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Accelerated cell sheet detachment by copolymerizing hydrophilic PEG side chains into PNIPAm nanocomposite hydrogels

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Abstract
One-end-connected short poly(ethylene glycol) (PEG) side chains were facilely introduced into the poly(N-isopropylacrylamide) (PNIPAm) nanocomposite hydrogel (NC gel) via in situ copolymerization of NIPAm monomer and PEG macromonomer in the aqueous suspension of hectorite clay Laponite XLS. The NC gels were characterized with Fourier transform infrared and x-ray photoelectron spectroscopy for the composition, DSC and transmittance for the phase separation temperature, dynamic mechanical spectra and swelling ratio for the interaction. Increasing the PEG content led to a small increase in the storage modulus and the lower critical solution temperature (LCST) of the copolymerized NC gels, and the LCST of the copolymerized NC gels was still below 37 °C. The L929 cell adhesion and proliferation on the surface of these NC gels were not suppressed by the incorporation of hydrophilic PEG side chains. By lowering temperature below the LCST, the cell sheet spontaneously detached from the copolymerized NC gels. The surface morphology and surface wettability of the NC gels were detected by atom force microscope and contact angle measurement. A rough and hydrophilic surface induced by a small amount of PEG side chains was found to be favorable to accelerate the cell sheet detachment, probably due to the enhanced water permeation into the gel–cell sheet interface.

(Some figures may appear in colour only in the online journal)

1. Introduction
Stimuli–response polymers, exhibiting rapid response to external stimuli, have been widely studied in many fields related to the biotechnology [1–5]. Among the many stimuli–response polymers, the thermosensitive poly(N-isopropylacrylamide) (PNIPAm) hydrogel has received lots of attention for the cell culture because the cell sheet can be harvested facilely by changing temperature without trypsin treatment [6, 7]. The PNIPAm aqueous solution has the lower critical solution temperature (LCST) at about 32 °C. Above the LCST, PNIPAm hydrogel exhibits a hydrophobic surface and cells can attach and proliferate on it. With decreasing temperature below the LCST, the hydrogel surface becomes hydrophilic and the cell sheet detaches spontaneously from the surface without enzyme digestion, which may damage the cell membrane [8]. The harvested cell sheet maintains the cell-to-cell junctions [9] and extracellular matrix underneath the cell sheet [4, 7, 10–12]. The noninvasively detached cell sheets show attractive properties to construct three-dimensional tissue-like structures by layering the cell sheets. Therefore, the complication associated with traditional tissue
engineering, such as significant inflammatory responses due to the degraded products of the implanted scaffolds may be avoided [13, 14].

Okano et al. reported that cells or cell sheets, cultivated on tissue-culture polystyrene (TCP) dishes modified by grafting PNIPAm chains, can be detached by decreasing the temperature below the LCST [12, 15–17]. However, PNIPAm hydrogel becomes soft and weak at temperatures lower than the LCST, losing its strength. For extending the application of PNIPAm hydrogel as the cell culture substrate, the nanocomposite hydrogel (NC gel) was adopted to improve poor mechanical properties of the conventional chemically cross-linked PNIPAm hydrogels [18, 19]. The NC gel was prepared by in situ polymerization of NIPAm monomer in aqueous suspension of hectorite clay Laponite, showing high mechanical properties and high transparency [20–24]. The Laponite XLS platelets worked as multifunctional cross-linkers in the NC gel to connect polymer chains together without any chemical cross-linkers. The incorporation of clay increases the hydrophilicity of the PNIPAm gel above the LCST, and the mechanically strong culture substrates benefit possible application of building three-dimensional scaffolds in future tissue engineering. Besides, the anionic charges on the exfoliated clay surfaces promote cell adhesion. Consequently, the NC gels exhibit better mechanical properties and cell attachability than the conventional PNIPAm hydrogels. Haraguchi et al. reported the cell cultivation and cell sheet detachment from the surface of the PNIPAm-clay NC gels [18]. Grunlan et al. observed the cell releasing behavior on the hydrogel consisting of PNIPAm and polysiloxane nanoparticles [19].

Cultured cell sheet can detach from the aqueous PNIPAm surface spontaneously, but the process is slow. Rapid recovery of cell sheet is significant to maintain the cell biological function and viability and reduce the time necessary for the practical assembly of tissue structure. The fast hydration of PNIPAm gel surface during lowering temperature is essential for rapid cell sheet detachment and several approaches have been attempted, such as grafting PNIPAm chains on porous membrane [25], developing comb-type grafted PNIPAm on TCP [17] and introducing hydrophilic components into PNIPAm gels [26–28]. Incorporation of hydrophilic components into PNIPAm hydrogels speeded up the cell sheet detachment by accelerating the permeation of cold incubation medium into the gel–cell sheet interface, resulting in a rapid hydration of the PNIPAm chains already aggregated at the incubation temperature of 37 °C [26]. Recently, Wang et al. reported that the time consumption for the cell sheet detachment was greatly reduced from 30 to 15 min when a small amount (0.2 w/v%) of alginate was added into the PNIPAm NC gel to form a semi-interpenetrating network (semi-IPN) [29]. However, the interpenetrated alginate chains may be washed out during the purification and incubation processes.

In this work, we are trying to introduce a hydrophilic component into the PNIPAm NC gel by copolymerization. Poly(ethylene glycol) (PEG) is chosen for its hydrophilic nature, good biocompatibility and resistance to protein adsorption [30, 31]. Another merit to use PEG is that the poly(ethylene glycol methacrylate) macromonomer with a double bond at one end of each PEG chain can be incorporated into the PNIPAm NC gel as side chains. The cell proliferation and cell sheet spontaneous detachment from this PEG copolymerized PNIPAm NC gel have been investigated.

2. Materials and methods

2.1. Materials

N-isopropylacrylamide (NIPAm, Acros, 1% stabilizer) was purified by recrystallization from a toluene/n-hexane mixture, followed by drying in vacuum at 40 °C. PEG macromonomer poly(ethylene glycol methacrylate) with MW of 526 g mol\(^{-1}\) (H\(_2\)C = C(CH\(_3\))CO(OCH\(_2\)CH\(_2\))\(_6\)OH, Aldrich) and \(N,N,N',N'\text{-tetramethylethlenediamine}\) (TEMED) were used as received. Potassium peroxydisulfate (KPS) was recrystallized from deionized water before use. Synthetic hectorite clay of sol-forming grade Laponite XLS (Rockwood Co., 92.3 wt% of Mg\(_5\)Si\(_6\)O\(_{20}\)(OH)\(_4\)Na\(_{0.66}\) and 7.7 wt% of Na\(_2\)P\(_2\)O\(_7\)) was provided by Rockwood and used after drying at 125 °C for 2 h. Water was purified by deionization and filtration with a Millipore purification apparatus (resistivity >18.2 MΩ cm) and bubbled with argon for more than 1 h prior to use.

2.2. Synthesis of NC gel

The NC gel was synthesized through in situ radical copolymerization of NIPAm and PEG macromonomer in the aqueous suspension of Laponite XLS. First, the suspension was prepared by dispersing the Laponite powder into water at a desired concentration under continuous stirring for about 3 h to allow the Laponite platelets to be completely exfoliated and evenly distributed. Second, a known volume of PEG macromonomer solution was added into the Laponite suspension and stirred for 2 h. Then, the NIPAm monomer was added and stirred for another 2 h. Finally, after the solution was bubbled with argon for 5 min, the solutions of initiator KPS (20 mg mL\(^{-1}\)) and catalyst TEMED were subsequently added to the system under continuous stirring. The solution was poured into the glass tube of 6.0 mm (diameter) × 120 mm (length) and a laboratory-made mold of two flat glasses separated by a rubber spacer with 80 mm (width) × 80 mm (length) × 2 mm (thickness). The reaction was carried out at 20 °C for 24 h. The NC gel sheet prepared in the mold was used for cell culture, and the rod sample synthesized in the glass tube was used for swelling measurement.

In all cases, the mole ratio of monomer to initiator to catalyst was kept at 100:0.370:0.638. The total monomer concentration was fixed at 1 mol L\(^{-1}\), the concentration of PEG macromonomer in all monomers was varied from 0.5 to 10 mol% and the Laponite concentration in all the NC gels was fixed at 6 w/v% (clay-to-water). The NC gel samples were referred to as S6PEG\(_n\), where S6 stood for 6 w/v% of Laponite XLS, \(n\) for the mol% of the PEG macromonomer in all monomers. For example, S6PEG\(_1\) means that a NC gel
contains 1 mol% of PEG macromonomer in all monomers and 6 w/v% of Laponite XLS. All hydrogels for cell culture were purified prior to use in a way similar to that reported by Haraguchi et al [18]. The NC gels were carefully taken out from the mold and immersed in an excess of deionized water at 20 and 45 °C for one week with daily refreshment to extract impurities. The NC gels were prepared and purified with sterilized water and handled under aseptic condition throughout the experiment for the cell culture.

2.3. Swelling experiments

The swelling experiment was performed at 20 °C by immersing the NC gel (initial size of 6 mm (diameter) × 20 mm (length)) in a large excess of water to reach equilibrium. Deionized water was refreshed every day. The swelling ratio was defined as the gel volume at the swelling equilibrium V related to its original volume V0 of the as-prepared one. Assuming isotropic swelling, V/V0 was calculated as (d/d0)3, where d and d0 denoted the corresponding diameter of the gel sample.

2.4. NC gel characterization

The Fourier transform infrared (FT-IR) spectra were recorded on a Bruker Vector 33 FT-IR spectrometer for dried and milled gel samples by the conventional KBr pellet method at room temperature. The gel surface composition was analyzed by an XPS (x-ray photoelectron spectroscopy, Axis Ultra DLD, Kratos) with a monochromatized Al Kα x-ray source. Surface elemental composition was calculated from the integrated element peak area.

The phase transition temperatures of the NC gels were determined by a differential scanning calorimeter (NETZSCH DSC 204 F1). A small piece of sample was sealed in a hermetic pan and the temperature was increased from 20 to 50 °C at a rate of 2 °C min⁻¹ for two cycles under dry nitrogen atmosphere. The LCST was defined as the onset temperature of the endotherm during the second heating [32].

The transmittance of the NC gels was detected as a function of temperature with a UV/Vis spectrophotometer (Hitachi U-3010) at 600 nm. Data were collected after the NC gels equilibrated at desired temperature for 10 min.

Rheology measurements were carried out on the as-synthesized NC gel with a strain-controlled rheometer ARES-RFS using parallel plates of diameter of 25 mm. Silicone oil was laid on the edge of the fixture plates to prevent solvent evaporation. First, the dynamic strain sweep was carried out at an angular frequency of 5 rad s⁻¹ to determine the linear viscoelasticity region. Then, the frequency sweep was performed over the range of 0.01–10 rad s⁻¹ at a constant shear strain of 0.5%. All rheology measurements were carried out at 25 ± 0.1 °C controlled by a Peltier plate.

The surface morphology of the NC gel was observed by an atomic force microscopy (AFM, SPI 3800N, SII Nano Technology Inc.) under the ambient environmental condition with the tapping mode. The detection angle and scan speed were 90° and 1 Hz, respectively. The average root-mean-square roughness Ra was adopted to evaluate the surface morphology.

The temperature-dependent aqueous wettability of the NC gel was determined by static water contact angle θw with a contact angle measuring system (OCA20, Dataphysics Instrument) using the sessile-drop method at 22 °C (below the LCST) and 37 °C (above the LCST). The NC gels were immersed in a temperature-controlled water bath and equilibrated for at least 30 min. Then, the NC gel was removed from the water bath, gently blotted with filter paper to wipe out the surface water and placed in a thermostatic chamber keeping at the designated temperature during the measurement. Data were read at 1 min after deposition of 10 μL of distilled water droplet. θw value was calculated as the average of at least three measurements with standard deviation (SD).

2.5. Cell culture and cell sheet detachment

The mouse fibroblast L929 was cultured on the conventional TCP dish using Dulbecco’s modified Eagle medium (DMEM, GIBCO, Invitrogen Co.) supplemented with 10% fetal bovine serum (FBS, GIBCO, Invitrogen Co.) at 37 °C in a humidified atmosphere of 5% CO2. The L929 cells were harvested from the dish by treatment with 0.25% trypsin–EDTA in PBS (GIBCO, Invitrogen Co.).

For cell culture, swollen NC gel discs of constant dimension (20 mm diameter) were punched from the NC gel sheet and transferred to Petri dishes containing DMEM medium. The dishes were then incubated at 37 °C for one day to equilibrate the NC gels with the cell culture medium. The suspended L929 cells were seeded onto each NC gel surface at an initial density of 1.5 × 10⁴ cells cm⁻² and cultured at 37 °C under a humidified atmosphere of 5% CO2. Throughout the cell culture process, the medium was replaced every two days. Cell morphology was photographed with a phase-contrast microscope and a digital camera at definite time intervals. The L929 cells that grew on the surface were dissociated by treating with 0.25% trypsin–EDTA solution after a prescribed time, and the number of cells was counted with a hemocytometer from four trials to produce an average value with ± SD.

After the L929 cell was cultured to obtain confluent cell sheet on the surface of NC gels, the medium temperature was decreased by exchanging with a cold medium and kept at 20 °C. The detachment behavior was monitored under a microscope with a digital camera. The photographs were taken to measure detaching area of each cell sheet by the NIH image software. The percentage of area for the detached cell sheet relative to the confluent cell monolayer area was estimated from the average of three trials with ± SD.

3. Results and discussion

3.1. Properties of copolymerized NC gels

Figure 1 illustrates FT-IR spectra of the NC gels S6PEG5 and S6PEG0, NIPAm, PEG macromonomer and Laponite XLS. The characteristic bands of the amide I at ca. 1660 cm⁻¹ and the amide II at ca. 1550 cm⁻¹ were observed owing to the amide

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group of the NIPAm unit in both the S6PEG0 and S6PEG5 NC gels. In the spectrum of S6PEG5, a new band at ca. 1720 cm\(^{-1}\) was attributed to C=O stretching of ester groups in the PEG macromonomer. Furthermore, the disappearance of C=C stretching at ca. 1636 cm\(^{-1}\) for the PEG macromonomer and ca. 1621 cm\(^{-1}\) for the NIPAm monomer indicated the copolymerization of the PEG macromonomer with NIPAm.

Elemental compositions of the purified NC gels were analyzed with XPS and the data were summarized in Table 1. Comparing with the NC gel S6PEG0, the oxygen atom content in the S6PEG5 gel is higher, confirming that the PEG chains were introduced into the copolymerized NC gel.

DSC thermograms of the NC gels in temperature range of 25–50 °C are shown in Figure 2. The LCST of the NC gel S6PEG0 is 33.2 °C, while that of the copolymerized NC gels slightly increases with the increase of PEG content. The introduction of hydrophilic PEG chains into the NC gel enhances its hydrophilicity and suppresses the occurrence of the phase transition, resulting in the increase of the LCST. Meanwhile, the DSC results indicate that the LCST of the copolymerized NC gels is below 37 °C, which is required because the hydrophobic gel surface at 37 °C is favorable for cell adhesion and proliferation [12]. By decreasing temperature below the LCST, the cell sheet can spontaneously detach from the hydrophilic gel surface due to the PNIPAm phase transition.

Temperature dependence of transparency of the NC gels is depicted in Figure 3. The transparency at temperature below the LCST decreases with increasing PEG content in the copolymerized NC gels due to the immiscibility of PEG and PNIPAm [33]. All the NC gels demonstrate a drop in transparency starting from ca. 33.5 °C, corresponding to the phase separation, similar to that determined by DSC. The sharpness of the transition appearing in Figures 2 and 3 is weakened with the increase of PEG content because addition of PEG strengthens the affinity of the gel to water and further suppresses the transition.

The equilibrium swelling ratio \(V/V_0\) is a fundamental property to evaluate the NC gel, which was measured in pure water at 20 °C. Figure 4 displays the equilibrium swelling ratio \(V/V_0\) of the NC gels with different PEG concentrations. The equilibrium swelling ratio decreases with increasing PEG content due to the increase of hydrogen bonding between the PEG side chains and the PNIPAm backbone.

In order to confirm the enhancement of PEG to the PNIPAm NC gels, dynamic modulus was detected by rheology measurements. Figure 5 shows the angular frequency \(\omega\) dependence of the storage modulus \(G'\) and loss modulus \(G''\) for the NC gels with different PEG contents. \(G'\) is always a plateau and higher than \(G''\) for all the samples, indicating a
cross-linked network in the NC gel. $G'$ of the copolymerized NC gels is higher than that of the PNIPAm NC gel S6PEG0 and increases with the PEG content. The hydrogen bonds are formed between the PEG side chains and PNIPAm chains, resulting in additional cross-linkers in the gels and therefore, the NC gels exhibit an increase in the storage modulus. This is coincident with the decrease of the equilibrium swelling ratio with increasing PEG content in figure 4.

### 3.2. Cell culture and cell sheet detachment

Photographs of the L929 cells that grew on the surfaces of different NC gels after one day and three days incubation at 37 °C were taken with a phase-contrast microscope and shown in figure 6. The cells spread and proliferate well on the copolymerized NC gels, indicating a good cell compatibility of these NC gels. The cell proliferation on the NC gel surfaces was further investigated by quantifying the number of cells with a hemacytometer. Figure 7 illustrates the number of cells attached on the NC gels as a function of culture time. The cell numbers on the copolymerized NC gels and PNIPAm gels are almost identical, considering the experimental error. This tendency implies that the introduction of hydrophilic PEG side chains into NC gels has no adverse effect on cell attachment and proliferation. Actually, we found that the
copolymized NC gel with 20 mol% of PEG was generally supportive for cell adhesion and proliferation to confluence. The L929 cell density on this NC gel achieved \(4.6 \times 10^5\) cell cm\(^{-2}\) after being cultured for seven days (data are not shown here). However Okano et al found that cell attachment was suppressed on grafted surfaces with only more than 1 wt% of PEG and that cells could not form confluent monolayer on the surface area [27]. This difference seems to be caused by the Laponite platelets in the copolymerized NC gels, which carry negative charges on the surface to promote cell adhesion and proliferation.

The L929 cell was cultured to reach confluence on the NC gels for about 10–12 days varying with PEG contents. Then, the spontaneous detachment of the cell sheet was realized by lowering temperature below the LCST to produce a hydrophilic gel surface. Figure 8 demonstrates the process of the L929 cell sheet spontaneously detaching from S6PEG1 gel surface by decreasing temperature to 20°C to produce an intact cell sheet. The percentage of detached area for the cell sheet recovered from the NC gel by lowering temperature is plotted against the detaching time in figure 9. Cell sheet detachment was accelerated on the copolymerized NC gel surface as compared to the PNIPAm gel surface. The time required for a complete cell sheet detachment is \(\sim 13\) min for S6PEG0.5, \(\sim 17\) min for S6PEG1 and \(\sim 18\) min for S6PEG5, much shorter than that for PNIPAm NC gel (\(\sim 23\) min for S6PEG0). A dramatically rapid cell sheet detachment was achieved from the NC gel containing only 0.5 mol% of PEG. The reported time consumption for the spontaneous cell sheet detachment from the thermoresponsive PNIPAm NC gel without hydrophilic additives was 20 min [18], but the clay content was lower than that in the present NC gels. In this study, rapid cell sheet harvest was actualized by copolymerizing PEG side chains into PNIPAm NC gels.

3.3. Surface characterization of NC gels

In order to understand the reason that a small amount of PEG in the NC gel speeded up the cell sheet detachment, we further examined the morphology and surface wettability of the copolymerized NC gels. The surface morphology observed by AFM is displayed in figure 10. AFM images clearly manifest the difference in roughness of these NC gels. The PNIPAm NC gel has a smooth surface with the average root-mean-square roughness \(R_a\) of 3.8 nm. In contrast, the copolymerized NC gels have rougher surfaces with \(R_a\) of 48.5 nm for S6PEG0.5, 29.3 nm for S6PEG1 and 4.7 nm for S6PEG5. This fact implies that the rough surface is favorable for the rapid cell sheet detachment, possibly due to easy permeation of water into the gel–cell sheet interface during decreasing temperature.
Table 2. Water contact angle of the NC gel surface at 22 and 37 °C.

<table>
<thead>
<tr>
<th>NC gel</th>
<th>22 °C</th>
<th>37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>S6PEG0</td>
<td>102.2 ± 1.0</td>
<td>109.6 ± 2.2</td>
</tr>
<tr>
<td>S6PEG0.5</td>
<td>103.3 ± 0.6</td>
<td>110.2 ± 0.7</td>
</tr>
<tr>
<td>S6PEG1</td>
<td>100.2 ± 0.8</td>
<td>108.8 ± 0.1</td>
</tr>
<tr>
<td>S6PEG5</td>
<td>94.2 ± 1.0</td>
<td>105.1 ± 0.9</td>
</tr>
</tbody>
</table>

Static water contact angle on the NC gels was detected using the sessile-drop method at 22 and 37 °C, respectively, and listed in table 2. The NC gels exhibit extraordinarily high \( \theta_w \) values even at temperature of 22 °C below the LCST, though the NC gels consisted of hydrophilic components of NIPAm, PEG macromonomer and hectorite clay with large amounts of water. The similar behavior was reported for the PNIAPm NC gel by Haraguchi et al [34, 35]. They attributed the high \( \theta_w \) value to the isopropyl group contribution, which appeared at the interface of the NC gel and air. The copolymerized NC gel would also behave somewhat hydrophobicity in air. For the present NC gels, the contact angle only slightly decreases with the increase of PEG content, except for S6PEG0.5, because the end-connected PEG side chains increase the hydrophilicity of the NC gels. By increasing temperature to 37 °C, the NC gels exhibit increasing contact angle due to the hydrophobic aggregation of the PNIAPm chains at the temperature above the LCST. For the NC gel S6PEG0.5, its rough surface (figure 10) appears to increase the water contact angle due to the possible existence of air-containing nanoporous [36].

Taking the above facts into account, the reason for S6PEG0.5 providing a rapid cell sheet detachment is mainly due to the rough surface induced by the copolymerization of 0.5 mol% end-connected PEG side chains. The significant effect of PEG side chains on promoting cell sheet detachment can be understood from the heterogeneity on the gel surface caused by microscopic phase separation due to the immiscibility of PEG and PNIAPm. Recently, Wang et al suggested that a rough surface of the semi-IPN NC gel induced by heterogeneity with addition of a small amount of alginate accelerated the cell sheet detachment [29]. The addition of alginate also induced microscopic phase separation in the PNIAPm NC gels with a rough surface due to the immiscibility with PNIAPm. We consider that the hydrophilicity is necessary for the cell sheet detachment from the gel substrates, which is realized by lowering temperature of the PNIAPm NC gel below the LCST. Furthermore, the microscopic heterogeneous surface is helpful to accelerate cell sheet detachment by speeding up the water permeation into the gel–cell sheet interface through rough and micro-heterogeneous gel surface. Addition of a small amount of hydrophilic and immiscible polymer into the PNIAPm NC gel, e.g., PEG and alginate, would induce this expected surface through microscopic phase separation. But excess addition of immiscible polymer will cause macroscopic phase separation and cannot form the optimal microscopic heterogeneity on the NC gel surface.

4. Conclusions

One-end-connected short PEG side chains were facilely introduced into the thermoresponsive nanocomposite hydrogels (NC) via in situ copolymerization in the aqueous suspension of hectorite clay Laponite XLS. Increasing the PEG content led to a small increase in storage modulus and the LCST of the copolymerized NC gels due to the interaction of the PEG and NIPAm chains. However, the LCST of the copolymerized NC gels was still below 37 °C required for cell culture. The L929 cell adhesion and proliferation on the surfaces of these NC gels were not suppressed by the incorporation of hydrophilic PEG side chains. By lowering temperature below the LCST, the cell sheet spontaneously detached from the copolymerized NC gels. A rough and hydrophilic surface induced by a small amount of PEG side chains was found to be favorable to accelerate the cell sheet detachment, probably due to the enhanced water permeation into the gel–cell sheet interface. The detached intact cell sheets provide the possibility to build three-dimensional tissues.

Acknowledgments

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