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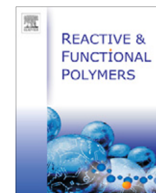

Promoted cell proliferation and mechanical relaxation of nanocomposite hydrogels prepared in cell culture medium

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ABSTRACT

Nanocomposite hydrogels (NC gels) were synthesized through *in situ* polymerization of *N*-isopropylacrylamide (NIPAm) in the hectorite clay suspension made from cell culture medium Dulbecco's modified Eagle medium (DMEM). Cell cultured on these NC gels (D-NC gels) surface proliferated faster compared with that on the NC gels synthesized in water due to the nutrients in the D-NC gels. Cells attached and proliferated faster on the D-NC gels with higher modulus. In addition, cell sheets with good viability spontaneously detached from the gel surface by lowering temperature. Their tensile elongation at break was higher than 7 and the true strength σ_{true} was up to 800 kPa. The stress–strain curves of the D-NC gels were described quantitatively with the Mooney–Rivlin equation. A stretched exponential stress equation was adopted to express the stress relaxation of the D-NC gels with an average relaxation time τ ($\sim 10^2$ s) estimated from data fitting, showing a broad distribution (polydispersity $k \sim 0.4$). The τ value, which was used in simulation for the tensile creep compliance of the D-NC gels, became shorter with increasing clay concentration due to the decrease in the network chain length. The relaxation process was similar to the natural articular cartilage. This work provides a facile way to produce hydrogels with improved cell compatibility and satisfactory mechanical properties for biomedical applications.

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1. Introduction

The nanocomposite hydrogel (NC gel) was synthesized by *in situ* polymerization of the acrylamide derivative monomers in the aqueous suspension of hectorite clay, where the inorganic clay platelets acted as multifunctional cross-linkers [1]. The NC gels showed ultrahigh extensibility, high strength, and high transparency, which overcame the weak and brittle shortcomings of the chemically cross-linked conventional hydrogels, promising a new sort of biomaterials [2,3]. The NC gels had been applied in cell culture and cell sheet detachment to obtain intact cell sheets [4,5].

Dulbecco's modified Eagle medium (DMEM) is a standard medium for cell culture with a broad suitability for various human and animal cells. DMEM contains inorganic salts, amino acids, vitamins and other components. It is a variant of Eagle's minimum essential medium (EMEM) and contains more nutrients. Chitosan hydrogel premixed with DMEM was reported to promote wound epithelization [6]. DMEM was also used to prepare hydrogels, such as alginate gels for *in situ* three dimensional (3D) cell culture [7,8].

Mechanical properties of hydrogels play a significant role in the application of biomaterials. For instance, the mechanical property or the structure of matrix environment was reported to regulate the stem cell fate [9,10]; hydrogels, as matrices to grow tissues *in vivo*, are subject to force from bones, muscles, and blood vessels. Hydrogels developed for tissue regeneration would be much more effective if their mechanical properties match those of the corresponding native tissue [11]. For example, bone plate with stress relaxation effectively improved the micro-circulation of the tissue adjacent to the prosthesis, avoiding bone resorption and mechanical strength deterioration [12].

Our previous work has focused on the tensile, compressive and viscoelastic properties of the NC gel before and after swelling. The PNIPAm–clay NC gels exhibited obvious strain hardening at large deformation ratio, the stress–strain curves at low strain were described quantitatively with the Mooney–Rivlin model. On the other hand, the strain hardening behavior was predicted by the Creton's model [13]. The PNIPAm–clay NC gels were used in cell culture and addition of small amount of alginate improved the cell compatibility and accelerated the cell sheet detachment [14].

In the present study, we prepare the NC gels in DMEM solution (marked as D-NC gel) instead of in deionized water to improve the biocompatibility of the NC gels by introducing nutrients during preparation. Swelling, thermo-responsibility, as well as cell culture and mechanical properties of the D-NC gels are investigated. Rubber elastic theory is used to interpret the tensile and relaxation phenomena.

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2. Experimental section

2.1. Materials

Monomer *N*-isopropylacrylamide (NIPAm, TCI, ⩾98%) was recrystallized from toluene/*n*-hexane mixture and dried in vacuum at 40 °C. *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED, Sinopharm Group Chemical Reagent Co. Ltd.) and Dulbecco's modified Eagle medium (DMEM, GIBCO, Invitrogen Co.) were used as received. Synthetic hectorite clay of sol-forming grade LAPONITE® XLS (92.32 wt% of $Mg_{5.34}Li_{0.66}Si_8O_{20}(OH)_4Na_{0.66}$ and 7.68 wt% of $Na_4P_2O_7$), a layered silicate with low heavy metal content and modified with inorganic polyphosphate, was kindly provided by Rockwood Ltd. and used after dried at 125 °C for 2 h. Initiator potassium peroxydisulfate ($K_2S_2O_8$) was recrystallized from deionized water and dried under vacuum at room temperature. Pure water was produced by deionization and filtration with a Millipore purification apparatus (resistivity >18.2 MΩ cm) and bubbled with argon gas for more than 1 h prior to use in the gel preparation.

2.2. Synthesis of NC gels

The PNIPAm–clay D-NC gel was prepared by *in situ* free radical polymerization of NIPAm in clay suspension of the DMEM solution. The procedure was similar to that used in our previous works [13,14]. The difference was that water was replaced by the DMEM solution. First, the clay was dispersed in DMEM solution under stirring for 2–3 h to produce a uniform suspension. Then, monomer NIPAm was added and stirred in an ice water bath for another 2 h. Finally, desired amount of initiator KPS solution of 20 mg/mL and catalyst TEMED were added to the suspension under stirring in the ice water bath. The polymerization was carried out at 20 °C for 24 h. The cylindrical NC gel samples were prepared in glass tubes of 6.0 mm diameter × 120 mm length. The NC gel film for cell culture was synthesized in a laboratory-made mold of 80 mm width × 80 mm long × 2 mm thickness, which was made of glass plates with a rubber spacer between the plates. The NC gels polymerized in deionized water were also prepared for comparison, which were marked as W-NC gels.

In this paper, the D-NC and W-NC gel samples were referred to as D-N1S*m* and W-N1S*n*, respectively, with 1 mol/L of NIPAm in the suspension, where D stood for DMEM and W for deionized water, *m* and *n* stood for 100 × clay/water (w/v), varying from 2 to 8. In all suspensions, the mole ratio of NIPAm monomer to initiator to catalyst was kept at 100:0.370:0.638.

2.3. Swelling and thermoresponse

For swelling in water, the cylindrical NC gel samples of 6.0 mm diameter and 20 mm length were soaked in a large amount of water at 20 °C with daily replacement for at least 1 week to remove impurities and to achieve the swelling equilibrium. The equilibrium swelling volume ratio V/V_0 of the swollen gel to the *as-prepared* (not dried) one was determined from gel diameter ratio d/d_0 as $V/V_0 = (d/d_0)^3$ with the isotropic swelling assumption, where d and d_0 were the gel diameter of swollen equilibrated and *as-prepared*, respectively.

Heat flow of the D-NC gels at swelling equilibrium in water was measured with a differential scanning calorimeter (Netzsch DSC 204 F1) at the rate of 2 °C/min. Heating from 20 to 50 °C and cooling from 50 to 20 °C were performed in nitrogen atmosphere. Phase transition temperature of the D-NC gels was determined as the onset temperature of the endotherm peak during the second heating.

Transmittance of the D-NC gels as a function of temperature was monitored at wavelength of 600 nm with a UV–Vis spectrophotometer (Hitachi U-3010). The swollen gel was put into a quartz cell (10 mm × 10 mm × 40 mm) with a cap. The sample was allowed to equilibrate at the test temperature for 15 min before data collection.

2.4. Cell culture and cell sheet detachment

The D-NC and W-NC gels were used in the cell culture for comparison. The gels were carefully taken out from the mold and purified by soaking in spacious deionized water at 20 °C for 4 days. Then, the gels were allowed to swell equilibrated at 45 °C and 20 °C repeatedly for 1 week to remove impurities further. During this period, the water was changed every day. The gels were not sterilized, but prepared and purified with sterilized water and handled under aseptic condition throughout the experiments.

Fibroblast (L929) was used for cell culture in the medium of DMEM supplemented with 10% fetal bovine serum. The cells, routinely sub-cultured in TCPS dishes at 37 °C in atmosphere of 5% CO_2, were harvested by treatment with 0.25% trypsin–EDTA solution (GIBCO; Invitrogen Co.).

The NC gel film for cell culture was cut into disks (10 mm diameter) at room temperature and pre-cultured in Petri dishes containing DMEM for one day in CO_2 incubator (37 °C and 5% CO_2). Before seeding cells, the gel film disks were put into 12-well TCPS plates (Cellstar, Greiner Bio-One). Then, L929 cells were inoculated on the gel surface at the density of 1.5×10^4 cell/cm^2 and incubated in 5% CO_2 atmosphere at 37 °C. During the culture process, the medium was changed every other day. Cell adhesion and proliferation were observed with photomicrography using an inverted biological microscope. The cells that grew on the gel surface were dissociated with 0.25% trypsin–EDTA at 37 °C for 5 min and counted using a hemocytometer every 2 days.

When fibroblast L929 cells were cultured almost confluence on the NC gel surface after 8 days, the medium temperature was decreased to 10–20 °C by exchanging cold medium and then kept at room temperature (∼20 °C). The cell sheet obtained was transferred to a fresh cell culture dish without breaking. The transferred cell sheet was seeded on a new culture dish without the NC gel and incubated again to test the cell viability.

2.5. Mechanical properties

Dynamic moduli of the D-NC gels after the purification were measured with a strain controlled rheometer ARES-RFS (TA) using a parallel plate fixture of diameter of 25 mm at 25 °C, because the gels became dehydrated during measurement at 37 °C after the volume phase transition at 32.3 °C [15]. Silicone oil was laid on the edge of the fixture plates to prevent water evaporation. In order to determine the linear viscoelasticity regime, the shear strain γ dependence of the complex modulus G^* was tested at 5.0 rad/s. G^* was independent of γ over the γ range from 0.01% to 10% for the NC gels. Therefore, the frequency sweep was performed over 0.1–10 rad/s at strain of 0.5%. The temperature was controlled by a Peltier plate. The equilibrium shear modulus G_e was taken from the plateau G' value at low frequency.

Elongation, relaxation, and creep were detected on the *as-prepared* cylindrical D-NC gels of diameter of 6.0 mm with a Shimadzu AG-X plus 50 N testing system at 25 °C. The crosshead speed was 100 mm/min (the maximum strain rate <0.083 s^{-1}). The elongation ratio λ was taken as the deformed length l related to the original one l_0, $\lambda = l/l_0$. The nominal stress was estimated as $\sigma = F/A_0$, where F was the force and A_0 was the cross-section area of the NC gel before deformation. To rule out the effect of the area change on the stress during deformation, the true stress σ_{true} was adopted

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