J Mater Sci: Mater Med (2009) 20:583–590
DOI 10.1007/s10856-008-3593-0

Cell adhesion and accelerated detachment on the surface of temperature-sensitive chitosan and poly(*N*-isopropylacrylamide) hydrogels

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Received: 11 June 2008 / Accepted: 10 September 2008 / Published online: 14 October 2008

Abstract A series of temperature-sensitive poly (NIPAAm-*co*-CSA) hydrogels were synthesized by the copolymerization of acrylic acid-derivatized Chitosan (CSA) and *N*-isopropylacrylamide (NIPAAm) in aqueous solution. Their swelling properties and L929 cell adhesion and detachment behaviors were studied. It was found that poly(NIPAAm-*co*-CSA) hydrogels were temperature-sensitive associated with the roles of the component PNIPAAm. Most significantly, poly(NIPAAm-*co*-CSA) hydrogels exhibited simultaneously good swelling properties. The investigation of L929 cell adhesion and detachment of poly(NIPAAm-*co*-CSA) hydrogels indicated the cell adhesion and spreading was higher on the surface of poly(NIPAAm-*co*-CSA) hydrogels than that of PNIPAAm hydrogel at 37°C due to the incorporation of CS, which having excellent cell affinity. Poly(NIPAAm-*co*-CSA) hydrogels showed more rapid detachment of cell sheet compared to PNIPAAm hydrogel because of the highly hydrophilic and hygroscopic nature of CS chains when reducing the culture temperature from 37°C to 20°C.

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1 Introduction

Nowadays, a polymer-grafted culture surface with temperature-sensitivity has been utilized for non-invasive recovery of several distinct to culture cell monolayers by manipulating the culture temperature without enzyme or chelator addition [1, 2]. Among the many intelligent polymers, poly(*N*-isopropylacrylamide) (PNIPAAm) is exploited for these applications because it exhibits coil-*to*-glouble changes with temperature in aqueous solution across its lower critical solution temperature (LCST) of 32°C [3, 4]. PNIPAAm is fully hydrated with an extended chain conformation in aqueous solution below 32°C and is extensively dehydrated and compact above 32°C. The LCST of NIPAAm copolymers can be controlled over a broad temperature range by adding copolymer compositions, making these polymers very interesting for medical and biotechnological applications. Due to unique thermal property, PNIPAAm and its copolymers have been widely studied in the fields of separation [5, 6], drug delivery [7–9], cell and tissue engineering [10, 11], and other switching devices [12].

Okano et al. has introduced PNIPAAm molecules onto solid surfaces to induce temperature-responsive hydrophilic/hydrophobic surface property alterations for several different applications [13–15]. On PNIPAAm-grafted surfaces, various cell types adhere and proliferate at 37°C. Upon reducing the culture temperature to 20°C, culture surfaces become hydrophilic, and cells adhered spontaneously detach along with their deposited extracellular matrix [16–18]. However, PNIPAAm is not good biocompatible, cell attach is restricted. Therefore, its biocompatility should be improved by fabricating PNIPAAm copolymers. Furthermore, spontaneous cell sheet detachment from surfaces of NIPAAm-grafted TCPS is relatively a slow

process, occurring gradually from the sheet periphery toward the interior. Thus, significant incubation time at reduced temperature is required to lift up an intact cell sheet completely. Rapid detachment of cultured cell sheets is a very important recovery method that permits facile manipulation of the sheets. The rate-limiting step to cell sheet recovery is the hydration of the underlying PNIPAAm molecules grafted on the surface. Incorporation of a highly water permeable substrate to interface between the cell sheets and the thermo responsive polymer surfaces could accelerate the hydration of the hydrophobized PNIPAAm segments interacting with the cell sheet.

Chitosan (CS) is a positively charged specific polysaccharide, which stimulates cell growth and protein adsorption. It was reported that, besides its good biocompatility, CS has an excellent cell affinity [19, 20], and has good cellular proliferation and attachment properties in chondrogenic human mesenchymal stem cells (MSC). On the other hand, the hydrogels with porous conformation could accelerate the hydration of the material. In the present study, CS was introduced into PNIPAAm gel and the copolymerized hydrogels poly(NIPAAm-*co*-CSA) were prepared by the free radical copolymerization of NIPAAm and macromer CSA to achieve a much more cell attachment and growth, also a much more rapid cell sheet detachment in comparison with only PNIPAAm hydrogel.

2 Materials and methods

2.1 Materials

NIPAAm was purchased from Kohjin Co. Ltd., Japan and used after recrystallization from *n*-hexane. Chitosan (CS, Mr = 50,000, percentage of deacetylation degree: 85%) was provided by Shanghai Concachem Co., Ltd. Acrylic acid was obtained from Tianjin Chemical Reagent and purified by distillation. TCPS, Trypsin−EDTA solution, streptomycin, penicillin, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Shanghai Sangon Biological Engineering Technology & Sevices Co., Ltd. All other reagents, including 1-ethyl-3-(3-dimethyl-aminopropyl)-carboiimide (EDC), peroxydisulfate (APS), *N,N,N′,N′*-tetra-methylenediamine (TEMED), *N,N′*-methylenebis(acrylamide) (MBAA), ethanol, etc., were of analytic grade and made in China, and were used as received without further purification.

2.2 Synthesis of CSA

To obtain a CS-based reactive monomer that can be copolymerized with NIPAAm to form a hydrogel, macromer CSA was synthesized. 1 g CS was dissolved in 100 ml hydrochloric acid (HCl) (1 wt.%) followed by the addition of 0.5 g of EDC and 0.1 ml TEMED, 15 ml acrylic acid was then added to the solution. The reaction mixture was stirred for 30 h at room temperature. After neutralization of acrylic acid and HCl with NaOH, the solution was purged by distilled water with stirring. The precipitate was collected by filtration and washed twice with ethanol for purification. The macromer CSA was dried under vacuum at room temperature for 24 h.

2.3 Synthesis of poly(NIPAAm-*co*-CSA) hydrogels

Poly(NIPAAm-*co*-CSA) hydrogels were prepared by the free radical copolymerization of NIPAAm and CSA in HCl solution using APS and TEMED as an initiating system. Specifically, CSA and NIPAAm were dissolved according to a weight ratio *r*, in the feed (NIPAAm/CSA) of 3, 4, 5, 6, and 7 respectively. APS (0.2 wt.%) was then added. After bubbling with nitrogen to remove oxygen, TEMED (0.5 wt.%) was added with stirring and the copolymerization proceeded at room temperature for 24 h. As a comparison, pure PNIPAAm hydrogel was prepared in the presence of MBAA (2 wt.%). The obtained hydrogels were cut into thin disks of 24 mm in diameter, and then immersed in distilled water for 1 week to remove the unreacted monomer.

2.4 Measurements

CS, CSA and Poly(NIPAAm-*co*-CSA) hydrogels were powdered with KBr and pressed into pellets under reduced pressure to determine infrared spectra (Vector 22 FTIR, BRUKER Co., Germany). The same sample was analyzed by XPS (Perkin Elmer 5600 ESCA system).

The hydrogels were immersed in distilled water to swell for 24 h over a range of temperatures from 20°C to 50°C. The equilibrated swelling ratio (SR) was defined as W_s/W_d, where W_s and W_d were the weights of the swollen and dry gels, respectively.

To study the reswelling kinetics, hydrogels after equilibrium swelling at 37°C were immersed into distilled water of 20°C quickly. The hydrogels were weighed at different times. The reswelling ratio (RSR) was calculated by $(W_t - W_0)/W_d$, where W_0 and W_t were the weight of swollen gel at initial and t times, respectively.

The SR and RSR in cell culture medium were obtained as in water.

2.5 Cell culture on poly(NIPAAm-*co*-CSA) hydrogels

The ability of poly(NIPAAm-*co*-CSA) hydrogels to support cell attachment was tested using L929 cells in 12 well plates. The L929 cells were provided by Shanghai Queen &

King Biochem Co,.LTD, and a cell bank was first created by expanding the cells on 25 cm^2 flasks in a humidified environment of 95/5% air/CO_2, and freezed the cells at 7.0×10^4 cells/ml (1 ml) in cryogenic vials. Cell freezing was achieved at a rate of 1°C/min and the cells were stored in liquid N_2 until use.

For each experiment, a vial of frozen cells was thawed and seeded on 25 cm^2 flasks. The cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin until they reached about 80% confluence, and trypsinized once more for seeding onto polymer surfaces. The trypsinized cells were suspended in tissue culture medium and were diluted, then 2 ml of cell suspension was added to the hydrogels and TCPS to give 1×10^4 cells/cm^2. Cell studies were typically completed during the subsequent 8 days with culture medium changes every 2 or 3 days.

The cell morphology was observed by phase-contrast microscopy (OLYMPUS ckx31, Japan). For the opacity hydrogels, the cell morphology was observed by Scanning Electron Microscope (SEM) (Quanta 200, FEI Co.). Since the cell seeding onto hydrogel surfaces could not sustain lyophilization and low-temperature treatment, sample preparations of the cell–hydrogel complexes for SEM were modified as follows. The specimens were first rinsed with 37°C PBS buffer and fixed in 2.5% glutaraldehyde (in 0.1 M phosphate buffer) at 37°C. After fixation for 24 h, the samples were washed in PBS buffer (37°C, 10 min) three times. Finally, the specimens were dried using a critical point dryer in liquid carbon dioxide, sputter-coated with gold, and visualized by SEM.

The number of cells adhered to a specific surface was quantified using a hemacytometer. For this method, the excess culture media was drained and 0.5 ml of trypsin/EDTA was added to each well. After about 5 min, 1 ml of fresh culture medium was added, cells were counted manually from five different fields and averaged, giving the number of cells/ml.

The 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide (MTT) assay was used to quantify metabolic activity in each well. At the desired time point, the culture medium were drained and re-supplied with 1 ml of fresh culture medium. MTT dissolved in HBSS (5 mg/ml) was added to each well. After incubating at 37°C for 4 h, excess medium was removed. The cells were then dissolved in 1.2 ml DMSO and the OD value of each well was determined by Auto microplate reader (Σ960, Metertech Co. America) while the wavelength was selected at 490 nm.

2.6 Detachment of single L929 cell

Detachment of single L929 cell was achieved using low temperature treatment after incubation at 37°C for 24 h. L929 cells were plated on each surface at a density of 3×10^4 cells/cm^2 and cultured for 24 h to allow attachment and spreading on each polymer surface. For low temperature treatment, the spread cells were transferred to a CO_2 incubation equipped with a cooling unit fixed at 20°C. After 5, 10, 15, 20, 25, 30, 35, and 40 min incubation, the sing cell detached was counted using a hemacytometer.

2.7 Detachment of confluently cultured L929 cell sheets

L929 cells were plated onto each surface at a density of 5×10^4 and cultured at 37°C. After 24 h cultivation, unattached cells were removed by medium change. Cells were cultured for 6 days after reaching confluence, and each plate was transferred to the CO_2 incubator equipped with a cooling unit fixed at 20°C, and periodically the cells detached were counted using a hemacytometer. All procedures were carefully carried out not to alter the incubator temperature because the cell culture surfaces used in this experiment were all thermo responsive.

3 Results and discussion

3.1 Characterization of CSA and poly(NIPAAm-*co*-CSA) hydrogel

A series of poly(NIPAAm-*co*-CSA) hydrogels were synthesized by the copolymerization of acrylic acid-derivatized Chitosan (CSA) and *N*-isopropylacrylamide (NIPAAm) in aqueous solution. The structures of CSA and poly(NIPAAm-*co*-CSA) hydrogels were determined by IR and XPS analysis. For IR measurements (Fig. 1), the absorption bands with peak positions at 1,640 cm^{-1} and 1,572 cm^{-1} displayed common spectrum with that of CS, were due to the –CONH group. And it was stronger at 1,640 cm^{-1} in the CSA spectrum because the –CONH groups of CS as well as those groups came from –COOH of AAc polymerized with $–NH_2$ of CS. Furthermore, the characteristic peaks of NIPAAm at 1,648 cm^{-1} and 1,547 cm^{-1} showed a relatively strong absorption band with peak position at 1,645 cm^{-1} and the other band at 1,547 cm^{-1} in the copolymer poly(NIPAAm-*co*-CSA). The C=C of NIPAAm at 1,622 cm^{-1} disappeared in the copolymer as well.

The XPS analysis results showed the N composition of CSA (3.6) was lower than that of CS (4.8), C composition of CSA (72.4) was higher than that of CS (69.4) because of the introduction of AAc, indicating that the reaction between CS and AAc occurred. The degree of substitution of CSA (DS, the molar ratio of AAc per CS unit), determined by XPS was 2.2 in this study [21].

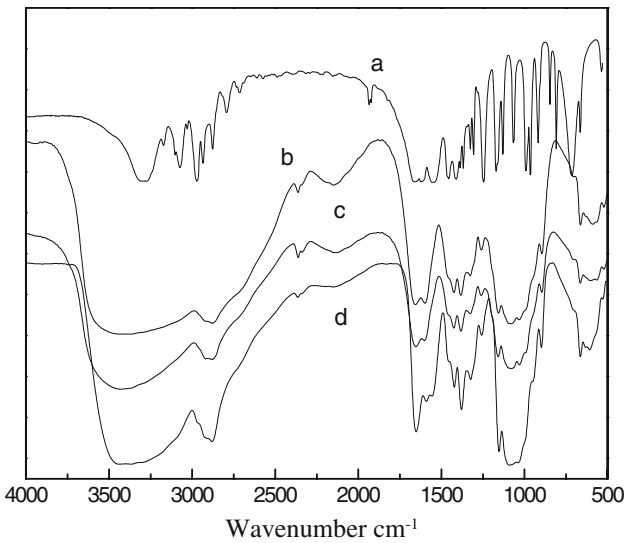

Fig. 1 FTIR spectra of a NIPAAm; b CS; c CSA; d Poly(NIPAAm-*co*-CSA)

3.2 Thermo-responsive properties of hydrogels

The swelling behaviors of poly(NIPAAm-*co*-CSA) hydrogels with different r were investigated at various temperatures in water and the results were shown in Fig. 2a. It could be seen that the SR of all hydrogels decreased with increasing temperature, suggesting that poly(NIPAAm-*co*-CSA) hydrogels were temperature-sensitive due to the coil-*to*-globule transition of macromolecular chain and the PNIPAAm component played a dominant role for the volume change of the hydrogels in response to the temperature. It was also found that the SR of poly(NIPAAm-*co*-CSA) hydrogel was larger than that of PNIPAAm gel and increased with the decrease of r (that was the increase of CSA). This indicated that the indroduction of –OH, –CONH– groups from CS resulted in an increase in hydrophilicity of the hydrogels. The LCST of poly(NIPAAm-*co*-CSA) hydrogel was lower than that of PNIPAAm gel and decreases with the decrease of r, e.g., LCST of poly(NIPAAm-*co*-CSA) ($r = 3$) was about 23°C, which was far lower than 32°C of PNIPAAm. The swelling behaviors of poly(NIPAAm-*co*-CSA) hydrogels in cell culture medium were also studied for further cell culture, and the similar temperature-sensitive character (Fig. 2b) was observed, but the SR value was lower. It might be associated with the electrostatic interaction between hydrogels and the ions (e.g., OH^-\$HCO_3^-$\$Na^+$\$Cl^-$), which were presence in cell culture medium. A similar result can be found from the study of Kim et al. [22] upon IPN hydrogels synthesized by graft polymerization or blending of CS and PNIPAAm.

Figure 3 showed the reswelling behaviors of gels in distilled water and cell culture medium. As seen here, the reswelling was observed with all these hydrogels, and the reswelling speed was fast in the initial time, then became slowly. The reswelling ratio (RSR) of poly(NIPAAm-*co*-CSA) hydrogels ($r = 3$, 5) was higher than that of PNIPAAm hydrogel, and increased with the decrease of r, on account of the increase of the amount of hydrophilic CSA. The reswelling behaviors of poly(NIPAAm-*co*-CSA) hydrogels were similar in the two mediums, though, which showed a little slower in cell culture medium due to the presence of the ions.

3.3 Cell attachment and growth

The cell compatibility of the poly(NIPAAm-*co*-CSA) hydrogels were examined by morphology and assessing the cellular adhesion. Figure 4 showed the SEM images of L929 cells cultured 8 days on the surface of hydrogels. The images clearly showed the cell adhered well and began to grow on the hydrogels, indicating good cell compatibility of hydrogels. The cell adhesion and spreading was higher on poly(NIPAAm-*co*-CSA) hydrogels compared with PNIPAAm hydrogel.

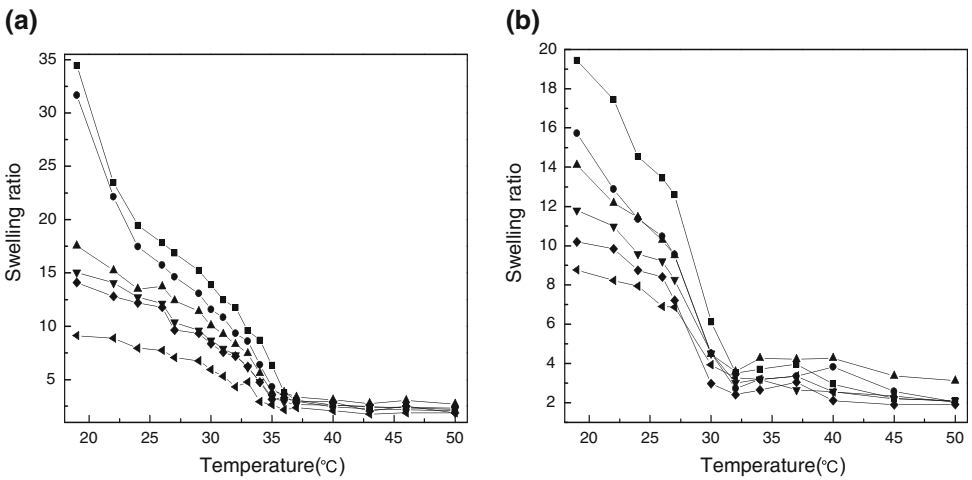

Fig. 2 Temperature dependence of the SR of Poly(NIPAAm-*co*-CSA) hydrogels with $r = 3$ (■), $r = 4$ (●), $r = 5$ (▲), $r = 6$ (▼), $r = 7$ (◆), and PNIPAAm (◀) in **a** distilled water and **b** cell culture medium

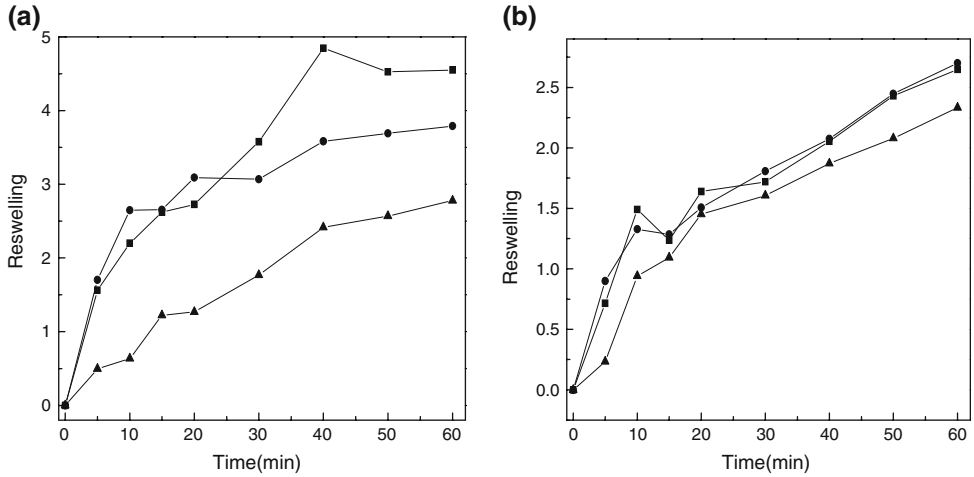

Fig. 3 Reswelling of Poly(NIPAAm-*co*-CSA) hydrogels with $r = 3$ (■), $r = 5$ (●), and PNIPAAm (▲) in **a** distilled water and **b** cell culture medium

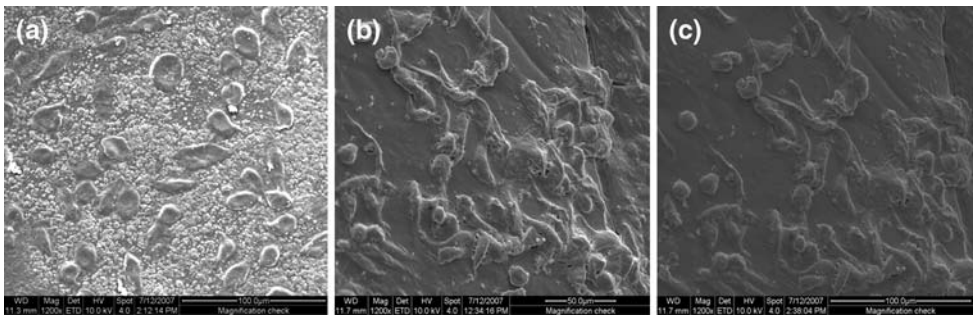

Fig. 4 The SEM photograph of cell attachment of **a** PNIPAAm hydrogels and Poly(NIPAAm-*co*-CSA) hydrogels with **b** $r = 5$ and **c** $r = 3$. Seeding density: 1×10^4 cells/cm^2

The difference in cell attachment was further revealed by cell growth quantified using a hemacytometer. Figure 5 showed the proliferation curves of L929 cell on the surfaces of poly(NIPAAm-*co*-CSA) hydrogels, PNIPAAm hydrogel, and TCPS. It can be seen that the cell number abruptly increased in 2–6 days and then saturated for hydrogels and TCPS. Based on cell counts, poly(NIPAAm-*co*-CSA) hydrogel surfaces supported higher numbers of cells than that of PNIPAAm hydrogel. It is noted that the cell on the surface of poly(NIPAAm-*co*-CSA) hydrogel with $r = 3$ grown more rapidly than that on poly(NIPAAm-*co*-CSA) with $r = 5$. A similar result was obtained by the total metabolic activity. As shown in Fig. 6, at all time points measured, the cell adhesion to poly(NIPAAm-*co*-CSA) surface was higher than the PNIPAAm surface, but lower than the TCPS control surface. The increase of cell number is due to the incorporation of CS, which has an excellent cell affinity and stimulates cell growth.

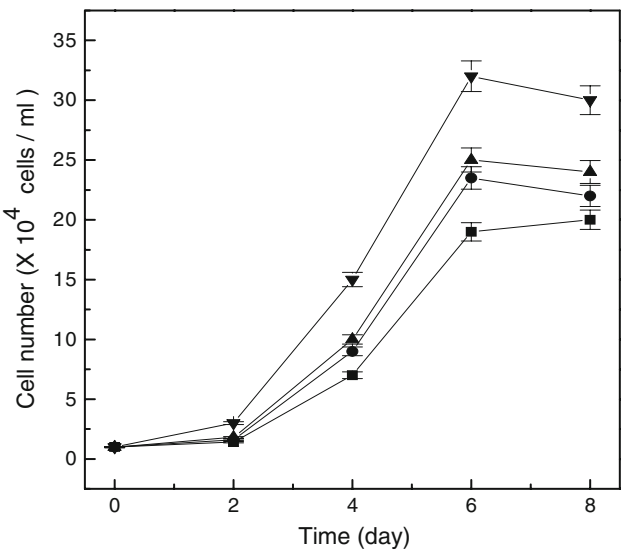

Fig. 5 Proliferation curves of L929 cell on Poly(NIPAAm-*co*-CSA) hydrogels with $r = 3$ (▲) and $r = 5$ (●), PNIPAAm (■) and TCPS (▼)

3.4 Single cell detachment

Since poly(NIPAAm-*co*-CSA) hydrogels exhibit well-defined temperature sensitivity, it is expected that cells cultured on poly(NIPAAm-*co*-CSA) hydrogels could be detached simply by decreasing the temperature from 37°C (hydrophobic) to 20°C (hydrophilic). It was observed that when the culture temperature was reduced to 20°C after 24 h incubation at 37°C during which almost all of the seeded cells were attached and spread on those surfaces, the spread cells were rounded and detached from the surfaces.

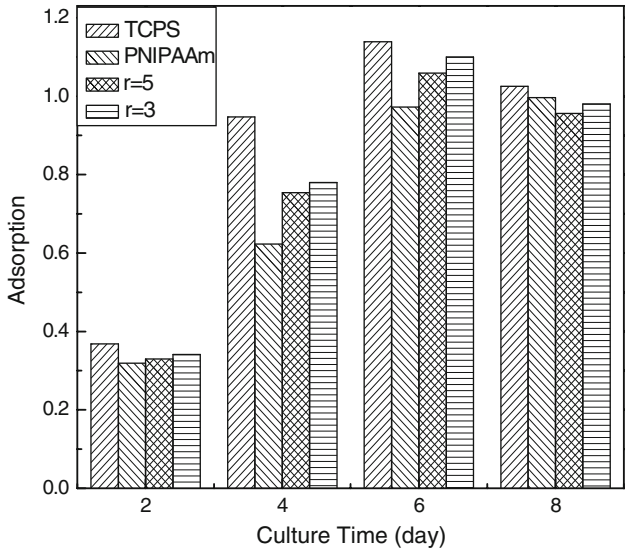

Fig. 6 Cell growth on hydrogels and TCPS using MTT assay. Cells were seeded at 1×10^4 cells/cm^2 in a medium with 10% FBS. After 2 days, the cells were washed with HBSS and subjected to the MTT assay as described in methods

Figure 7 showed the percentage of detached single cells from the surfaces of PNIPAAm hydrogel, poly(NIPAAm-*co*-CSA) hydrogels ($r = 3$, $r = 5$), TCPS as a function of lower culture temperature treatment time. It can be seen that there were no cells detached from TCPS because of no surface property alteration, while the cells could detach from these thermosensitive hydrogels by reducing the temperature. This cell detachment is attributed to the formation of an expanded, swollen, and hydrophilic surface,

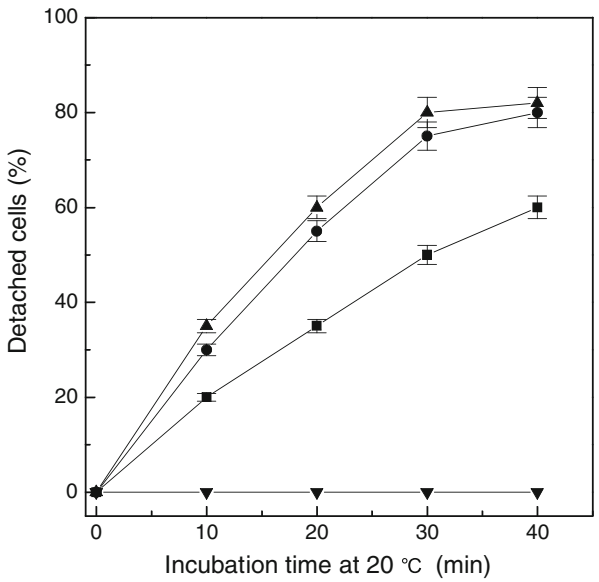

Fig. 7 Percentage of detached single cell from Poly(NIPAAm-*co*-CSA) hydrogels with $r = 3$ (▲) and $r = 5$ (●), PNIPAAm (■) and TCPS (▼) as a function of incubation time in culture medium at 20°C

which weakened cellular adhesion and resulting in spontaneous cell detachment due to the hydration of PNIPAAm below its LCST [23]. One should be noted that the spread cells on poly(NIPAAm-*co*-CSA) hydrogels showed more rapid detachment behavior than that on PNIPAAm hydrogel. The percentage of detached cells were about 35% and 30% from poly(NIPAAm-*co*-CSA) hydrogels with $r = 3$ and $r = 5$, but was about 20% from PNIPAAm hydrogel after initial 10 min incubation at 20°C. This could be associated with the reswelling behaviors of hydrogels. It is known that hydrogel has porous conformation. Just this porous conformation could accelerate the hydration of the material. When the temperature decreased from 37°C to 20°C, the hydrogels occurred reswelling due to the hydration of PNIPAAm as shown in Fig. 3. The reswelling of hydrogel supplied water molecules not only from the periphery of each cell but also through pores underneath the adherent cells. The water uptaken increased with the increase of the amount of hydrophilic CSA due to the highly hydrophilic and hygroscopic nature of CS chains. Introducing CS to PNIPAAm enhanced water diffusion to the entire poly(NIPAAm-*co*-CSA) hydrogel, and supplied more rapid and an extensive water for cell detachment, therefore, accelerated single cell detachment. This result is consistent with the study of Kwon et al. [23], which prepared membranes of PNIPAAm grafted with PEG for accelerated cell sheet recovery.

3.5 Detachment of cell sheets

After cells reached confluence, a continuous monolayer cell sheet formed on the surface of the poly(NIPAAm-*co*-CSA) hydrogel. When the grown cell sheet was placed at 20°C, it detached gradually from the surface of the thermosensitive hydrogel spontaneously, without treating with any enzymes. Figure 8 showed changes in L929 cell sheets detached on PNIPAAm hydrogel, poly(NIPAAm-*co*-CSA) hydrogels ($r = 3$, $r = 5$) and TCPS as a function of reduced temperature treatment time. The detachment of the cell sheets occured more rapidly from the surfaces of poly(NIPAAm-*co*-CSA) with $r = 5$ and $r = 3$ within 35 and 30 min incubation, respectively, while that on PNIPAAm surfaces slowly, required about 45 min. These results could be attributed to the existence of co-polymerized CS chains, accelerating cultured cell sheet detachment effectively, and enhancing diffusion of water molecules to cell substrate interface to hydrate the chains of PNIPAAm. In contrast, in the case of conventional TCPS dishes, cell could not be detached using the same treatment. Thus, the resulting cells and cell sheets detached from poly(NIPAAm-*co*-CSA) hydrogel surfaces could maintain extra-cellular matrix (ECM) with no trypsin treatment. This procedure could be useful in culturing cells

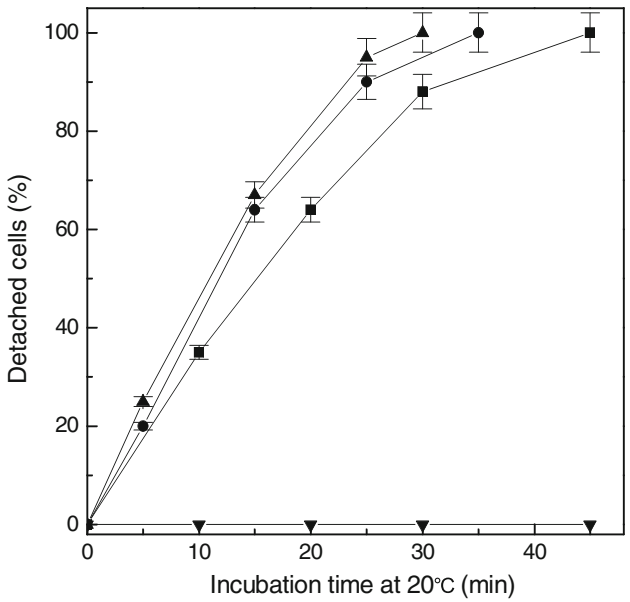

Fig. 8 Percentage of detached cell sheets from Poly(NIPAAm-*co*-CSA) hydrogels with $r = 3$ (▲) and $r = 5$ (●), PNIPAAm (■) and TCPS (▼) as a function of incubation time in culture medium at 20°C

for tissue engineering, such as transplantation or constructing two- or three-dimensional cell assemblies.

3.6 Cell transshipment

The characteristics of the detached cells on poly(NIPAAm-*co*-CSA) hydrogels (at 20°C) were examined by the total metabolic activity. The L929 cells detached from hydrogels and digested from TCPS were seeded respectively onto dishes at a cell density of 2×10^4 cells/cm^2.

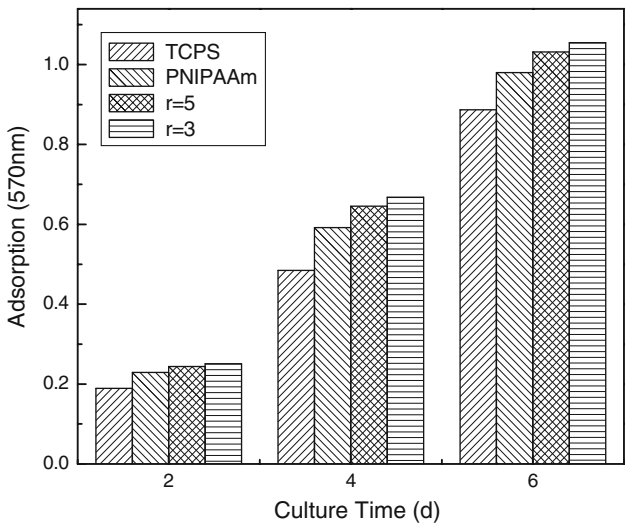

Fig. 9 Cell transshipment of Poly(NIPAAm-*co*-CSA) hydrogels with $r = 3$, $r = 5$, and PNIPAAm. Here they were compared with cells digested from TCPS

Figure 9 showed the proliferation of detached L929 cells when cultured at 37°C, we could see that the cell number also increased in 2–6 days and then saturated when cultured renewedly, showing the detached cells at 20°C were still alive and showed characteristics similar to attached condition at 37°C. At all time points measured, the cell detached from poly(NIPAAm-*co*-CSA) surface was higher than the PNIPAAm surface, and the cell from the surface of poly(NIPAAm-*co*-CSA) hydrogel with $r = 3$ grown more rapidly than that from poly(NIPAAm-*co*-CSA) with $r = 5$. All cells detached from hydrogels were higher than digested from TCPS control surface.

4 Conclusions

In this work, acrylic acid-derivatized Chitosan (CSA) and a series of poly(NIPAAm-*co*-CSA) hydrogels were successfully synthesized and their swelling behaviors were investigated. It was found that poly(NIPAAm-*co*-CSA) hydrogels were temperature-sensitive and exhibited much better swelling properties than the PNIPAAm gel.

A study of L929 cells cutured on the hydrogels indicated that the cell adhesion and spreading was higher on surfaces of poly(NIPAAm-*co*-CSA) hydrogels compared with PNIPAAm hydrogel, suggesting that the incorporation of CS were capable of enhancing the attachment of L929 cells. When the temperature decreased, the poly(NIPAAm-*co*-CSA) hydrogel showed hydrophilic and the cells spontaneously detached along with their deposited extracellular matrix. The existence of co-polymerized CS chains could accelerate cell detachment. This step protected the cell function well.

Acknowledgments This research was financially supported by the National Nature Science Foundation of China (Contract grant number: 20574051), and the Specialized Research Fund for the Doctoral Program of Higher Education (Contract grant number: 20050058001).

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