have been working towards the development of a new type of polymer hydrogel with a novel network structure. The resulting novel hydrogels, i.e., nanocomposite hydrogels (NC gels),have a unique organic/inorganic network structure formed by a polymer-clay combination.[10]Also, by extending the concept of NC gels and their synthesis to the field of solid polymer nanocomposites, a new transparent, soft nanocomposite (M-NC) consisting of a hydrophobic polymer and inorganic clay was developed.[11] Furthermore, a novel series of thermoresponsive NC gels (MD-NC gels) consisting of inorganic clay and copolymers with different chemical affinities for water were developed.[12]

There are a number of properties that are considered to be desirable for materials that are to be used for cell culture applications such as tissue engineering and regenerative medicine. These include transparency, flexibility, elasticity, and, most importantly, biocompatibility. Materials that are capable of being formed into various shapes, such as thin films, rods, spheres, and hollow tubes, are even more sought-after as they increase the potential for being utilized in many *in vivo* or *in vitro* applications under both static and dynamic conditions. One biomedical application that is currently being explored is the production of an enzyme-free cell-harvesting system, using thermoresponsive polymers. Materials reported thus far have been composed of particle monolayers [13], honeycomb-patterned thin films[14], or thin coatings on rigid substrates such as polystyrene dishes.[15] Some culture systems are composed of soft hydrogels: nanocomposite hydrogel consisting of a PNIPA/clay network[16] or polyethylene glycol (PEG)/clay network[17],thermoresponsive chitosan-PNIPA hydrogels [18], protein conjugated PEG [19]or polyacrylamide [20]hydrogel; highly negatively charged poly(2-acrylamido-2-methyl-1-propanesulfonic acid) hydrogels. [21]

Material Chemistry Laboratory, Kawamura Institute of Chemical Research, 631 Sakado, Sakura, Chiba 285-0078, Japan

Received 07 Feb 2012; accepted 11 Feb 2012; published online 14 Apr 2012

Here, we report novel soft, transparent, and elastomeric hydrophilic and hydrophobic substrates that satisfy all of the requirements for a biomaterial described above. [16, 22-24] In addition, these materials are shown to be suitable for cell culture and subsequent enzyme-free cell detachment. The materials described are soft and transparent organic/inorganic nanocomposite hydrogels or solid films (NC gel, MD-NC gel, M-NC film), consisting of the organic polymer poly(*N*-isopropylacryamide) (PNIPA) or poly(2-methoxyethyl acrylate) (PMEA) and inorganic clay (hectorite: Laponite XLG). In the present review, we show the synthesis and characteristics of these recently developed soft nanocomposite materials and demonstrate their great potential for applications in cell culture and bioengineering.

2. Synthesis and characterization of the nanocomposite materials

2.1. Nanocomposite hydrogels (NC gels)

The NC gels were synthesized by *in-situ* free-radical polymerization of *N*-alkylacrylamidemonomersin the presence of exfoliated clay platelets (hectorite)uniformly dispersed in aqueous media. [9,25]NC gels could be prepared in various forms and sizes at high yields (>99.9%) under mild conditions such as 20°C without stirring. [8] Here, disk-like clay nanoparticles functioned as crosslinkers within the organic polymer to form new types of organic/inorganic network structures. [26] In this work, synthetic hectorite("Laponite XLG": Rockwood Ltd., UK; $[Mg_{5.34}Li_{0.66}Si_8O_{20}(OH)_4]Na_{0.66}$: layer size =~30 nm in diameter × 1 nm in thickness, cation exchange capacity = 104 mequiv/100 g) (**Figure 1**) was used as the inorganic clay. By using *N*-isopropylacrylamide (NIPA) or*N,N*-dimethylacrylamide (DMAA) as a monomer, thermoresponsive or thermostable NC gels were prepared, respectively. In the case of NIPA monomer, the resulting NC gels (N-NC gels) showed a large volume change when heated to 32 °C due to the coil-to-globule transition of PNIPA that is known to occur at this temperature (lower critical solution temperature, LCST). [9] The N-NC gels showed remarkable swelling/deswelling behavior and hydrophilic to hydrophobic change in surface wettability by simply increasing the temperature. On the other hand, gels prepared using the DMAA monomer and clay (D-NC gels) were hydrophilic regardless of the surrounding temperature and did not show any drastic change in gel volume or surface wettability. [27]

The NC gels obtained are shown in **Figure 2**. The materials were transparent, uniform hydrogels containing ≥90wt% water and could be prepared in various shapes, including sheets, thin films, spheres, rods, hollow tubes, and bellows) [8] and could also be modified by micrometer-scale surface patterning using replica molding. [28] It was found that NC gels show an extraordinarily high mechanical toughness, with an ultrahigh elongation at break (>1000%) and widely controllable strength and modulus [29], in addition to excellent swelling and stimulus sensitivity. [30] Furthermore, NC gels exhibit numerous unique characteristics related to optical anisotropy[31], polymer/clay morphology[32], biocompatibility[24], stimuli-sensitive surfaces[33], ultrahigh hydrophobicity[34-36], and self-healing capabilities. [37]

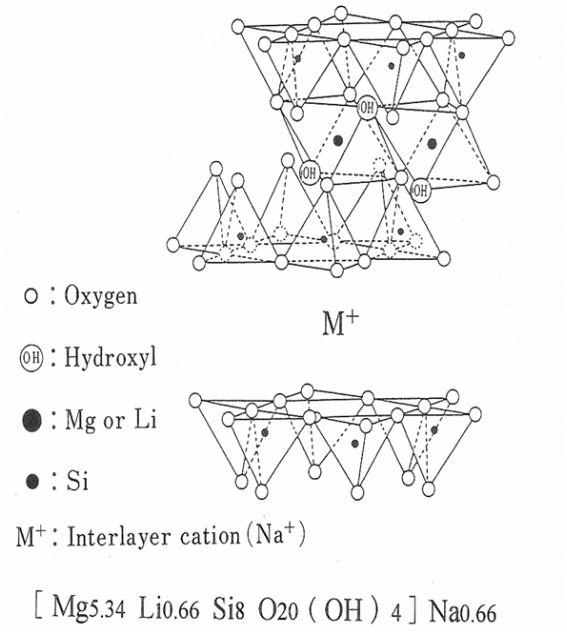

Figure 1. Inorganic clay: Synthetic hectorite ($[Mg_{5.34}Li_{0.66}Si_8O_{20}(OH)_4]Na_{0.66}$,layer size =~30 nm in diameter × 1 nm in thickness, cation exchange capacity = 104 mequiv/100 g)

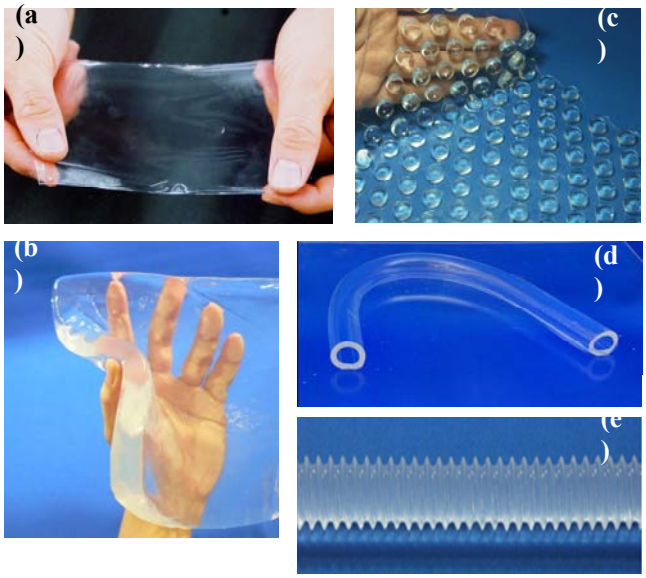

Figure 2. NC gels with various shapes: (a) thin film, (b) sheet, (c) uneven sheet, (d) hollow tube and (e) bellow.[45]

2.2. Soft nanocomposites (M-NCs)

PMEA, the organic polymer constituent of the soft nanocomposite (M-NC), is a hydrophobic polymer with a low glass transition temperature (-34°C). The PMEA is a promising material for use in blood-contacting medical devices, such as those used in cardiopulmonary bypass surgery [38-40], as it demonstrates low protein adsorption and platelet adhesion [41-44]. However, in practical applications, neither linear PMEA (M-LR), nor chemically crosslinked PMEA (M-OR) has ever been used as a self-standing film as they are mechanically weak and difficult to process. On the other hand, the newly developed soft nanocomposite (M-NC), consisting of hydrophobic PMEA and hydrophilic inorganic clay (hectorite), can be obtained as a uniform transparent film (**Figure 3**) that exhibits excellent optical and mechanical properties. [11]

The M-NCs were synthesized in a similar way to the NC gels, by *in-situ* free-radical polymerization of water-soluble 2-methoxyethylacrylate (MEA) monomer in the presence of exfoliated clay. The crucial aspect of the synthesis is that the monomer-to-polymer (hydrophilic to hydrophobic) transitions occur with micro- and macro-phase separations. Consequently, uniform, colorless, and transparent (>90% transmittance) M-NC films (**Figure 3**) were obtained with a wide range of C_{clay}, e.g. 5–50 wt%. The, M-NCs could also

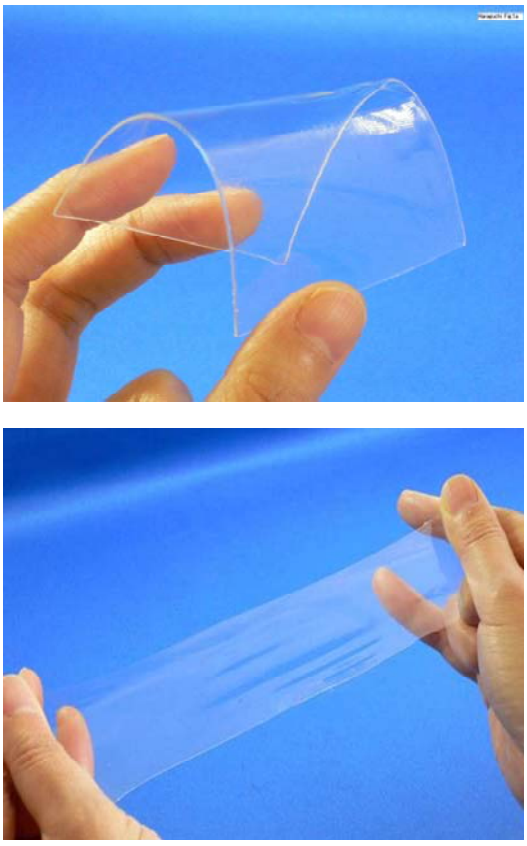

Figure 3 (a) Transparent, soft, elastomeric nanocomposite film (M-NC), consisting of PMEA and 23wt % inorganic clay.[11](b) Elongated M-NC film.

properties compared to conventional polymeric materials (M-LR and M-OR), such as ultrahigh reversible extensibility and well defined yielding behavior, despite their high clay contents. [11] Thus, a number of serious disadvantages, including intractability, mechanical fragility, optical turbidity, poor processing ability, and low stimulus sensitivity, associated with conventional, chemically crosslinked polymeric materials were overcome in the NC gels and M-NCs. [45]

2.3. Copolymer-based nanocomposite hydrogels (MD-NC gels)

A class of highly versatile, thermoresponsive nanocomposite hydrogels (MD-NC gels) has also been developed. [12] These gels consist of clay (hectorite) and specific copolymers with different chemical affinities for water, i.e., copolymers composed of hydrophobic MEA and hydrophilic DMAA units. The resulting MD-NC gels showed outstanding stimuli sensitivities in response to changes in temperature, pH, salt concentration, and solvents in the surrounding aqueous solution (**Figure 4A**) as well as excellent, composition-dependent mechanical properties. **Figure 4B** shows the reversible change of MD-NC gel size (length) in response to alternating temperature changes.

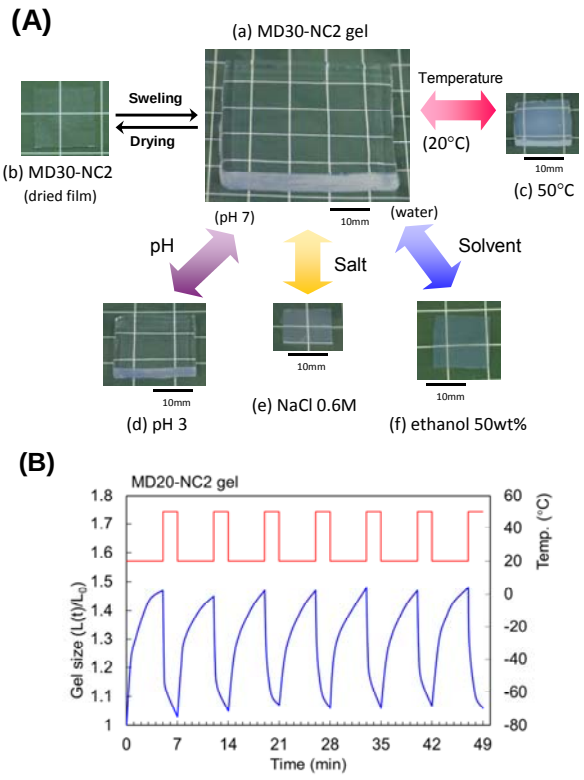

Figure 4 (A).(a) Transparent and mechanically tough MD-NC gel and (b) the soft nanocomposite (MD-NC) dried there from. The MD-NC gel exhibits stimuli-sensitivity in response to (c) temperature (20 ↔50 °C), (d) pH (7 ↔3), (e) salt concentration (NaCl, 0 ↔0.6M), and (f) solvent (ethanol, 0 ↔50wt%) in the surrounding aqueous solution. Here, Images in (a)–(f) are different views of the same sample under different conditions.(B) Thermo-reversible change in the gel size (length) of an MD-NC gel by alternating the temperature between 20 and 50 °C.[12)]

2.4. Polymer/clay network structures

The network structure consisting of polymer and inorganic clay is totally different in NC gels and M-NCs. The NC gels possess a unique organic (polymer) /inorganic (clay) network structure (**Figure 5**) in which exfoliated clay nanoparticles are interlinked by a number of flexible polymer chains. [9,10,26,27,46] The interaction is ascribed to non-covalent bonds, mainly hydrogen bonds between the amide side groups on the polymer and the surface of the clay. It was hypothesized that a number of polymer chains interact with a single clay platelet and that each polymer chain may interact with the clay surface at multiple points.

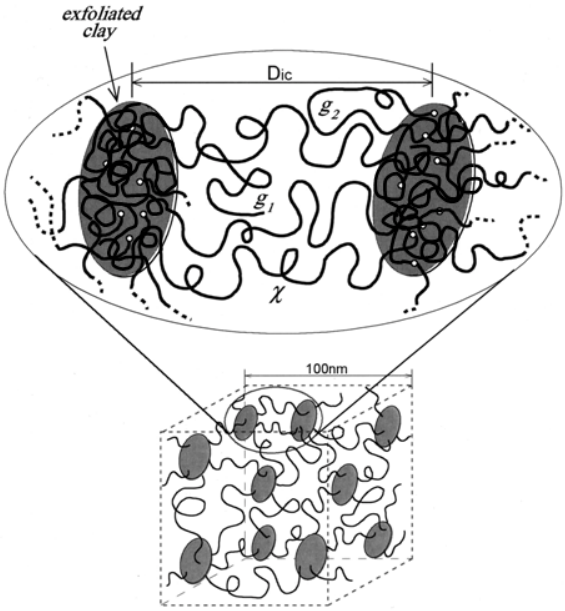

Figure 5 Schematic representation of the structural model with organic (polymer)/inorganic (clay) networks in the NC gel. D_{ic} is inter-particle distance of exfoliated clay sheets. χ, g_1 and g_2 represent crosslinked chain, grafted chain and looped chain. In the model, only a small number of polymer chains are depicted for simplicity.[45]

This was supported by the molecular characteristics of PNIPA separated from NC gels [47,48]and contrast-variation small angle neutron scattering measurements. [49] In contrast, M-NCs possess a clay-network morphology consisting of a large number of hollow spheres of aggregated clay platelets (thickness of clay shell is about 20 nm) with PMEA chains packed inside (**Figure 6**)[11].

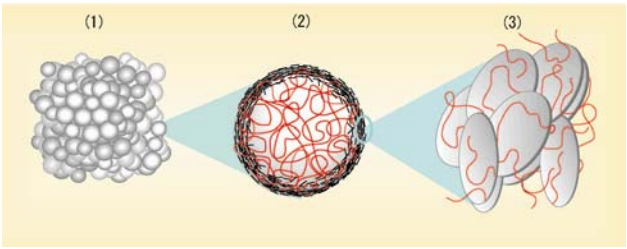

Figure 6 Schematic illustrations of clay network morphology and (b) the deformation process for M-NC11. (1) clay network structure. (2) a clay/PMEA sphere. (3) clay outer shell.[11]

MD-NC gels have an intermediate network structure between those of NC gels and M-NCs, which depends on the copolymer ratio of MEA and DMAA. [12]

3. Cytocompatibility of nanocomposite hydrogels (N-NC gels)

From the extensive studies of cell culture on PNIPA carried out by Okano and colleagues [50-55], it was revealed that cells could be cultured on the thin PNIPA layer grafted to the surface of a tissue culture polystyrene dish. The cells could then be harvested as a cell-sheet by utilizing the hydrophobic to hydrophilic transition behavior of PNIPA that occurs when the temperature is reduced to below its LCST. It was found that cells could only attach and proliferate if the PNIPA layer was very thin, in particular, less than 30 nm in thickness. [54]

The PNIPA hydrogels developed in this work (N-NC gels) were assessed for their potential as cell culture substrates.[16] Alongside the N-NC gel, a chemically crosslinked PNIPA gel (N-OR gel) was also investigated. In the case of conventional N-OR gels, it was almost impossible to culture cells on their surfaces. In contrast, it was found that cells could be cultured to confluence on the N-NC gels. **Figure 7a-c** and **Figure7d** show phase contrast micrographs of HepG2 cells after different culture times on the surfaces of N-NC and N-OR gels, respectively. It can be clearly seen that the cells became attached and began spreading on N-NC gel after just 12 h (**Fig. 7a**), with more extensive spreading evident at 24 h(**Fig. 7b**). Almost complete cell coverage was observed within a 5 day culture period (**Fig.7c**). In contrast, little adhesion and no proliferation of the HepG2 cells were observed on the surface of N-OR gel throughout the 7 day culture period (**Fig. 7d)**. Taking the structural differences of these PNIPA hydrogels into consideration, the remarkable improvement of the N-NC gel, compared with that of the N-OR gel, was attributed to the specific PNIPA/clay network structure of the N-NC gel. In order to confirm the effects of the materials on cultured cells, two primary cell types were also investigated, normal human dermal fibroblasts (NHDFs)

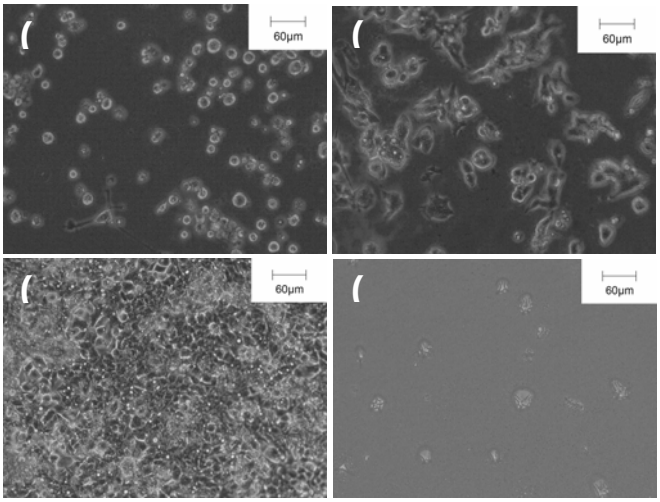

Figure 7 Phase-contrast photomicrographs of HepG2 cultures on the surfaces of (a) ~ (c) N-NC6 gels and (d) N-OR1 gel, after (a) 12 h, (b) 24 h, (c) and (d) 5 days.

and human umbilical vein endothelial cells (HUVECs). [16] It was observed that both cell types could adhere to and spread well on the N-NC gel, in a similar manner to the HepG2s. They were also seen to proliferate almost to confluence, as shown in **Figure 8a** (NHDF) and **8b** (HUVEC). Both cell types only partially adhered to the N-OR gel and did not spread at all over the 7 day culture period, as was found with the HepG2s. Thus, it was concluded that the conventional PNIPA hydrogel (N-OR gel) was generally non-supportive for cell adhesion and spreading, whereas the nanocomposite PNIPA hydrogel (N-NC gel) was capable of culturing various types of cells. This difference is likely due to the combination of the negatively charged clay particles with the hydrophobic polymer chains making the culture surface more favorable.

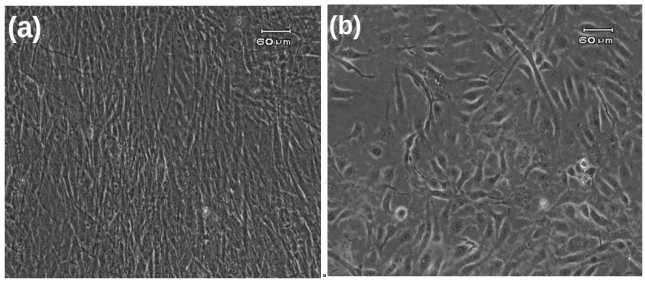

Figure 8 Phase-contrast photomicrographs of fibroblast (a) and HUVEC (b) proliferated on N-NC6 gel after culturing for 5 days.[16)]

Some of the factors influencing cell cultivation on N-NC gels, namely, the effects of clay content (C_{clay}), water content (C_{H2O}), and gel thickness were clarified. [16] It was found that the most preferable clay concentration for cell culture is around C_{clay}= 6 × 10^{-2} mole/L-H_2O for HepG2, NHDF, and HUVEC. It was found that the $C_{H2O}$of a satisfactory substratum could 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 of hydrophobicity. With regards to the gel thickness, it was concluded from experiments using N-NC gels of0.5–5 mm thick in their as-prepared state that cell cultures always grew to confluence, regardless of the gel thickness.

To clarify the reason for the totally different cell culture behaviors on the two types of PNIPA hydrogels, the adsorption of bovine serum albumin (BSA) on their surfaces was examined using an aqueous solution of FTIC-BSA (0.1 mM in Tris-HCl, pH7.4) at 37 °C for 3 hours. [16] It was found that, in general, NC gels adsorbed large quantities of BSA, whereas the OR gels adsorbed a lot less. This suggests that the PNIPA/clay network structure plays an important role in protein adsorption, thereby enabling the cells to adhere and proliferate. In the case of the OR gels, the absence of a sufficient amount of adsorbed protein from the culture medium meant that cells were not able to adhere.

For the two types of PDMAA hydrogels, D-NC and D-OR gels, which do not exhibit temperature dependent behavior, and so are not hydrophobic at culture temperature, it was found that few cells of any kind adhered to or proliferated on their surfaces, regardless of the type of network structure. [16] This is probably because both D-NC and D-OR gels are totally hydrophilic under the cultivation conditions(at 37 °C). In fact, they swelled in the medium up to 2090 wt% and 1050 wt% for D-NC6 gel and D-OR3 gel, respectively, by the third day.

4. Detachment of cell sheets from nanocomposite hydrogels (N-NC gels)

Okano *et al* reported that cells, or cell sheets, cultivated on TCPS dishes modified by grafted PNIPA, could be detached just by decreasing the temperature below the PNIPALCST. [54] Since N-NC gels exhibit the same well-defined temperature sensitivity, it was hypothesized that cells cultured on these gels could be detached in the same way. It was observed that HepG2 cell sheets cultured on N-NC gel separated from the surface by decreasing the temperature followed by slight agitation of the medium at the cell-gel interface using a pipette. [16] **Figure 9(a)–(c)** shows fibroblast cell sheets detached from the substrate within 20 min simply by decreasing the temperature to 10–20 °C.

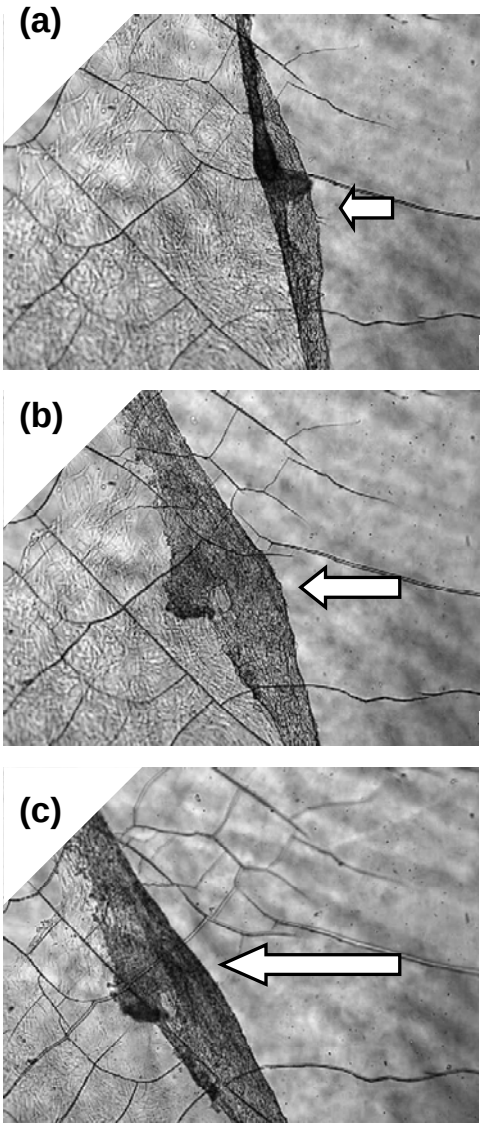

Figure 9 Cell sheet detachment of fibroblast by decreasing the temperature to 10 ~ 20 °C. (a) ~ (c) Changes of cell sheet detachment from dried N-NC6 gels by time intervals of 1 min. for (a) to (b) and 3 min. for (b) to (c).[16)]

Subsequently, we found that the continued culture of cells that had been obtained by temperature-induced detachment was possible, indicating that there was no adverse effect on their viability. As trypsin is not necessary when culturing cells on the N-NC gels, the maintenance of the extracellular matrix synthesized by the cells is possible. Therefore, this procedure could be useful in culturing cells for tissue engineering, such as transplantation or constructing two- or three-dimensional cell assemblies. There are a number of advantages of the N-NC gels compared to TCPS dishes modified by grafted PNIPA. (1) The materials are transparent, soft and deformable (with a high mechanical toughness); (2) they are available in various forms, such as rod, hollow tube, thin film, sheet, etc. with widely different thicknesses; (3) there is the possibility of making uneven or patterned surfaces or chemically modified surfaces; (4) they display good temperature sensitivity (controlled LCST behavior).

N-NC gels can also be synthesized by free-radical polymerization initiated by a photo-initiator and UV light irradiation[56]. The resulting photopolymerized NC gels (photo-NC gels) were uniform, transparent, and exhibited excellent mechanical properties similar to those of the NC gels prepared by thermal redox-initiated free-radical polymerization described above. The photo-NC gels can exist in various forms including thin films and large monoliths with a wide range of thicknesses (10^{-3}–10^{2} mm) and can even form thin coatings on a substrate. Many outstanding characteristics have been demonstrated for the photo-NC gel coatings, such as an anti-fogging property, thermosensitive sliding frictional behavior, flow control in microfluidic channels, formation of patterned NC gels by UV light and cell harvesting without enzymatic treatment. **Figure 10** shows a phase-contrast photomicrograph of NHDFs cultured on the surface of a polystyrene dish coated with photo-NC gel. It was observed that the NHDFs attached and could be cultured to confluence (Figure **10-i**) and that the cell sheet could subsequently be detached without the use of trypsin, by decreasing the temperature to 10 °C for 5 min (**10-ii**). Similar cell cultivation and subsequent cell detachment was also observed for other cells, such as HepG2s and HUVECs[56]. These cell harvestings on photo-NC-gel-coated polystyrene dishes are another example of the versatility of these nanocomposite materials.

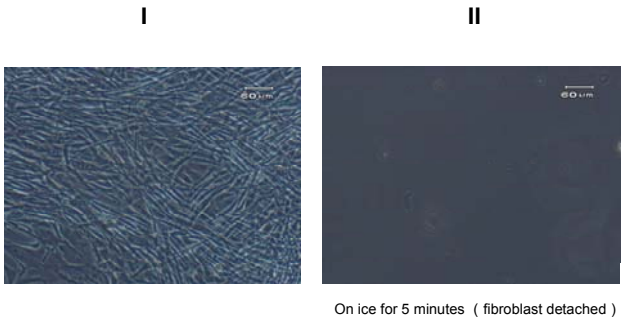

Figure 10 Phase-contrast photomicrographs of NHDF cultures (i) on photo-NC5 gel coating on polystyrene dish and (ii) after detaching cell sheet by decreasing temperature to 10 °C for 5 min.[56]

5. Cytocompatibility of soft nanocomposites (M-NCs)

Soft, transparent, and mechanically tough nanocomposite(M-NC) films were also studied as substrates for cell culture[22]. It was found that M-NC films are supportive of the adhesion and proliferation of different types of cells. **Figure 11a** shows phase-contrast micrographs of various cells (3T3,NHDF, HUVEC, and BAEC) cultured on M-NC films (C_{clay}= 15 wt%). It is clear that all cells adhered to and proliferated on the film surfaces in a similar manner to those on the TCPS dish (control).Almost complete cell coverage was observed for both substrata within the five day culture period. It was also observed that the cells were cultured to confluence on the surface of M-NC films, almost regardless of clay content (C_{clay}= 5–50 wt%). In contrast, little adhesion and no proliferation of cells were observed on the surface of the chemically crosslinked M-OR film, regardless of crosslink density, for any of the cells used. Also, for the linear PMEA (M-LR) film, cells barely grew on the surface, although it was difficult to obtain precise data on cell culture, since it was difficult to prepare a reliable and uniform M-LR thin film. Quantitative data on the number of cells cultured on the M-NC film, M-OR film, and TCPS dish are shown in **Figure 11b**. Thus, it was concluded that various types of cells could securely adhere to, and successfully grow to confluence on the surfaces of soft M-NC films in contrast to the non-adhesive conventional M-OR and M-LR films.

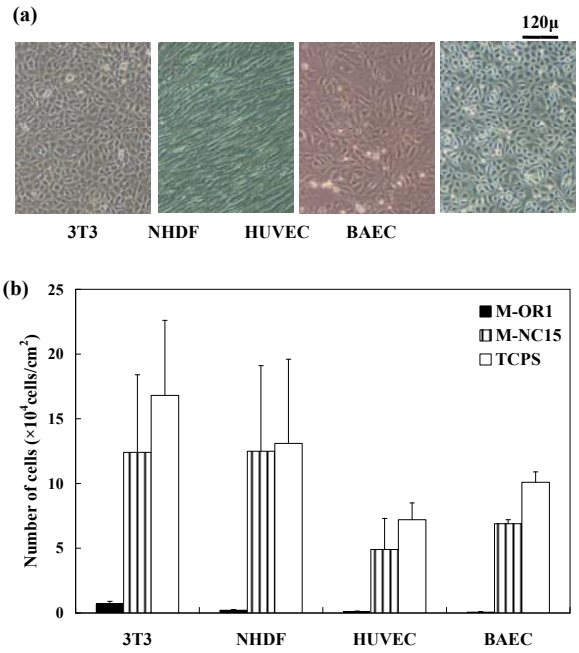

Figure 11.(a) Phase-contrast photomicrographs of 3T3, NHDF, HUVEC, and BAEC cultured on the surfaces of M-NC, after a five-day culture period (three days for 3T3). No cell type cultured well on the M-OR1 film. (b) Densities of various cells cultured on the surface of the M-OR film, M-NC film, and TCPS dish, after a five-day culture period (three days for 3T3). Cells: 3T3, NHDF, HUVEC, and BAEC.[22]

6. Cell harvesting from soft nanocomposites(M-NCs)

It was found that, after cells were cultured on M-NC films, the resulting cells or cell sheet spontaneously detached from the surface when the temperature of the medium was reduced [22]. **Figure 12a-1** shows a phase-contrast micrograph of 3T3 cells cultured for three days to confluence on an M-NC film and **Figure 12a-2** shows the cells after reducing the temperature for 30 min. Some of these cells can be seen to have changed morphology to become round, as shown in the inset (magnification) of **Figure 12a-2**. This indicates that, although almost all of the cells were still attached to the surface at this stage, some cells had begun to lift off, probably because cell-surface adhesion was weakened by reducing the temperature. By subsequently using gentle pipetting, most of the cells became detached, with only a few remaining on the surface (**Figure 12a-3**).

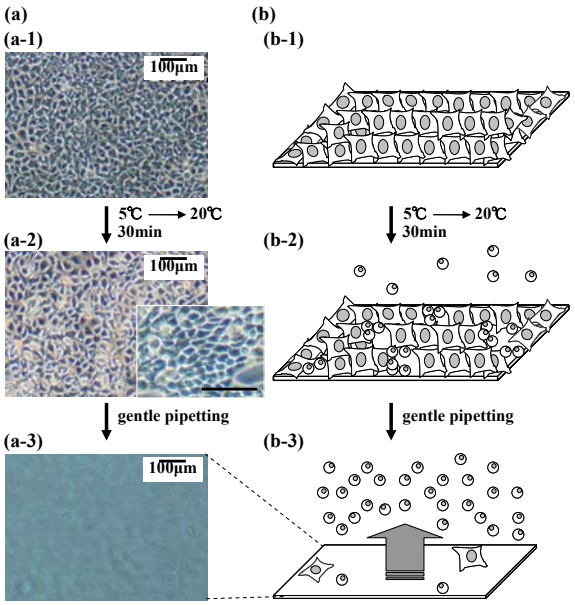

Figure 12. Cell detachment of 3T3 cultured on the surface of M-NC11 film, by reducing the medium temperature to 10~20°C and using gentle pipetting. (a-1) 3T3 cultured to be confluent on M-NC11 film. (a-2) 3T3 on M-NC11 film, after reducing temperature for 30 min. The inset is a magnification of a part of the micrograph, to show the change in cell morphology. (a-3) The surface of M-NC11 film after gentle pipetting for (a-2). (b-1)–(b-3) Models depict cell detachment behavior corresponding to (a-1)–(a-3).[22)]

The single cell suspension obtained is shown in **Figure 13a,** with a phase contrast image of the cells shown in **13a(i)**. Models for these phenomena are depicted in **Figure 12b**. Next, re-culture of the cells obtained by this procedure was examined in a new TCPS dish. It was found that the 3T3 cells could be sub-cultured as usual, as shown in **Figure 13b**. These findings indicate that the detached cells from the surface of the M-NC film retained high viability and would likely have retained the extracellular proteins on their surface because no enzymatic digestion was required for harvesting them. The image in **Figure 13a(ii)**shows that this temperature-dependent behavior was not limited to 3T3 cells.

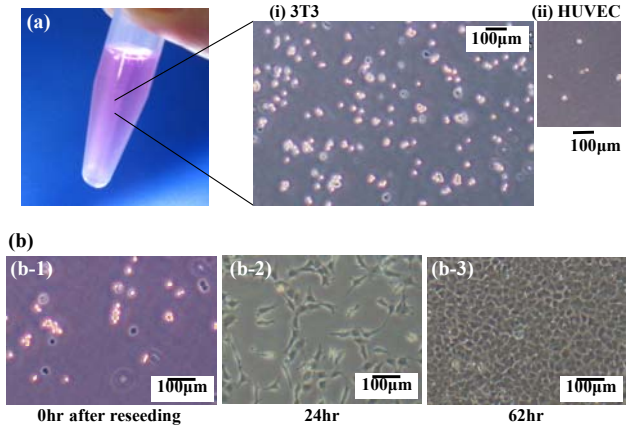

Figure 13.(a)A suspension and phase-contrast micrograph of cells detached from M-NC11 film, by reducing temperature and using gentle pipetting: (i) 3T3 and (ii) HUVEC. (b) Phase-contrast micrographs of 3T3 during the re-culturing of detached cells: (b-1) Reseeding of detached cell; (b-2) Re-cultured for 24 h; (b-3) Re-cultured for 62 h.[22)]

Here, HUVECs were detached from the surface of an M-NC film and again, formed a single cell suspension. It was occasionally observed that the cells detached from the film surface in the form of a sheet rather than as single cells, depending on the composition of the material or the cell type. These variations are likely due to slight differences in balance between cell-cell and cell-substrate interactions. In cases where cell-cell or cell-substrate interactions predominate, cell-sheet or single-cell detachment will occur, respectively. As expected, it was found that the cells cultured in TCPS dishes (3T3 and HUVEC) showed almost no change when treated in this manner, owing to the lack of temperature sensitivity of the TCPS surface. Thus, it was confirmed that M-NC has great potential to be used as a new substrate for an enzyme-free, living cell-harvesting system.

The results of these cell culture experiments were attributed to the combined effects of the organic PMEA (hydrophobic, low protein-adsorptive, non-cell-adhesive) and inorganic clay nanoparticles (hydrophilic and cell-adhesive), in the form of a PMEA/clay network structure. Given the differences in cell adhesion and proliferation on the surfaces of the M-NC and M-OR films (**Fig. 11**), it is thought that the existence of clay nanoparticles plays an important role in producing the cell compatibility of PMEA-based materials. In **Table1**, some surface properties of M-NC and M-OR films, clay, and the TCPS dish are listed[22]. The water contact angle (θ_w) for the clay was shown to be 35° in the dried state due to its hydrophilic nature. Here, θ_w changed very little on altering the measuring temperature. On the other hand, the M-NC films with different C_{clay} (11 and 15 wt%) showed intermediate values, 59° and 56.8°, respectively, which are close to that of TCPS (61.7°), compared with that of a typical hydrophobic polymer (e.g., polystyrene which is non-adhesive towards cells (ADVANTEC PETRI DISH: Toyo Roshi Ltd.) θ_w= 94.1°) or hydrophilic polymer (e.g.,poly(acrylic acid) θ_w= 40°also non-adhesive for cells).

Table 1

	M-NC11	M-NC15	M-OR1 (M-OR0.01)	Clay	TCPS
θ_w(°)	59.0	56.8	80→20.5	35.0	61.7
Ads. of IgG37°C	48.5	41.3	(2.4)	21.0	30.6
(ng/cm^2) 4°C	12.1	8.9	(1.3)	34.0	26.0
W_{water}/W_{dry} 37°C	6.4	11.9	29.8	–	–
(%) 10°C	11.7	25.1	29.7	–	–

Table 1 Surface-contact angles for water (θ_w), IgG adsorption (Ads. of IgG), and water absorption (W_{water}/ W_{dry}) for the M-NC11, M-NC15, and M-OR1 (or M-OR0.01) films, dried clay gel and TCPS dishes. The changes in IgG adsorption and W_{water}/W_{dry} due to alteration in temperature were also measured. [22)]

This suggests that an intermediate θ_w is the most favorable for cell cultivation. On the other hand, θ_w for M-OR film was initially quite high (80°) and decreased to 20.5°within a few minutes. This instability was probably due to its irregular, rough surface. In fact, in SEM micrographs, an irregular, rough surface with small holes was observed on aM-OR film, while M-NC films have smooth, flat surfaces, regardless of C_{clay}.

As protein adsorption to a surface is essential for cell adhesion, IgG was used as a model protein to investigate the effects of the different materials. It was found that M-NC films adsorb a large amount of IgG (40 - 50 ng/cm^2) at 37 °C, compared to that of PMEA (2 ng/cm^2), as shown in **Table 1**. This is consistent with the results of PMEA coatings, whose surfaces have shown low protein (bovine serum albumin) adsorption. Interestingly, it was observed that the amount of IgG adsorbed on M-NC decreased considerably on decreasing the temperature, i.e., from 40–50 ng/cm^2 at 37 °C to approximately 10 ng/cm^2 at 4 °C. Both PMEA and TCPS showed very little change in adsorption behavior (**Table 1**). This thermoresponsive behavior of protein adsorption on M-NC films is likely to contribute to the temperature-induced cell detachment.

Another contributing factor is likely to be the level of water absorption by the films. It was observed that water absorption (W_{water}/W_{dry}) increased with decreasing temperature (**Table 1**). This indicates that, in water, the M-NC surface changes from hydrophobic at 37 °C to relatively hydrophilic at 10–20 °C. This may cause cell detachment, because cells are generally less likely to adhere to hydrophilic surfaces. Thus, it is concluded that the incorporation of clay nanoparticles transforms the normally cell-resistant PMEA into a cell-adhesive material owing to the formation of a PMEA/clay network structure, and that this composite undergoes surface changes that result in cell detachment in response to a reduction in temperature.

7. Conclusions and future prospects

Thus, it was found that cells could be cultured on the surfaces of N-NC gels, N-NC gel coatings, and M-NC films, and that the cultured cells could be detached, without any enzymatic digestion, by decreasing the medium temperature and simultaneously using gentle pipetting. The detached cells were obtained as a single cell suspension or a contiguous cell sheet, both of which were viable and could be re-cultured. From the compositions and surface properties of the materials, it was hypothesized that the cell culture and subsequent cell detachment were due to the unique PNIPA/clay and PMEA/clay networks. These new soft nanocomposites, N-NC gels and M-NC films, are potentially very promising materials for tissue engineering, because they are soft, elastomeric, have distinguished mechanical and optical properties and can be readily fabricated in various shapes and surface forms. It is very recently found that a new class of thermoresponsive nanocomposite hydrogels (MD-NC gels) also shows the well-controlled cell culture and cell harvestings by temperature decrease[23].

When comparing the three materials that have been described in this review, namely, NC gels, M-NCs, and photo-NC gel coatings on TCPS, they all show a number of similar characteristics. However, it should be noted that these are completely different materials and each has its own unique advantages. NC gels are swollen hydrogels consisting of thermo-sensitive PNIPA, clay, and water. M-NCs are soft, solid films consisting of hydrophobic PMEA and clay. Photo-NC gel coatings on TCPS are hard, brittle, dried N-NC gels coated onto a cell culture dish. The fact that the three systems, with different compositions and different properties, can all be used in a living cell-harvesting system is also highly advantageous.

In future studies, the most suitable system could be selected depending on the kind of cells used and the harvesting conditions. Quantitative evaluations of the three systems and the mechanisms governing the harvesting of single cells or cell sheets are topics of ongoing research. Furthermore, since soft M-NCs and N-NC gels fabricated in various forms exhibit large and reversible extensibility, they can be used *in vivo* or *in vitro* under various static and dynamic stress conditions. This versatility provides the potential for these materials to be used for the engineering or regeneration of many different tissue types.

In order to apply these novel materials to biomedical engineering applications, it is important to confirm their safety. Firstly, concerning the safety of inorganic clay, synthetic hectorite (“Laponite XLG”), it is well known that Laponite XLG is used as a raw material for various cosmetics and tooth powder. Also, the safety of purified Laponite XLG has been confirmed in a cytotoxicity test. To evaluate the safety of the NC gels, biological testing of medical devices was performed, which involved a sensitization test, irritation test, intracutaneous test, and *in vitro* cytotoxicity tests.

Consequently, the safety of the NC gel in all of these tests was confirmed[24]. The examples of cytotoxicity tests for MD-NC gels and M-NC are shown in **Figure 14**[12].Further, we carried out an *in vivo* investigation of the interaction of living tissue with the NC gel and M-NC films by implantation in rabbits and goats for 1–180 days[24,57]. We found that neither inflammation nor concrescence occurred around the NC gel during the implantation *in vivo*.

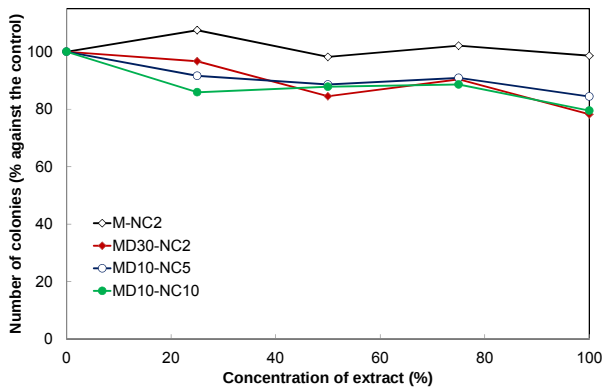

Figure 14.*In vitro* cytotoxicity tests of MD-NCs (30-NC2, MD10-NC5, MD10-NC10) and M-NC (M-NC2) using V79 cells. The change in number of colonies by altering the concentration (0–100 %) of extract added in culture medium was represented in percentage against the number of colonies obtained in culture medium without sample extract (0 %).[12)]

We evaluated the potential of NC gels, modified NC gels, MD-NC gels, and M-NC films as soft, transparent, mechanically tough and/or absorbing materials for biomedical applications such as wound-dressing materials, implant materials, anti adhesive materials, and dressings for pressure ulcers. For example, we used NC gels as dressing materials for healing various types of trauma in animal experiments involving epidermis wounds, full-depth skin wounds, and decubitus (pressure sore) models. We concluded that NC gels can comfortably be used as dressing materials for wound healing while simultaneously preventing the formation of intractable granulomatous tissues[57].

Thus, these newly developed transparent, soft nanocomposites are extremely promising functional soft materials that can be used in basic research and applications for cytology, medical devices, cell-based regenerative medicine, and tissue engineering.

References

1. DeRossi D, Kajiwara K, Osada Y, Yamauchi, A (Eds), Polymers Gels; Plenum: New York, 1991.
2. Okano T (Ed), Biorelated Polymers and Gels; Academic: Boston, 1998.
3. Lee KY, Mooney DJ. Hydrogels for tissue engineering. Chem. Rev. 2001.101: 1869-1879.
4. Osada Y, Khokhlov A (Eds), Polymers Gels and Networks; Marcel Dekker: New York, 2002.
5. Hoffman AS. Hydrogels for biomedical applications. Adv. Drug Deliv.Rev.2002; 54: 3-12.
6. Yui N, Mrsny R, Park K (Eds), Reflexive Polymers and Hydrogels; CRC: New York, 2004.
7. Gerald G, Karl-Friedrich A (Eds), Hydrogel Sensors and Actuators; Springer: Boston, 2009.
8. Haraguchi K. Nanocomposite gels: new advanced functional soft materials. Macromol. Symp.2007; 256: 120-130.
9. Haraguchi K, Takehisa T, Fan S. Effects of clay content on the properties of nanocomposite hydrogels composed of poly(N-isopropylacrylamide) and clay.Macromolecules2002; 35: 10162-10171.
10. Haraguchi K, Takehisa T. Nanocomposite hydrogels: a unique organic-inorganic network structure with extraordinary mechanical, optical, and swelling/de-swelling properties. Adv. Mater.2002; 14: 1120-1124.
11. Haraguchi K, Ebato M, Takehisa T. Polymer-clay nanocomposites exhibiting abnormal necking phenomena accompanied by extremely large reversible elongations and excellent transparency.Adv. Mater.2006; 18: 2250-2254.
12. Haraguchi K, Murata K, Takehisa T. Stimuli-responsive nanocomposite gels and soft nanocomposites consisting of inorganic clays and copolymers with different chemical affinities. Macromolecules2012; 45: 385–391.
13. Miura M, Fujimoto K. Formation and recovery of a cell sheet by a particle monolayer with the surface roughness. Colloids Surf. B 2008; 66: 125-133.
14. Tanaka M, Yoshizawa K, Tsuruma A, Sunami H, Yamamoto S, Shimomura M. Formation of hydroxyapatite on a self-organized 3D honeycomb-patterned biodegradable polymer film. Colloids Surf. 2008; 313-314: 515-519.
15. Hirose M, Kwon OH, Yamato M, Kikuchi A, Okano T. Creation of designed shape cell sheets that are noninvasively harvested and moved onto another surface. Biomacromolecules2000; 1: 377-381.
16. Haraguchi K, Ebato M, Takehisa T. Control of cell cultivation and cell sheet detachment on the surface of polymer/clay nanocomposite hydrogels. Biomacromolecules 2006; 7: 3267-3275.
17. Chang CW, van Spreeuwel A, Zhang C, Varghese S. PEG/clay nanocomposite hydrogel: a mechanically robust tissue engineering scaffold. Soft Matter 2010; 6: 5157-5164.
18. Chen JP, Yang TF. Applications of chitosan-based thermo-sensitive copolymers for harvesting living cell sheet. Appl. Surf.Sci.2008; 255: 297-300.
19. Gilbert PM, Havenstrite KL, Magnusson KE, Sacco A, Leonardi NA, Kraft P, Nguyen NK, Thrun S, Lutolf MP, Blau HM. Substrate elasticity regulates skeletal muscle stem cell self-renewal in culture. Science 2010; 329: 1078-1081.
20. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell 2006; 126: 677-689.
21. Kwon HJ, Yasuda K, Ohmiya Y, Honma K, Chen YM, Gong JP. In vitro differentiation of chondrogenic ATDC5 cells is enhanced by culturing on synthetic hydrogels with various charge densities. ActaBiomater.2010; 6: 494-501.
22. Haraguchi K, Masatoshi S, Kotobuki N, Murata K. Thermo responsible cell adhesion/detachment on transparent nanocomposite films consisting of poly(2-Methoxyethyl acrylate) and clay. J.Biomater. Sci.Polym.Ed.2011; 22: 2389-2406.
23. Kotobuki N, Murata K, Haraguchi K., to be submitted.
24. Haraguchi K, Takehisa T. Novel manufacturing process of nanocomposite hydrogel for bio-applications. Proc. ASME2005 IMECE.2005; 80533.
25. Haraguchi K, Li HJ. Control of the coil-to-globule transition and ultrahigh mechanical properties of PNIPA in nanocompositehydrogels.Angew.Chem.Int.Ed.2005; 44: 6500-6504.
26. Shibayama M, Karino T, Miyazaki S, Okabe S, Takehisa T,Haraguchi K. Small-angle neutron scattering study on uniaxially-stretched poly(N-isopropylacrylamide)-clay nanocomposite gels. Macromolecules 2005; 38: 10772-10781.

27. Haraguchi K, Farnworth R, Ohbayashi R, Takehisa T. Compositional effects on mechanical properties of nanocomposite hydrogels composed of poly (N,N-dimethylacrylamide) and clay. Macromolecules 2003; 36: 5732-5741.
28. Song L, Zhu M, Chen Y, Haraguchi K. Surface-patterning of nanocomposite hydrogel film by direct replica molding and subsequent change in pattern size. Polym. J. 2008; 40: 800-805.
29. Haraguchi K, Li HJ. Mechanical properties and structure of polymer-clay nanocomposite gels with high clay content. Macromolecules 2006; 39: 1898-1905.
30. Haraguchi K, Li HJ, Song L, Murata K. Tunable optical and swelling/deswelling properties associated with control of the coil-to-globule transition of poly(N-isopropylacrylamide) in polymer-clay nanocomposite gels. Macromolecules 2007; 40: 6973-6980.
31. Murata K, Haraguchi K, Optical anisotropy in polymer- clay nanocomposite hydrogel and its change on uniaxial deformation. J. Mater. Chem. 2007; 17: 3385-3388.
32. Haraguchi K, Matsuda K. Spontaneous formation of characteristic layered morphologies in porous nanocomposites prepared from nanocomposite hydrogels. Chem. Mater.2005; 17: 931-934.
33. Haraguchi K, Takada T. Characteristic sliding frictional behavior on the surface of nanocomposite hydrogels consisting of organic - inorganic network structure. Macromol. Chem. Phys. 2005; 206: 1530-1540.
34. Haraguchi K, Li HJ, Okumura N. Hydrogels with hydrophobic surfaces: abnormally high contact angles for water on PNIPA nanocomposite hydrogels. Macromolecules 2007; 40: 2299-2302.
35. Haraguchi K, Li HJ, Song L. Unusually high hydrophobicity and its changes observed on the newly-created surfaces of PNIPA/clay nanocomposite hydrogels. J. Colloid Interface Sci.2008; 326: 41-50.
36. Haraguchi K, Li HJ. Hydrophobic surface characteristics of nanocomposite hydrogels. Macromol. Symp.2010; 291-292: 159-167.
37. Haraguchi K, Uyama K, Tanimoto H. Self-healing in nanocomposite hydrogels. Macromol. Rapid Commun.2011; 32: 1253-1258.
38. Saito N, Motoyama S, Sawamoto J. Effects of new polymer-coated extracorporeal circuits on biocompatibility during cardiopulmonary bypass. Artificial Organs2000; 24: 547-554.
39. Rubens FD. Cardiopulmonary bypass technology transfer: musings of a cardiac surgeon. J. Biomater. Sci., Polym.Ed.2002; 13: 485-499.
40. Mueller XM, Jegger D, Augstburger M, Horisberger J, von Segesser LK. Poly2-methoxyethylacrylate (PMEA) coated oxygenator: an ex vivo study. Int. J. Artif. Organs.2002; 25: 223-229.
41. Tanaka M, Motomura T, Kawada M, Anzai T, Kasori Y, Shiroya T, Shimura K, Onishi M, Mochizuki A. Blood compatible aspects of Poly(2-methoxyethylacrylate) (PMEA) -Relationship between protein adsorption and platelet adhesion on PMEA surface-. Biomaterials 2000; 21: 1471-1481.
42. Baykut D, Bernet F, Wehrle J, Weichelt K, Schwartz P, Zerkowski HR. New surface biopolymers for oxygenators: an in vitro hemocompatibility test of poly(2-methoxyethylacrylate)Eur. J. Med. Res.2001; 6: 297-305.
43. Tanaka M, Mochizuki A, Ishii N, Motomura T, Hatakeyama T.Study of blood compatibility with Poly(2-methoxyethyl acrylate). Relationship between water structure and platelet compatibility in Poly(2-methoxyethylacrylate-co-2-hydroxyethylmethacrylate)Biomacromolecules2002; 3: 36-41.
44. Tanaka M, Mochizuki A. Effect of water structure on blood compatibility - Thermal analysis of water in poly(meth)acrylate. J. Biomed. Mater. Res.2004; 68A: 684-695.
45. Haraguchi K. Synthesis and properties of soft nanocomposite materials with novel organic/inorganic network structures. Polym. J. 2011; 43: 223-241.
46. Haraguchi K, Li HJ, Ren HY, Zhu M. Modification of nanocomposite gels by irreversible rearrangement of polymer/clay network structure through drying. Macromolecules2010; 43: 9848-9853.
47. Haraguchi K, Xu Y, Li G. Molecular characteristics of poly(N-isopropylacrylamide) separated from nanocomposite gels by removal of clay from the polymer/clay network. Macromol. Rapid Commun.2010; 31: 718-723.
48. Xu Y, Li G, Haraguchi K. Gel formation and molecular characteristics of poly(N-isopropylacrylamide) prepared by free-radical redox polymerization in aqueous solution. Macromol. Chem. Phys.2010; 211: 977-987.
49. Miyazaki S, Endo H, Karino T, Haraguchi K, Shibayama M. Gelation mechanism of poly (N-isopropylacrylamide)-clay nanocomposite gels. Macromolecules2007; 40: 4287-4295.
50. Okano T, Yamada N, Sakai H, Sakurai Y. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly (N-isopropylacrylamide)J. Biomed. Mater.Res. 1993; 27: 1243-1251.
51. Okano T, Yamada N, Okuhara M, Sakai H, Sakurai Y. Mechanism of cell detachment from temperature-modulated, hydrophilic-hydrophobic polymer surfaces. Biomaterials 1995; 16: 297-303.
52. Kushida A, Yamato M, Konno C, Kikuchi A, Sakurai Y, Okano T. Decrease in culture temperature releases monolayer endothelial cell sheets together with deposited fibronectin matrix from temperature-responsive culture surfaces. J. Biomed. Mater.Res. 1999; 45: 355-362.
53. Shimizu T, Yamato M, Kikuchi A, Okano T. Two-dimensional manipulation of cardiac myocyte sheets utilizing temperature-responsive culture dishes augments the pulsatile amplitude. Tissue Engineering 2001; 7: 141-151.
54. Akiyama Y, Kikuchi A, Yamato M, Okano T. Ultrathin poly(N-isopropylacrylamide) grafted layer on polystyrene surfaces for cell adhesion/detachment control. Langmuir 2004; 20: 5506-5511.
55. Yamato M, Okano T. Cell sheet engineering. Materials today 2004; May: 42-47.
56. Haraguchi K, Takada T. Synthesis and characteristics of nanocomposite gels prepared by in situ photopolymerization in an aqueous system. Macromolecules 2010; 43: 4294-4299.
57. Haraguchi K, et al. Development of technology for producing new medical devices using nanocomposite gel (NC gel) to be submitted.

Correspondence : Dr. K. Haraguchi, Material Chemistry Laboratory, Kawamura Institute of Chemical Research, 631 Sakado, Sakura, Chiba 285-0078, Japan. Tel: +81-43-498-2111, FAX: +81-43- 498-2182, E-mail: hara@kicr.or.jp