Cellular Compatibility of Copolymer Hydrogels Based on Site-Selectively-Modified Chitosan with Poly(*N*-isopropyl acrylamide)

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ABSTRACT: This research synthesized graft copolymers of chitosan-*g*-poly(*N*-isopropyl acrylamide) (CS-*g*-PNIPAAm) by UV-initiated free-radical polymerization of NIPAAm monomer to CS selectively at the C-6 position of pyranose ring. First, amino groups in CS were protected by reaction with phthalic anhydride (PA) to form PACS. The degree of phthaloylation was carefully controlled to ensure that most amino groups were protected, and only a very small amount of hydroxyl groups were reacted. In the second step, the vinyl functional group was introduced to the PACS by reaction with a vinyl compound containing an isocyanate group (3-isopropenyl-$\alpha\alpha'$-dimethylbenzyl isocyanate), through the urethane linkage with hydroxyl groups at the C-6 position. The phthaloyl groups were then removed by hydrazine to recover the amino groups in CS. Finally, PNIPAAm was grafted to the vinyl CS at the C-6 position by UV-initiated free-radical polymerization. The synthesized CS-*g*-PNIPAAm copolymers were confirmed to have a structure of an AB-crosslinked graft copolymer. Respectively, these copolymer hydrogels exhibited pH- and thermal-responsive swelling properties in an aqueous solution due to their CS and PNIPAAm components. The test of cell viability with L929 fibroblast revealed that the CS-*g*-PNIPAAm copolymers having a grafting ratio lower than 1.7 had cellular compatibility as good as pure CS. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 120: 1–12, 2011

Key words: chitosan; poly(*N*-isopropyl acrylamide); graft copolymer; hydrogel

INTRODUCTION

Environmentally stimuli-responsive hydrogels, or called smart hydrogels, can undergo a reversible and yet discontinuous volume change in response to environmental stimuli such as pH,[1] temperature,[2–4] ionic strength,[5] electric field[6,7] and so on. These stimuli-responsive hydrogels have potential applications in biomedical and pharmaceutical fields.[8] One of the most studied stimuli-responsive hydrogels is poly(*N*-isopropyl acrylamide) (PNIPAAm), a thermal-responsive polymer having a volume phase transition temperature (VPTT) at around 32°C in an aqueous solution.[9,10] Below VPTT, a crosslinked PNIPAAm hydrogel can swell greatly in water, but as temperature increases to above VPTT, it shrinks sharply due to the disruption of hydrogen bonding with water and the simultaneous increase of hydrophobic interactions among isopropyl groups.[11,12] In addition to the application in controlled drug release, PNIPAAm-grafted surfaces have been exploited for controlling cell adhesion/detachment by changing the incubation temperature above or below its VPTT, thus avoiding the usage of tripsin.[13]

Recently, great attention has been drawn to the development of stimuli-responsive hydrogels with unique properties such as biocompatibility, biodegradability and biological functionality for biomedical applications.[14–18] A number of polysaccharides thus have been considered to be combined with the thermo-responsive PNIPAAm to form smart hydrogels; and the one with the greatest potentiality is chitosan (CS), which is generally obtained from the deacetylation of chitin in a hot alkali solution. CS has been used in a variety of biomedical fields such as drug delivery carrier, surgical thread, and wound healing material.[19,20] Furthermore, CS exhibits pH-responsive behavior due to the protonation–deprotonation equilibrium of amino groups attached at the C2 position.[21,22] The combination of CS and PNIPAAm will therefore produce dual-stimuli-responsive hydrogels to be used as delivery vehicles in response to changes in environmental pH and temperature. Lorenzo et al.[18] reported the anionic drug affinity and release properties of CS/PNIPAAm interpenetrated polymer

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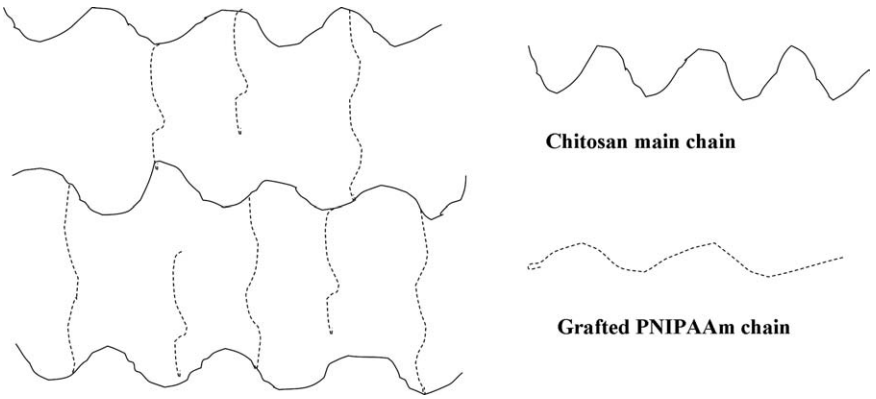

Figure 1 Structure of AB-crosslinked graft copolymer based on chitosan and PNIPAAm.[26]

network (IPN). The results showed that the pH sensitivity and improved drug loading capacity were due to the incorporation of chitosan component; temperature sensitivity and sustained release property stemmed from the inclusion of PNIPAAm component. This delivery system therefore can potentially be used for modulating the release kinetics of an array of anionic drugs and therapeutics. Brazel and Peppas[23] have found that dual stimuli-responsive polymers could be used in the pulsatile delivery of drugs by simultaneous pH and temperature alterations. The pulsatile delivery of the thrombolytic agent, streptokinase, by such a mechanism was found to be useful in its application at the site of blood clotting. However, the exploitation of CS/PNIPAAm copolymer hydrogels has not received much attention until recently.[24–32]

Another advantage of CS is that it has both reactive amino and hydroxyl groups that can be used to chemically alter its structure and thus change its properties. In our previous paper,[26] an AB-crosslinked graft copolymer of CS and PNIPAAm was synthesized by first introducing a vinyl functional group to the CS through the reaction of maleic anhydride and the amino group in CS (Fig. 1). Subsequently, PNIPAAm was grafted to the maleilated CS via free radical polymerization. The results showed that the synthesized CS-*g*-PNIPAAm copolymer exhibited the pH- and thermo-responsive property from its respective CS and PNIPAAm component. However, the cell compatibility evaluated by fibroblast viability was found to be poor. The poor cell compatibility is believed to be due to the unavoidable carboxylic acid groups generated from the reaction between maleic anhydride and amino group. The carboxylic acid group has negative effects on the viability of fibroblast cell. In addition, the decrease in the amount of amino group is also an unfavorable factor.

Therefore, the purpose of this study is to synthesize CS-*g*-PNIPAAm copolymer hydrogels without deteriorating the cell compatibility. To accomplish this goal, acid groups should not be introduced and amino groups should be preserved during the synthesis of the copolymer hydrogels. Therefore, the amino group of CS was protected first by reaction with phthalic anhydride (PA).[33] The PACS then underwent reaction with an isocyanate-containing vinyl compound to introduce the vinyl functional group to CS. Thereafter, the phthaloyl groups were removed by means of a hydrazine solution. The vinyl CS was finally graft-copolymerized with NIPAAm monomer via a UV photo-polymerization. This new strategy to synthesize CS-*g*-PNIPAAm graft copolymer is illustrated in Figure 2. The structure, swelling behavior, thermal properties, and cell compatibility of the CS-*g*-PNIPAAm were then evaluated and reported in this article.

EXPERIMENTAL

Material

Chitosan (TCI, Tokyo, Japan), CS, was purified by dissolution in an acetic acid solution. It was filtered and the filtrate was added with an alkali solution to precipitate out the CS. The precipitate was washed with an excess amount of water until it became neutral. Finally, it was lyophilized at −20°C for 72 h. The viscosity average molecular weight (M_v) of the purified CS, determined via a capillary viscometric method[34,35] was 2.64×10^5. The degree of deacetylation (DDA, i.e., moles of amino group per mole of pyranose ring) of CS was determined by nuclear magnetic resonance (NMR)[36] and found to be 0.83. Phthalic anhydride (PA) was purchased from Showa (Tokyo, Japan). *N*-isopropyl acrylamide (NIPAAm) monomer was supplied by ACROS (New Jersey). Chemical compounds, 3-isopropenyl-αα′-dimethylbenzyl isocyanate (also called *m*-tetramethylxylene isocyanate, *m*-TMI), dibutyltin dilaurate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

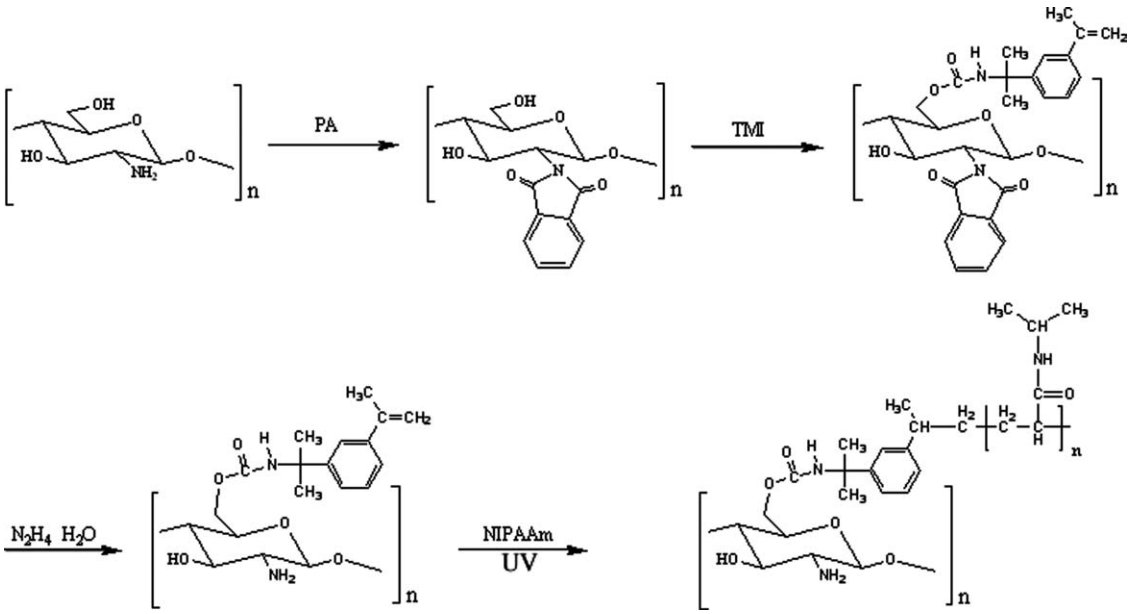

Figure 2 The reaction route to synthesize PNIPAAm-*g*-CS graft copolymer.

(MTT) were purchased from Sigma (St. Louis, MO). Hydrazine hydrate (80% in H_2O) was obtained from Riedel-de Haën (Hannover, Germany). The photo-initiator, 2-hydroxy-2-methylphenyl-1-propanone (HMPP), also called "Darocure 1173", for initiating graft polymerization of NIPAAm to CS was purchased from Merck (Darmstadt, Germany). Other chemical reagents are at least reagent grade and used without further purification.

Phthaloylation of CS

Phthaloylation was carried out in a homogeneous reaction system to protect the amino group in CS. First, 3.00 g CS (0.018 mol of amino group) was dissolved in formic acid (150 g) at room temperature for 12 h. The solution was then added into 300 mL dimethyl formamide (DMF) solution dissolved with 45.00 g of PA (0.304 mol). The homogeneous solution was heated in a nitrogen atmosphere at 130°C for 24 h. After reaction, the resulting pale tan solution was cooled down and poured into a large excess of acetone. The precipitate was collected on a filter, washed with acetone several times, and finally dried in a vacuum oven at 80°C to obtain the PACS as a pale tan powdery material.

Synthesis of Vinyl CS

For introducing vinyl functional group to PACS, the obtained PACS (5.42 g) was dissolved in 100 mL DMAc in a reactor purged with N_2 and then dibutyltin dilaurate (0.24 g, 3.8×10^{-4} mol) as a catalyst was added into the solution. The solution was heated up to 80°C and then added with the vinyl compound *m*-TMI (24.12 g, 0.0269 mol). The reaction was carried out for 24 h with a mechanical stirring. The reaction product, PACS-TMI, was precipitated out from the solution with the addition of five times volume of methanol. It was centrifuged to separate the precipitate and washed several times with methanol. Finally, it was dried and weighed. The phthaloyl groups in PACS-TMI were removed by reductive reaction with hydrazine in DMF/water solution at 80°C for 12 h under a nitrogen atmosphere. The CS-TMI, having both amino and vinyl groups, was then obtained by precipitation in acetone.

Synthesis of CS-*g*-PNIPAAm copolymers

The vinyl CS-TMI (W_{CS}) was dissolved in formic acid at a concentration of 10/100 (g/v), and the solution was then added with different amounts of NIPAAm monomer (W_m), and 1 wt % (based on monomer) of photo initiator (Darocure 1173). It was pre-baked at 50°C for 1 h to reach a solid content about 80–85%. Graft polymerization of NIPAAm to CS-TMI was then carried out under UV irradiation at an energy level of 900 mJ/cm^2. After reaction, the sample was post-dried at 50°C for 12 h. The obtained sample film was incubated in hot water at 50°C for the removal of unreacted monomer. It was then dried in vacuo and weighed (W_1). During polymerization, not only graft copolymer was formed but also the PNIPAAm homopolymer. The PNIPAAm homopolymer was extracted out of the sample film with cold water at 15°C. The final copolymer was obtained by oven-drying in vacuo at 50°C and weighed again (W_2). The monomer conversion (X, %), grafting efficiency (GE, %), and grafting ratio (GR) were all determined through

the gravimetric method according to the following equations.

$$X = (W_1 - W_{CS})/(W_m) \times 100 \quad (1)$$

$$GE = (W_2 - W_{CS})/(W_1 - W_{CS}) \times 100 \quad (2)$$

$$GR = (W_2 - W_{CS})/(W_{CS}) \quad (3)$$

For comparison, pure PNIPAAm was also synthesized in the same way by UV photo-initiation. However, *N*,*N*′-methylene-bisacrylamide as a crosslinking agent (1 wt % of monomer), was added to crosslink PNIPAAm.

Structure analysis

Structure analysis of intermediates and final copolymers was carried out using a Fourier transform infrared (FTIR) spectrophotometer (model 550, Nicolet). Sample was ground into powder, mixed with KBr and then pressed into a transparent disc. It was scanned from 4000 to 400 cm^{-1} for 32 times with a resolution of 4 cm^{-1}. Chemical structures of various samples were also analyzed with an NMR technique. Different solvents were used for dissolving different kinds of samples to obtain ^{1}H-NMR spectra: DCl/D_2O for pure CS, *d*-DMSO for PACS and PACS-TMI and CD_3COOD/D_2O for CS-TMI. Solid ^{13}C-NMR spectra (Bruker DSX400WB NMR, 100 MHz for ^{13}C nucleus) were also obtained for CS-*g*-PNIPAAm copolymers. The degree of phthaloylation (DS_{PA}, moles of phthaloyl group per mole of pyranose unit) in PACS, $(C_8H_{13}O_5N)_{0.17}(C_6H_{11}O_4N)_{0.83}(C_8H_2O_2)_x{\cdot}y(H_2O)$, and the degree of addition of *m*-TMI (DS_{TMI}, moles of *m*-TMI group per mole of pyranose unit) in TMI-CS, $(C_8H_{13}O_5N)_{0.17}(C_6H_{11}O_4N)_{0.83}(C_{13}H_{15}NO)_x$, were determined with an elementary analyzer (Elementar vario EL III, Germany).

Thermal properties

A differential scanning calorimeter (TA 2920 from TA Instruments) was used to observe the thermograms of copolymers. The sample (8–10 mg) was placed in an aluminum pan and scanned from 30 to 220°C at a heating rate of 10°C/min under a nitrogen atmosphere. Thermal degradation behavior of various samples was investigated using a thermal gravimetric analyzer (Hi-Res TGA 2950 from TA Instruments). The sample (8–12 mg) was placed in a platinum pan and heated to 100°C, held for 5 min to remove residual moisture, and then continuously heated to 800°C at a rate of 20°C/min under a nitrogen atmosphere.

Phase transition temperature and swelling ratio of copolymer gels

The swelling experiment was performed by immersing samples (~ 0.01 g, D = 2 cm) in 30.0 g buffer solutions at different pH values (pH = 2–12), as well as at different temperatures (15–50°C). After 24 h of incubation, equilibrium swelling being reached, the gel sample was taken out from the solution, blotted with a filter paper to remove free water from the surface, and then weighed. The swelling ratio (SR, g/g) was evaluated using the equation: $SR = (W_{wet})/(W_{dry})$, where W_{dry} and W_{wet} are weights of dry and wet samples, respectively. For comparison, a crosslinked PNIPAAm was also measured under the same conditions.

Cell culture and MTT assay

L929 (ATCC CCL 1) is a fibroblast-like cell line cloned from strain L. The parent strain was derived from normal subartaneous areolar and adipose tissue of a male C3H/An mouse. The prepared membranes with 16.4 mm in diameter were sterilized with UV irradiation and placed in wells of a 24-well tissue culture polystyrene plate (Corning, Action, MA). A sterilized silicone ring with the same diameter was placed on each of the tested samples in the well to prevent them from floating. Aliquots (1 mL) of L929 cell suspension with 5×10^4 cell/mL were layered on the sample membranes. Cells were cultured in a humidified incubator balanced with 5% CO_2 at 37°C up to 4 days. The viabilities of fibroblasts were determined by MTT assay. At each period of cultivation, 500 μL of media was decanted and 75 μL MTT solution was added to each well. After 45 min incubation at 37°C, acid-isopropanol (0.06 N) with an amount of 500 μL was added to dissolve the formazan crystals. The optical density of the formazan solution was detected by means of an ELISA plate reader (ELx 800, BIO-TEK, Winooski, VT) at 570 nm. The cell concentration was then obtained from the calibration curve. All experiments were performed in triplicate and results were averaged.

RESULTS AND DISCUSSION

Phthaloylation of CS

In the phthaloylation of CS for the protection of its amino group, we first followed Kurita's method[33,37] by mixing CS powder in a solution of PA in DMF at 120°C. It was a heterogeneous reaction system, but the PACS product could be gradually dissolved in the DMF medium. A reaction product with a degree of phthaloylation (DS_{PA}) of 1.41 was obtained from elemental analysis (C: 53.26%, N: 3.52%, H: 4.97%). The CS used in this study had a degree of deacetylation (DDA) of 0.83. This suggests that not only the *N*-phthaloylation occurred between the amino group and phthalic anhydride but also the *O*-phthaloylation between the hydroxyl group and phthalic

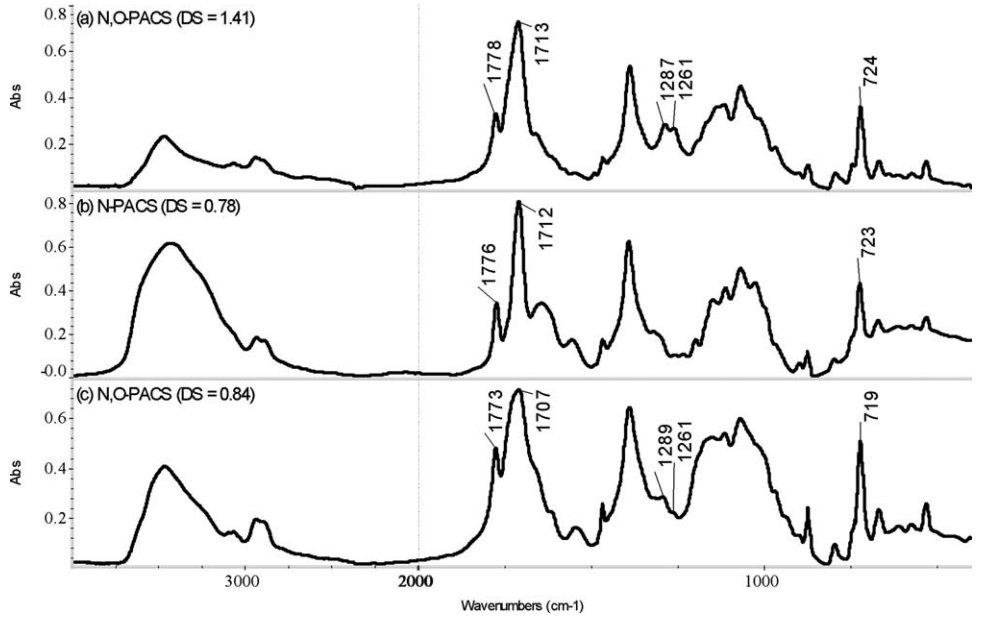

Figure 3 FTIR spectra of phthaloylated chitosan (PACS) with different degrees of phthaloylation (DS_{PA}). (a) *N,O*-PACS: $DS_{PA} = 1.41$; (b) *N*-PACS: $DS_{PA} = 0.78$; (c) *N,O*-PACS: $DS_{PA} = 0.84$.

anhydride. The structure of this *N,O*-phthaloylated CS was confirmed by the FTIR spectrum as shown in Figure 3(a), which revealed two strong absorption peaks at 1773 and 1709 cm^{-1} due to the imide group from *N*-phthaloylation, and medium ones at 1290 and 1260 cm^{-1} (ester group) with a weak band at 2600–2800 cm^{-1} (free carboxyl group) as a result of *O*-phthaloylation.[33] The absorption peak at 719 cm^{-1} is due to the phenyl ring of phthaloyl group. The *N,O*-phthaloylated CS could be dissolved in DMF, DMAc and DMSO, while pure CS can only be dissolved in the acid solution. The solubility in organic solvents is beneficial for the latter reaction with a coupling agent. The solubility is due to the addition of a number of phthaloyl groups to CS chains. The XRD pattern shows only a weak, broad peak at $2\theta = 18.6^{o}$ (data not shown here), indicating that the structure regularity was destructed due to the *N,O*-phthaloylation. In this study, it is desirable to preserve the hydroxyl groups in CS for the latter reaction with the vinyl compound. The partial *O*-phthaloylation could be prevented by using a mixed solvent like DMF/water (95/5) for the reaction as pointed out by Kurita.[37] The added water could hydrolyze the ester group formed from *O*-phthaloylation. The *N*-phthaloylated CS thus obtained by carrying out the reaction in the DMF/water (95/5) solvent had the DS_{PA} of 0.78 (C: 50.85%, N: 4.74%, H: 5.66%). Figure 3(b) shows that the *N*-phthaloylated CS did not have any ester group. However, in contrast to the *N,O*-phthaloylated CS, the *N*-phthaloylated CS was insoluble in DMF or DMSO. The XRD pattern reveals the *N*-phthaloylated had two peaks at $2\theta = 6.2^{\circ}$ (strong) and 11.9°. This indicates that the *N*-phthaloylated CS had a well ordered structure, agreed to the report from Gross and coworkers[38] that the *N*-phthaloylated CS could exhibit lyotropic mesophase. For the purpose of this study, the amino groups need to be protected; most hydroxyl groups in CS should be available as potential reaction sites; but the modified CS still could be soluble in some organic solvents to facilitate the future reaction with the coupling agent. This suggests that slight *O*-phthaloylation is necessary to give the PACS products soluble in organic solvents. Therefore, a different approach was adopted in this study that the phthaloylation of CS with phthalic anhydride was carried out in a homogeneous formic acid/DMF solution. The DS_{PA} of the as-prepared PACS was found to be 0.84 (C: 52.52%, N: 4.70%, H: 4.80%), slightly greater than the DDA value of the original CS. Figure 3(c) shows the FTIR spectrum of the obtained PACS product. It indicates a very small extent of *O*-phthaloylation, as there is nearly no carboxylic acid absorption and only a very weak band of ester group is observed at 1289 cm^{-1}. The prepared PACS was found to be soluble in DMF, DMSO and DMAc, but not in dioxane, tetrahydrofuran, water, acetone, methanol and ethanol.

The structures of various phthaloylated CS are further confirmed by solid-state NMR spectra. Figure 4 shows the solid-state ^{13}C-NMR spectra of the pure CS, *N*-phthaloylated CS ($DS_{PA} = 0.78$) and *N,O*-phthaloylated CS ($DS_{PA} = 0.84$). The *N*-acetyl group of pure CS causes absorption peaks at 23.7 ($\underline{C}H_3$) and 173.9 ($\underline{C}$=O) ppm.[26] After reaction with PA, the absorption peaks at 123.7, 131.2 and 134.8 ppm are found due to the phenylene ring. Most importantly, the *N*-

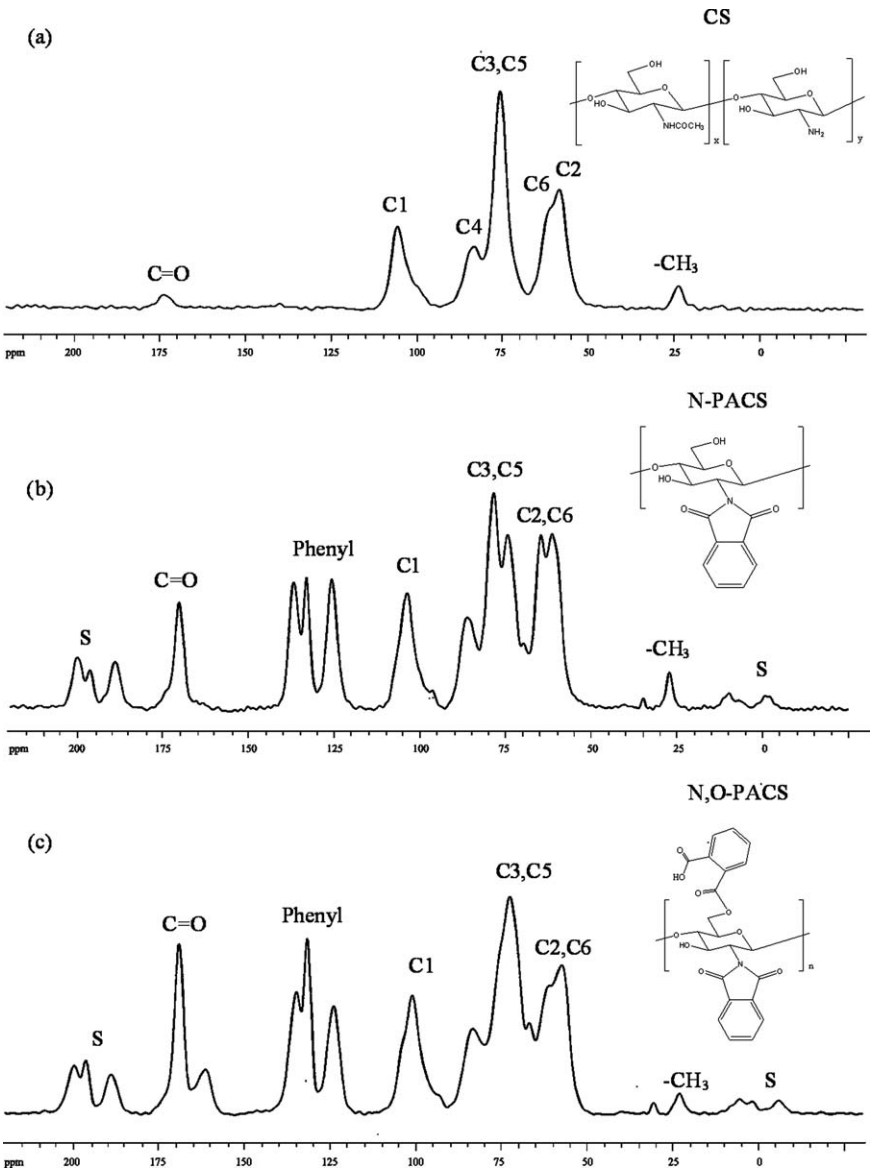

Figure 4 Solid-state ^{13}C-NMR spectra of (a) pure CS, (b) *N*-phthaloylated CS ($DS_{PA} = 0.78$) and (c) *N,O*-phthaloylated CS ($DS_{PA} = 0.84$). S indicates the spinning side-band in NMR.

phthaloylated CS has only one new C=O resonance absorption peak at 169 ppm due to the carbonyl group in imide, whereas the *N,O*-phthaloylated CS has two peaks at 169 and 161 ppm due to the carbonyl absorption in imide of *N*-phthaloyl group and in ester of *O*-phthaloyl group, respectively. Thus, it is concluded that in the reaction medium of formic acid/DMF, the obtained *N,O*-phthaloylated CS ($DS_{PA} = 0.84$) is soluble in some organic solvents due to a small extent of *O*-phthaloylation, and still leaves most hydroxyl groups free that can be used for further reaction with the vinyl coupling agent.

Preparation of vinyl CS

The PACS ($DS_{PA} = 0.84$) was then reacted with a vinyl coupling agent, 3-isopropenyl-αα′-dimethylbenzyl isocyanate (*m*-TMI), through the reaction of hydroxyl group in CS with the isocyanate group in *m*-TMI (compare Fig. 2). Figure 5(a) shows the FTIR spectrum of the reaction product, PACS-TMI. Compared with the spectrum of PACS in Figure 3, a new peak is observed at 1630 cm^{-1}, which is assigned to the stretching vibration of C=C. In addition, there is an increase in the intensity of methyl group due to the bonded *m*-TMI. This proves the successful formation of PACS-TMI. The ^{1}H-NMR spectrum also confirms the existence of C=C double bond from the observance of resonance peaks at 5.03 and 5.31 ppm (>C=CH$_2$).[26] Additional NMR peaks are 0.83 and 1.31 ppm from methyl group, as well as 7.14 and 7.26 ppm from phenylene group in *m*-TMI. It is also believed that the *m*-TMI prefers to react with the hydroxyl group at the C-6 position than

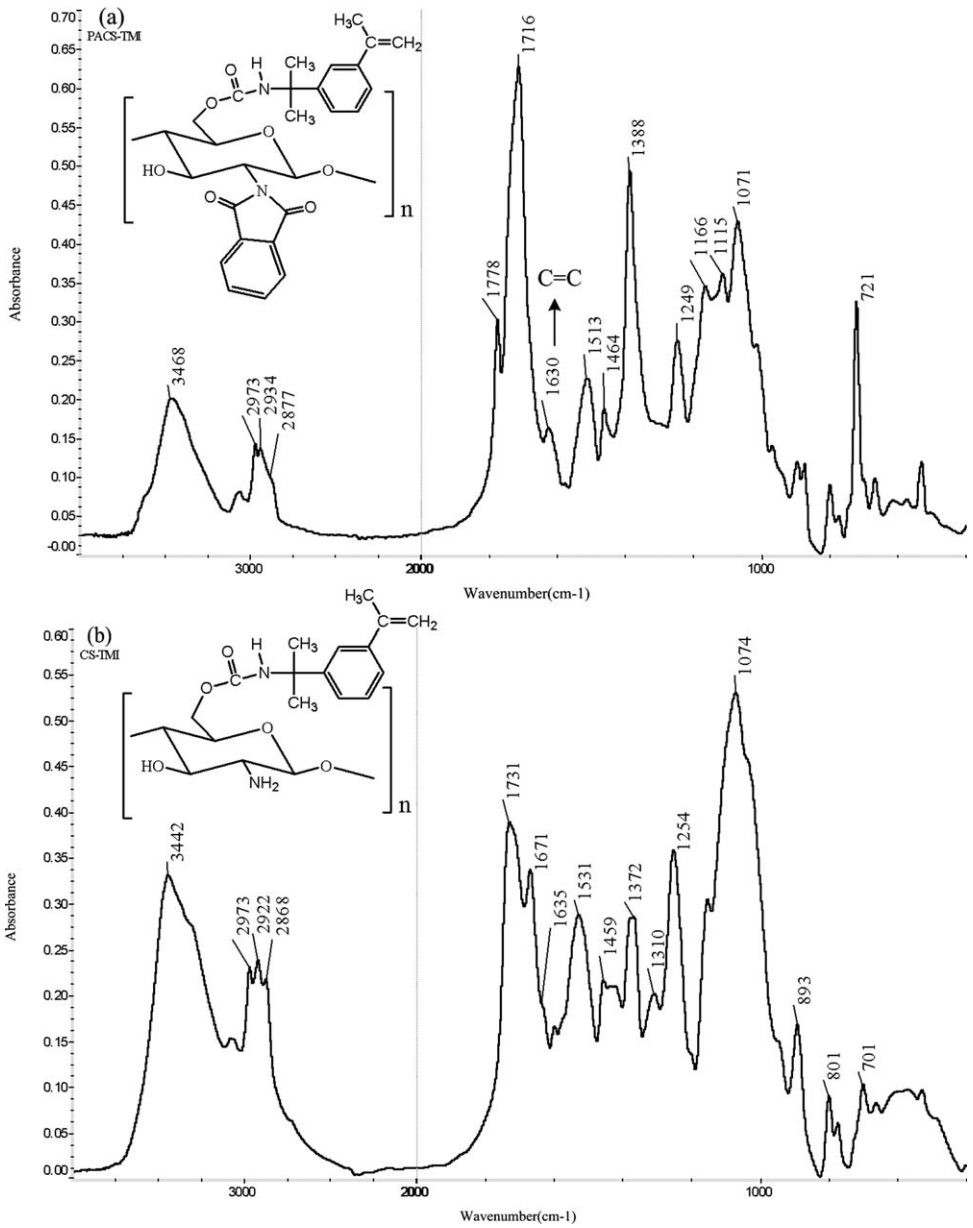

Figure 5 FTIR spectra of (a) TMI-substituted phthaloyl chitosan (PACS-TMI); (b) TMI-substituted chitosan (CS-TMI). The phthaloyl group was removed by hydrazine.

with that at the C-3 position due to the effect of steric hindrance. A bulky phthaloyl group is at the adjacent C-2 position that would prevent the approaching of *m*-TMI, also a bulky compound, to the C-3 position. After successfully incorporating the vinyl group to PACS, the phthaloyl groups of PACS-TMI were removed by reaction with hydrazine to obtain the vinyl CS, CS-TMI. The characteristic absorption peaks of imide (1778 and 1716 cm^{-1}) and phenylene (721 cm^{-1}) in phthaloyl group are no longer observed in the CS-TMI as shown in Figure 5(b). Though, it is possible that the hydrazine treatment could also cleave the carbamate linkage at C6 that would reduce the amount of the substituted *m*-TMI. Yet, FTIR spectrum in Figure 5(b) clearly shows that there still exists a strong absorption peak of C=O stretching of carbamate linkage at 1731 cm^{-1}. In addition, the weak absorption peak at 701 cm^{-1} is caused by phenylene ring of *m*-TMI. Most importantly, the C=C double bond is still observed at 1635 cm^{-1}, and also seen at 5.21 and 5.48 ppm in the ^{1}H-NMR spectrum. These results prove the substituted *m*-TMI still existed after the hydrazine treatment. By using elemental analysis on the CS-TMI product, the degree of addition of *m*-TMI (DS_{TMI}) was calculated to be 0.42 (C: 52.565%, N: 7.37%, H: 7.26%).

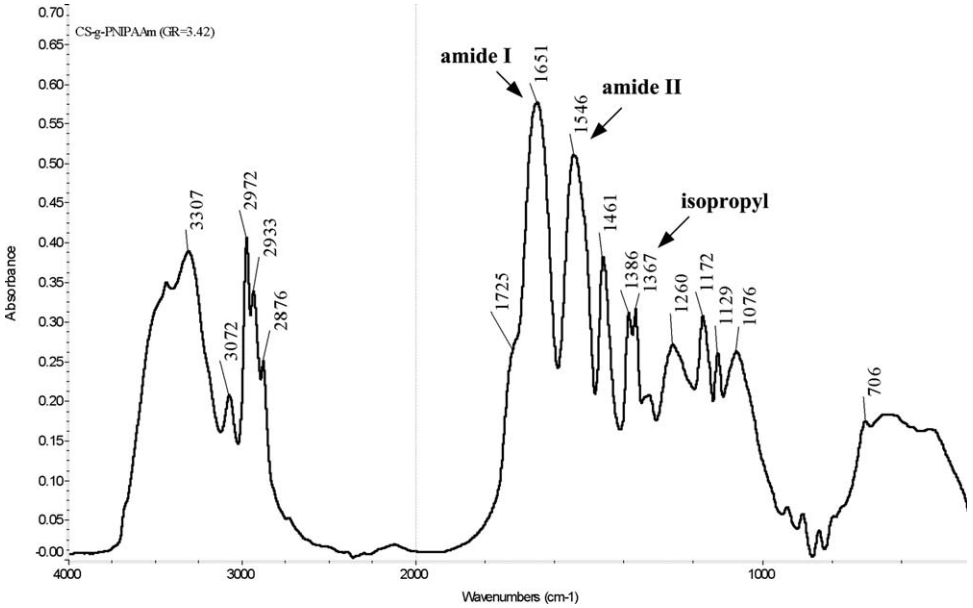

Figure 6 FTIR spectrum of a CS-*g*-PNIPAAm graft copolymer (CSPN5) with a grafting ratio of 3.42.

Synthesis of CS-*g*-PNIPAAm copolymers

For the grafting of PNIPAAm, CS-TMI was dissolved in formic acid together with the NIPAAm monomer and photo initiator. Upon UV irradiation, the photo initiator decomposed to free radicals and thereby NIPAAm monomer was able to be grafted to the vinyl CS chain to form CS-*g*-PNIPAAm copolymer by free radical polymerization. In addition to the formation of graft copolymer, PNIPAAm homopolymer was also produced during the reaction. Therefore, it is essential to remove the PNIPAAm homopolymer. After removing PNIPAAm homopolymer by cold water, Figure 6 shows a typical FTIR spectrum of the produced CS-*g*-PNIPAAm copolymer. The vinyl absorption peak at 1630 cm^{-1} is no longer observed and the absorption peaks at 1651 cm^{-1} (amide I) and 1546 cm^{-1} (amide II) are prominent due to the grafted PNIPAAm. In addition, the methyl group in PNIPAAm shows two strong absorption peaks at 2972 and 2876 cm^{-1}, in addition to the doublet peaks at 1386 and 1367 cm^{-1} due to the isopropyl group in PNIPAAm. Solid sate ^{13}C-NMR spectra were also obtained for CS-*g*-PNIPAAm copolymers. All spectra show the absorption peaks of PNIPAAm component at 22.9 ppm ($\underline{C}H_3$), 41.9 ppm (methine carbon in isopropyl group) and 175 ppm ($\underline{C}$=O in amide group). Therefore, both FTIR and NMR spectra prove that PNIPAAm was successfully grafted to CS. To obtain different extents of grafting, different amounts of NIPAAm monomer were fed into the reaction systems. Their monomer conversion (X), grafting ratio (GR) and grafting efficiency (GE) are listed in Table I. The lower monomer conversion in the present photo-initiated system compared to other polymerization systems using thermal-dissociated initiators or redox initiators is because of the high viscosity of the reaction medium and the nature of photo polymerization. Still, the monomer conversion was increased by increasing the feeding amount of NIPAAm, because of the increasing monomer concentration and the decreasing viscosity of the reaction medium. The grafting efficiencies are nearly the same for all systems, indicating the same probability for producing

TABLE I
Monomer Conversion (X), Grafting Ratio (GR) and Grafting Efficiency (GE) of Different Reaction Systems Polymerized via UV-Irradiation[a]

System	CSPN1	CSPN2	CSPN3	CSPN4	CSPN5
Vinyl CS (g)	0.302	0.301	0.302	0.301	0.301
NIPAAm (g)	0.302	0.602	0.905	1.204	1.504
X (%)	20.3	40.5	60.8	68.9	75.0
GR	0.18	0.73	1.71	2.49	3.42
GE (%)	91.1	90.1	93.9	90.2	92.0

[a] Photo-initiator was added in the amount of 1 wt % of NIPAAm monomer.

grafted PNIPAAm chains in all systems. As a result, the grafting ratio is increased with the feeding amount of NIPAAm monomer. These synthesized Cs-*g*-PNIPAAm graft copolymers were found to be insoluble in any solvents, even in acidic aqueous solutions which could dissolve both linear CS and PNIPAAm homopolymer originally. Their structure is therefore believed to be the type of AB-crosslinked graft copolymer, in which CS chains (i.e., A) are crosslinked by PNIPAAm chains (i.e., B) to form a network that swells but is not soluble in water as indicated in Figure 1. The structure will be further investigated by measuring their thermal properties and discussed in the following section. In conclusion, we have successfully grafted PNIPAAm chains preferably at the C-6 position and reserved all the amino groups at the C-2 position. This will be beneficial to the future biomedical applications.

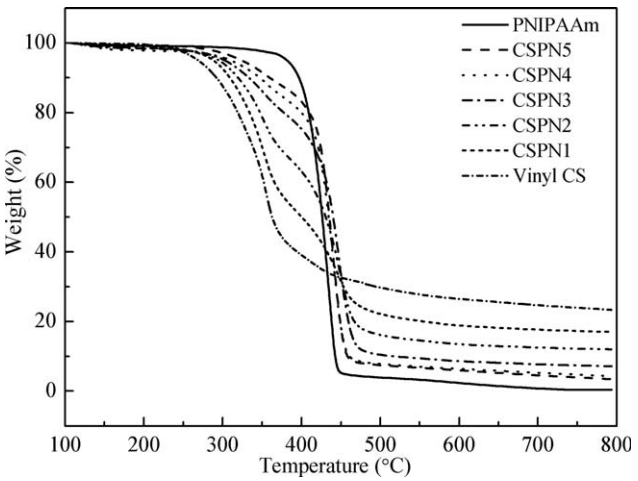

Figure 7 Thermal degradation behaviors of pure PNIPAAm, vinyl CS and various CS-*g*-PNIPAAm graft copolymers. Grafting ratio: CSPN1 (GR = 0.18); CSPN2 (GR = 0.73); CSPN3 (GR = 1.71); CSPN4 (GR = 2.49); CSPN5 (GR = 3.42).

Thermal properties

Thermal properties of CS-*g*-PNIPAAm copolymers were measured and discussed in the aspect of their structure. DSC thermal scans were recorded on pure PNIPAAm and various CS-*g*-PNIPAAm copolymers. After first run to evaporate all the moisture, the second run revealed the glass transition temperature of PNIPAAm. All the graft copolymers have approximately the same glass transition temperature at 138–140°C as the pure PNIPAAm, indicating the existence of an independent PNIPAAm chain segment in the copolymer. The transition is more obvious with higher grafting ratios.

Thermal degradation behavior of various CS-*g*-PNIPAAm copolymers under N_2 atmosphere was studied using a thermal gravimetric analyzer. Pure CS starts to degrade at 302°C (onset degradation temperature) and has a maximum-rate degradation temperature (T_{max}, first derivative peak temperature) at 333°C as reported by Don and Chen.[26] After addition of the *m*-TMI compound to form CS-TMI, the degradation curve becomes broader, starting to degrade from 225°C and having the T_{max} at 355°C as shown in Figure 7. In the Figure also shows that pure PNIPAAm has a one-stage degradation behavior with the T_{max} at 434°C. Combining CS-TMI and PNIPAAm into a graft copolymer, it exhibits a two-stage degradation behavior, where the first- and second-stage are correspondent to the degradation of CS-TMI and grafted PNIPAAm, respectively. It is also found that the ratio of degradation extent of these two stages is proportional to the grafting ratio. Though, the $T_{max,1}$ values of the first-stage degradation in various graft copolymers are almost the same as the T_{max} of CS-TMI, the $T_{max,2}$ values of the second-stage degradation of copolymers are about 5–15°C greater than the corresponding T_{max} of pure PNIPAAm. This is because the char produced from the earlier degradation of CS could protect the left PNIPAAm component and thus increase its thermal stability to some extent.

Table II lists the values of T_{max} and char yield (CY) of various samples. Pure CS has a high CY of 31% (residual at 800°C), whereas the CS-TMI has a lower CY of 23% because it contains the less thermally stable *m*-TMI group. On the contrary, a very little CY (0.43%) is found for the pure PNIPAAm. For graft copolymers, the CY decreases proportionally with increasing the grafting ratio. Table II also shows the theoretical values of char yield (CY_{theo}) calculated by using the linear addition law: $CY_{theo} = W_{CS\text{-}TMI} \times CY_{CS\text{-}TMI} + W_{PNIPAAm} \times CY_{PNIPAAm}$, where in the right hand side of equation, *W* and CY represent the weight fraction and char yield of pure components, respectively. The values are fairly in agreement with the experimental values, further proving the chemical nature of graft copolymers.

Swelling ratio and volume phase transition temperature

The environmentally responsive properties of CS-*g*-PNIPAAm copolymers were studied in the aspect of their swelling behavior. Figure 8 shows the swelling behavior of various samples in different buffer solutions. For the pure PNIPAAm, the transition is clearly observed at near 32°C and independent of pH value as shown in Figure 8(a). When grafting a small amount of PNIPAAm to CS as in the CSPN1 (GR = 0.18), it starts to show the volume-phase transition behavior, though not so obvious, Figure 8(b). The transition becomes more pronounced as the

TABLE II
First and Second Maximum-Rate Degradation Temperatures ($T_{max,1}$ and $T_{max,2}$) and Char Yield (CY, %) of Vinyl CS, PNIPAAm and Various Copolymer Samples

System	CS	Vinyl CS	CSPN1	CSPN2	CSPN3	CSPN4	CSPN5	PNIPAAm
$T_{max,1}$ (°C)	334	353	352	351	352	356	352	–
$T_{max,2}$ (°C)	–	–	447	449	448	439	439	434
CY (%)	31.3 (2.5)[a]	22.7 (1.3)	17.4 (1.2)	12.2 (1.1)	7.6 (0.9)	5.9 (1.2)	4.5 (0.4)	0.45 (0.06)
CY_{theo} (%)[b]	31.3	22.7	19.1	13.2	8.6	7.3	5.4	0.45

[a] The values in the parenthesis were the standard deviation of three measurements.
[b] Theoretical values of char yield CY_{theo} were calculated by $CY_{theo} = W_{Vinyl\ CS} \times CY_{Vinyl\ CS} + W_{PNIPAAm} \times CY_{PNIPAAm}$, where in the right hand side of equation, W and CY represent the weight fraction and char yield of pure components, respectively.

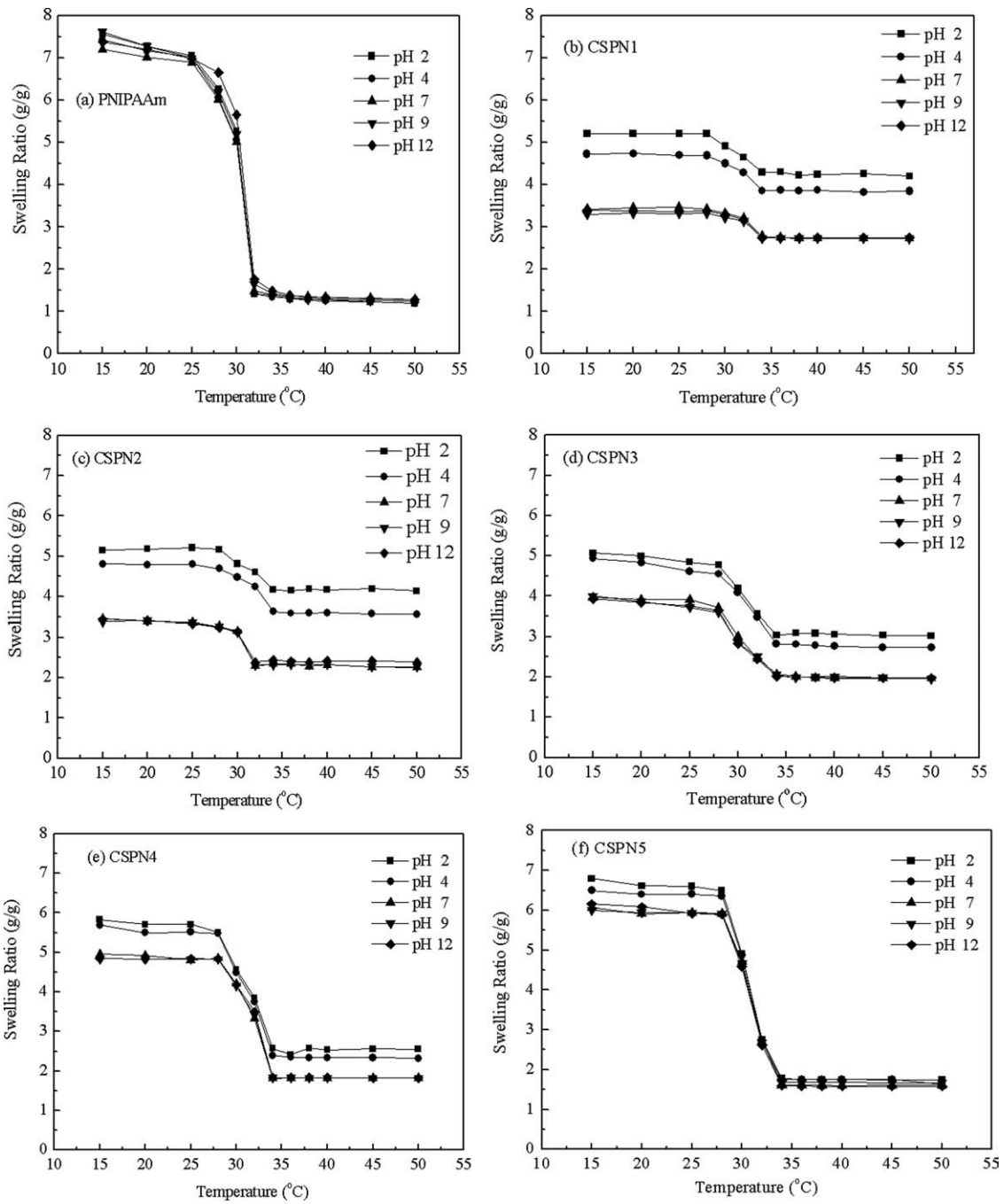

Figure 8 Swelling behaviors of pure PNIPAAm and various CS-*g*-PNIPAAm graft copolymers in different buffer solutions. (a) PNIPAAm; (b) CSPN1 (GR = 0.18); (c) CSPN2 (GR = 0.73); (d) CSPN3 (GR = 1.71); (e) CSPN4 (GR = 2.49); (f) CSPN5 (GR = 3.42).

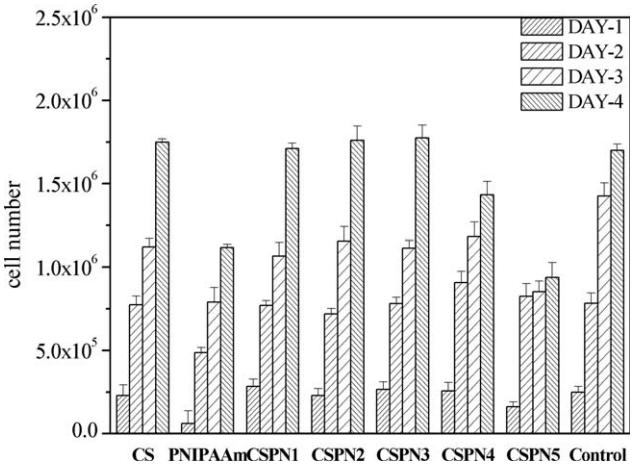

Figure 9 Time course of L929 cell-culture on gel membranes of CS-*g*-PNIPAAm graft copolymers, together with pure CS and PNIPAAm. Control group was the tissue-culture treated polystyrene. Grafting ratio, CSPN1: 0.18; CSPN2: 0.73; CSPN3: 1.71; CSPN4: 2.49; CSPN5: 3.42.

grafting ratio is increased as shown in Figure 8(c–f). All swelling ratios start to decline at 32°C due to the existence of the grafted PNIPAAm component. Furthermore, in this study, the PNIPAAm has higher swelling ratio than the CS when the temperature is below PNIPAAm's transition temperature (32°C). Therefore, by increasing the grafting ratio of PNIPAAm in the copolymer as from CSPN1 to CSPN5, the swelling ratio is consequently increased. For CSPN5 having a high grafting ratio of 3.42, its swelling ratio is only slightly less than the value of pure PNIPAAm. In addition to the thermo-responsive property, the swelling ratio is also dependent on the pH value. The values measured at pH 2 and 4 are obviously higher than those in the basic solutions. This is because of the existence of CS component. Linear CS easily dissolves in an acidic environment (pH $<$ 6) due to the protonation of amino groups at the C-2 position. The CS chains extend because of the repulsive force arising from positive charges along the chains. The degree of protonation of amino groups decreases with the pH value, leading to a decrease in the swelling ratio.

Cell culture and MTT assay

Figure 9 shows the time course of L929 cell culture on gel membranes of CS-*g*-PNIPAAm copolymers together with pure CS and PNIPAAm. Control group was the tissue-culture treated polystyrene plate. It can be seen that fibroblasts can adhere and grow very well on CS membrane, almost as good as that on the control group. This is because of its possession of amino groups which is advantageous for cell adhesion. Therefore, CS has been used as a biomaterial in tissue engineering and wound dressing for many years. However, the cell viability on PNIPAAm membrane is not as good as that on CS. After 4 days of culture, the L929 cell number was only about 60% of the value on CS membrane. Unlike the graft copolymers using maleic anhydride as the coupling agent, which have poor cell viability[26] as previously stated in Introduction; Figure 9 shows that CSPN1 to CSPN3 have almost the same cell viability as pure CS. This is because, for the present synthesized copolymers, there is no introduction of carboxylic acid group and all the amino groups are preserved. Therefore, we have successfully synthesized graft copolymers by selectively grafting PNIPAAm to CS, without deteriorating the cell compatibility of CS. Still, when the grafting amount is too high as in the samples CSPN4 and CSPN5, the cell viability starts to decrease.

CONCLUSIONS

By protecting the amino groups, we have grafted PNIPAAm to CS selectively at its C-6 position. The synthesized CS-*g*-PNIPAAm was proved to have a structure of an AB-crosslinked graft copolymer. In addition, these CS-*g*-PNIPAAm copolymers exhibit dual pH and thermo-responsive behaviors. They exhibit a volume-phase transition behavior with the same VPTT value as the pure PNIPAAm, and their swelling ratio increases as pH decreases due to the CS component. The higher the grafting ratio of PNIPAAm, the more obvious the phase transition is. These CS-*g*-PNIPAAm copolymers still containing amino groups are proved to have good cellular compatibility as pure CS, as long as the grafting ratio of PNIPAAm is below 1.7. Therefore, these CS-*g*-PNIPAAm graft copolymers can be used as smart hydrogels in the field of controlled drug release.

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