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# Preparation of environmental-responsive chitosan-based nanoparticles by self-assembly method

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ABSTRACT

In this research, environmental-responsive nanoparticles of chitosan-*graft*-poly(*N*-isopropylacrylamide) copolymers (CS-*g*-PNIPAAm) were prepared by the self-assembly method. The copolymer was first synthesized through polymerization of NIPAAm monomer in the presence of CS in an aqueous solution using cerium ammonium nitrate as the initiator. Then, the CS-*g*-PNIPAAm solution was diluted by deionized water and heated to a proper temperature for CS-*g*-PNIPAAm to undergo self-assembly. Micelles of CS-*g*-PNIPAAm were formed, and glutaraldehyde was added to reinforce the micelle structure to form nanoparticles. TEM images showed that a porous or hollow structure of nanoparticles was developed. The synthesized nanoparticles carried positive charges on the surface and their mean diameter could be manipulated by changing the temperature of environment. These nanoparticles with environmentally sensitive properties are expected to be utilized in hydrophilic drug delivery system.

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## 1. Introduction

In recent years, polymeric particles with hollow core morphology or cavity-containing structure have attracted great attention because of the broad potential applications (Gao, Lukyanov, Singhal, & Torchilin, 2002; Hu, Jiang, Ding, Chen, & Yang, 2004; Langer & Tirrell, 2004; Meier, 2000). Several different methods have been used to prepare polymeric hollow particles, such as the layer-by-layer deposition of polyelectrolyte on a template core, polymerizing monomers in lipid vesicles, self-assembly of block copolymers in an appropriate solvent and emulsion (or miniemulsion) polymerization (Berth, Voigt, Dautzenberg, Donath, & Mohwald, 2002; Caruso, Caruso, & Mohwald 1998; Caruso, Caruso, & Mohwald 1999; Donath, Sukhorukov, Caruso, Davis, & Mohwald, 1998; Hotz & Meier, 1998; Huang, Remsen, Kowalewski, & Wooley, 1999; Jang & Ha, 2002; Krafft et al., 2001; Stewart & Liu, 1999; Tiarks, Landfester, & Antonietti, 2001). For the biomedical applications, it would be better that polymeric hollow particles also have properties of biocompatibility, biodegradability and low cytotoxicity. Among numerous biocompatible and biodegradable materials, biopolymers are the suitable materials to synthesis hollow particles for biomedical applications because of its good biological properties.

Chitosan (CS) is a high-molecular-weight polysaccharide composed mainly of β-(1,4) linked 2-deoxy-2-amino-D-glucopyranose units and partially of β-(1,4) linked 2-deoxy-2-acetamido-D-glucopyranose. It is obtained by partial deacetylation of chitin, which is the most abundant natural biopolymer next to the cellulose and can be found in the skeletal materials of crustaceans and insects, and cell walls of bacteria and fungi. Differently from chitin, chitosan is soluble in acidic solution (pH < 6.4) as a result of the protonation of amino groups on the D-glucosamine residues (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). Because of its advantageous properties including biodegradability, biocompatibility, anti-bacteria and non-toxicity, CS can be used in the fields of wastewater treatment, food processing, cosmetics, pharmaceuticals, biomaterials and agriculture (Chen & Chen, 1998; Muzzarelli, 1977; Stevens, Rao, & Chandrkrachang, 1996). In the drug delivery field, the vesicles based on chitosan and derivatives can be used for transdermal, nasal, ocular, oral and parenteral administration and other application (Illum, Jabbal-Gill, Hinchcliffe, Fisher, & Davis, 2001; Thanou, Verhoef, & Junginger, 2001; Thein-Han & Stevens, 2004). Many methods have been developed to prepare CS particles including emulsion, spray-drying and emulsion-droplet coalescence technique. Recently, Cheng, Xia, and Chan (2006) prepared CS-polypyrrole hollow spheres by using AgBr crystal as the core template. Using different shapes of AgBr crystal, the size and morphology of hollow spheres could be controlled. Hu et al. (2004) reported the preparation of CS-PAA hollow spheres by an emulsion

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polymerization method without using a core template material. It is therefore possible to synthesize CS-based hollow spheres for biomedical application.

In our previous research, we found that chitosan could play a role as a surfactant when CS-PNIPAAm and CS-PAA-PNIPAAm nanoparticles were synthesized by the soapless dispersion polymerization (Chuang, Don, & Chiu, 2009; Lee, Wen, & Chiu, 2003; Lee, Wen, Lin, & Chiu, 2004). The surface charge of nanoparticles is positive. Recently, pH-sensitive micelles self-assembled by changing medium pH value to change the hydrophilicity of two pH-sensitive polymers have been successfully developed. Dou, Jiang, Peng, Chen, and Hong (2003) reported that the hydroxyethylcellulose-poly(acrylic acid) (HEC-PAA) hollow spheres could be prepared by pH-dependent self-assembly method. Based on these reports, we aim to synthesize well dispersed chitosan-based nanoparticles by the self-assembly method.

In this study, cerium ammonium nitrate (CAN) was used to initiate the polymerization of NIPAAm monomers in the presence of chitosan to form a CS-garft-PNIPAAm copolymer (CS-*g*-PNIPAAm) solution. In appropriate concentration and temperature, because PNIPAAm became hydrophobic when the environment temperature was higher than LCST, CS-*g*-PNIPAAm aggregated to form CS-PNIPAAm micelles. After crosslinking the shell region of micelles and cooling down the temperature, the CS-PNIPAAm porous or hollow nanoparticles were obtained. Structure, morphology, particle size, surface charge, thermo- and pH-responsive properties were examined.

## 2. Materials and methods

### 2.1. Materials

CS (degree of deacetylation 95%, average molecular weight 200,000 g/mol) was obtained from Kio-Tek (Taipei, Taiwan). NIPAAm and acetic acid (HAc) were obtained from Acros (Geel, Belgium). Ceric ammonium nitrate (CAN) was purchased from SHOWA (Tokyo, Japan). Doxycycline hyclate was obtained from Sigma–Aldrich (St. Louis, MO). All the other chemicals were analytical grade or above and used as received without further purification.

### 2.2. Synthesis of CS-g-PNIPAAm copolymer solution

Designed amount of CS was first dissolved in 20 mL HAc aqueous solution containing specific amount of NIPAAm. The solution was purged with nitrogen and heated to 40 °C. CAN was dissolved in 5 mL of water and pre-heated to 40 °C, before it was poured into the solution. After 6 h of reaction, a solution containing CS-*g*-PNIPAAm copolymers and PNIPAAm homopolymers was obtained. The solution was poured into acetone to precipitate the CS-*g*-PNIPAAm and PNIPAAm homopolymer. The characteristic data of CS-*g*-PNIPAAm copolymer sample were listed in Table 1. The grafting ratio (GR) was calculated by the gravimetric analysis using the following equation:

$$\mathrm{GR} = \frac{(W_2 - W_{CS})}{(W_{CS})} \quad (1)$$

In the equation, $W_{CS}$ is the initial weight of chitosan. Polymer mixture was extracted by methanol at 20 °C to remove the PNIPAAm homopolymers. After 48 h of extraction, the remaining CS-*g*-PNIPAAm copolymer was obtained by oven drying for 48 h at 60 °C in a circulation oven followed by another 48 h in a vacuum oven at 60 °C and weighted ($W_2$).

Molecular weight of PNIPAAm grafted on CS chains was characterized by a gel permeation chromatography (GPC) (Waters 2695, Waters, Milford, MA) analysis system with THF as the eluent at a flow rate of 1.0 mL/min and narrow-polydispersity polystyrene as the calibration standard. CS-*g*-PNIPAAm copolymer was hydrolyzed by 12 M $HCl_{(aq)}$ for 4 h, 40 °C to degrade the CS chain and obtain the grafted PNIPAAm. Grafted PNIPAAm was dissolved in THF to be analyzed by GPC. Average graft point on each CS chain was calculated by the following equation:

$$\text{Average graft point} = \frac{(W_2 - W_{CS})}{(M_w)} \div N_{CS} \quad (2)$$

In the equation, $M_w$ was the average molecular weight of grafted PNIPAAm, measured by GPC. $N_{CS}$ was the mole of CS chain.

### 2.3. Structure analysis of CS-g-PNIPAAm copolymers

Structure analysis was carried out with an NMR spectrometer (Avance-500, Bruker, Billerica, MA). CS-*g*-PNIPAAm copolymer was ground to fine powder and dissolved in 5% D-acetic acid/95% $D_2O$ solution to prepare the sample solution (500 MHz for $^1H$).

### 2.4. Preparation of CS-PNIPAAm nanoparticles

The dried CS-*g*-PNIPAAm copolymers were dissolved in design amount of HAc aqueous solution and the pH value of solution was 3.0. After that, the solution containing CS-*g*-PNIPAAm was heated to 40 °C for self-assembly. The solution became translucent, revealing the CS-PNIPAAm micelles were formed. After 24 h micellization, designed amount of glutaraldehyde (GA) as crosslinker was added to crosslink the micelles for 2 h at 40 °C (molar ratio of glucosamine unit in CS to glutaraldehyde, [CS]/[GA] = 2.0/1.0). After that, a dispersion solution containing CS-PNIPAAm nanoparticles was obtained. The reaction conditions and the sample code of CS-PNIPAAm nanoparticles were listed in Table 2.

### 2.5. Morphology observation

The morphologies of the CS-PNIPAAm nanoparticles were observed with TEM (JSM-1230 EX II, JEOL, Tokyo, Japan). One drop

**Table 1**
Characteristic data of CS-*g*-PNIPAAm copolymer.

| Copolymers | GR | | PNIPAAm grafts $M_w$ (g/mol) | Average graft point[a] |
|---|---|---|---|---|
| | Gravimetric method | $^1H$ NMR method | | |
| CS-*g*-PNIPAAm | 0.302 | 0.404 | 4725 | 17.8 |

[a] GR (gravimetric method) was used to calculate the average graft point.

**Table 2**
Sample code and characteristic data of CS-PNIPAAm nanoparticles.

| Nanoparticle sample | Copolymer concentration (mg/mL) | Self-assembly temperature (°C) | Solution pH | Particle size (nm)[a] | Average zeta potential (mV) |
|---|---|---|---|---|---|
| CS-PNIPAAm | 1.5 | 40 | 3.0 | 295 ± 65 | 16.1 |

[a] Based on intensity.

of CS-PNIPAAm nanoparticles dispersion was dropped on the Formvar carbon-coated Cu grid. The residual water was removed via blotting with filter paper from the bottom of the grid, and the grid was then air-dried at design temperature.

### 2.6. Particle size and zeta-potential of nanoparticles

The hydrodynamic diameter and size distribution of the synthesized nanoparticles were measured by a dynamic light scattering (DLS) method (Zetasizer Nano-ZS, Malvern, Malvern, UK). Before measurement, samples were adjusted to a proper concentration with 0.01 M $NaCl_{(aq)}$. The mean diameter (±SD) (based on intensity) was obtained from 20 determinations.

Zeta-potential of nanoparticles was obtained with Zetasizer Nano-ZS. Samples were diluted to a concentration of 1 wt% in 0.01 M $NaCl_{(aq)}$. All measurements were repeated three times and the average of three runs was taken as the result.

## 3. Results and discussion

### 3.1. Preparation of CS-g-PNIPAAm copolymers

CS-*g*-PNIPAAm copolymer solution was prepared by the polymerization of NIPAAm monomers in the CS solution. CAN is a strong redox initiator; which can oxidize the pyranose ring of polysaccharide and produce a free radical on it (Don, King, & Chiu, 2002; Don, King, & Chiu, 2006; Fernandez, Casinos, & Guzman, 1990; Graczyk & Hornof, 1988). Then, the graft copolymerization of NIPAAm monomer onto CS chains occurred. At the same time, the homopolymerization of NIPAAm monomer occurred because of the chain transfer reaction. Therefore, after the polymerization, a solution containing CS-*g*-PNIPAAm copolymers and PNIPAAm homopolymers was obtained.

Table 1 shows the characteristic data of CS-*g*-PNIPAAm copolymer sample. The grafting ratio GR of CS-*g*-PNIPAAm was 0.302 by gravimetric method. To calculate the average graft point and average molecular weight of grafted PNIPAAm on CS chains, HCl hydrolysis method was used to degrade the CS chains in CS-*g*-PNIPAAm copolymer (Varum, Ottoy, & Smidsrod, 2001). After HCl hydrolysis process and drying, grafted PNIPAAm was obtained and then dissolved in THF to analyze the molecular weight by GPC. Table 1 shows the average molecular weight of grafted PNIPAAm and the average graft point on CS chains of CS-*g*-PNIPAAm sample. We found that the average graft point of CS-*g*-PNIPAAm was 17.8 and the molecular weight of grafted PNIPAAm was 4725 g/mol.

$^1$H NMR spectrometer was used to observe the structure of CS-*g*-PNIPAAm copolymer, Fig. 1 displays the spectrum of CS-*g*-PNIPAAm and we found that the characteristic absorption peaks in CS and PNIPAAm were all observed in Fig. 1, which indicated that the CS-*g*-PNIPAAm copolymer was formed after the polymerization (Chung, Bae, Park, Lee, & Park, 2005; Guo, Yuan, & Gao, 2008; Trombotto, Ladaviere, Delolme, & Domard, 2008). In addition, $^1$H NMR spectrum was also used to calculate the GR of CS-*g*-PNIPAAm copolymer. From the area ratio of H2 to H11, the graft amount of NIPAAm monomer onto CS chains could be estimated and the GR value was listed in Table 1.

### 3.2. Preparation of CS-PNIPAAm nanoparticles

CS-PNIPAAm nanoparticles were prepared by self-assembly of CS-*g*-PNIPAAm copolymers in an appropriate condition. CS-*g*-PNIPAAm copolymers dissolved molecularly in acidic water at 25 °C and they self-assembled upon an appropriate temperature change. Micellization process of CS-*g*-PNIPAAm solution (copolymer concentration 1.5 mg/mL, pH = 3.0) was monitored by DLS and a result of the mean diameter as a function of temperature was shown in

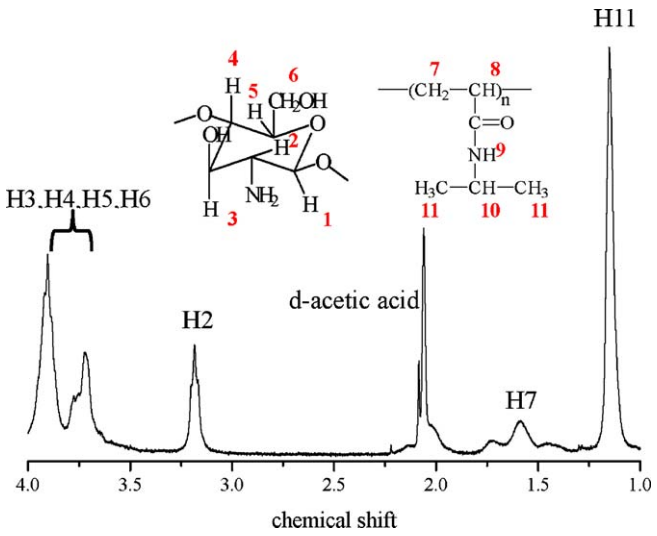


**Fig. 1.** $^1$H NMR spectrum of CS-*g*-PNIPAAm copolymer.

Fig. 2. It was clear that the mean diameter was very small and remained invariant in the temperature range of 25–32 °C. The solution was transparent. However, when the solution temperature increased to 35 °C, it was found that particle size had a dramatic increase and the solution became translucent. Obviously, CS-*g*-PNIPAAm experienced an aggregation caused by the temperature change and the thermo-induced micelles were formed. Afterwards, the mean diameter decreased and kept constant at about 440 nm, indicating that the micelles became more compact with the temperature increase. Crosslinking agent GA was added to the reaction system to reinforce the structure of micelles. After cooling the reaction system to room temperature, a solution containing CS-PNIPAAm nanoparticles was prepared.

### 3.3. Morphology observation of CS-PNIPAAm nanoparticles

The morphology of CS-PNIPAAm nanoparticles was studied by TEM observation. Fig. 3 shows TEM photographs of CS-PNIPAAm nanoparticles prepared with temperature-induced self-assembly. We found that the diameters of CS-PNIPAAm nanoparticles ranged from 120 to 400 nm and a difference in contrast was observed over the nanoparticles. In Fig. 3(a), the latex dispersion of CS-

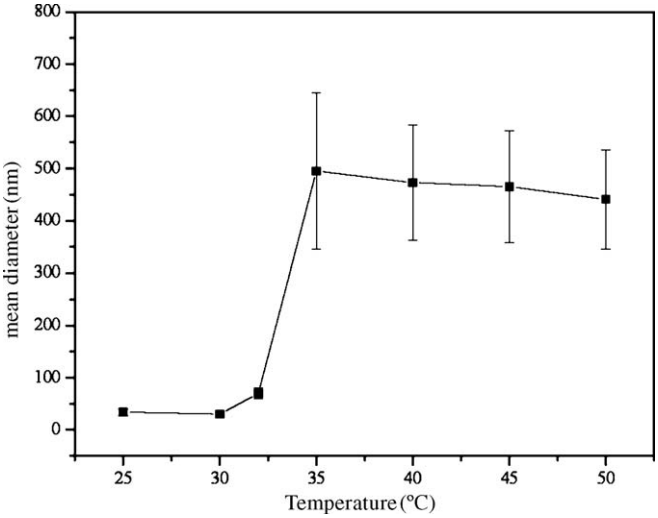


**Fig. 2.** Mean diameter (based on intensity) of CS-*g*-PNIPAAm copolymer as a function of temperature at pH = 3.0.

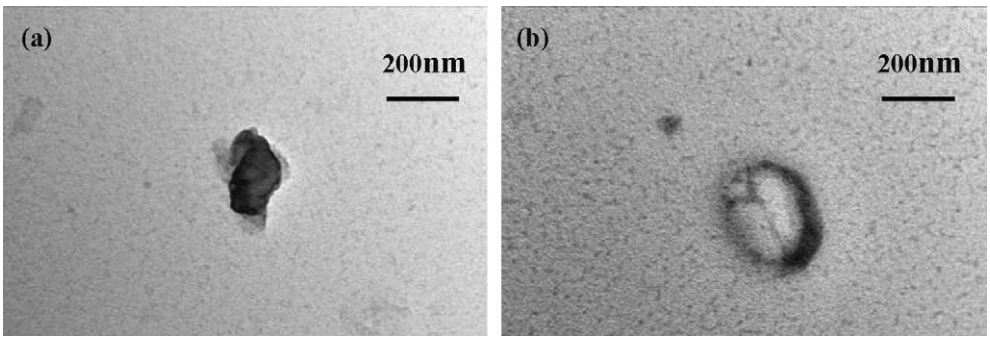


**Fig. 3.** TEM images of CS-PNIPAAm nanoparticles at different drying conditions: (a) CS-PNIPAAm (room temperature dry) and (b) CS-PNIPAAm (50 °C dry).

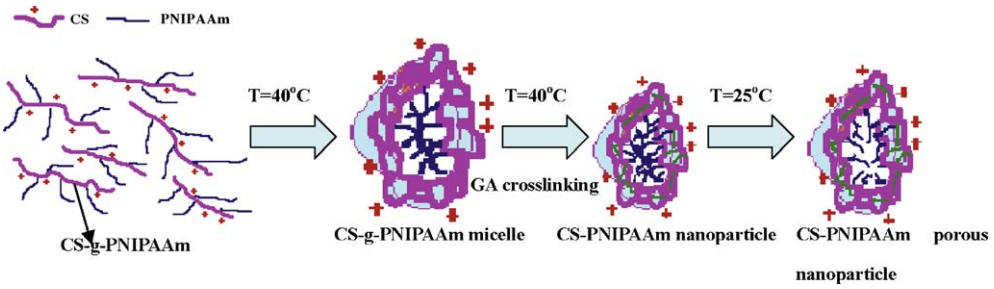


**Fig. 4.** Schematic illustration of formation mechanism of CS-PNIPAAm nanoparticles.

PNIPAAm nanoparticles was dropped on Cu grid and dried at room temperature for TEM observation. Polymer chains were swelling at room temperature, but shrinking with the removal of water gradually. Therefore, a porous structure was observed by TEM. However, when the latex dispersion of CS-PNIPAAm nanoparticles was heated to 50 °C, then one drop of dispersion was dropped on the Cu grid and dried at 50 °C to prepare the nanoparticle sample for TEM observation, Fig. 3(b) shows that the nanoparticles has a hollow sphere structure. It was because that PNIPAAm chains shrank abruptly above the LCST and adhered on the shell of nanoparticles, the interior of nanoparticles was occupied by water. After the removal of water quickly, a cavity-like hollow structure was observed by TEM.

The formation mechanism of CS-PNIPAAm porous nanoparticles could be schematically shown in Fig. 4. First, the CS-*g*-PNIPAAm copolymers were well dissolved in the solution at 25 °C. When the solution was heated to 35 °C, PNIPAAm became hydrophobic because the temperature of solution was higher than LCST of PNIPAAm. The shrinkage of PNIPAAm on CS-*g*-PNIPAAm induced the self-assembly of CS-*g*-PNIPAAm to form a micelle. The hydrophobic PNIPAAm was in the interior of CS-PNIPAAm micelles and hydrophilic CS was on the shell of micelles. GA was used to crosslink the CS chains in the micelles that could efficiently lock the integrality of micelle nanoparticles. After cooling the reaction system to room temperature, a solution containing CS-PNIPAAm porous nanoparticles was prepared.

### 3.4. Mean size and zeta potential of CS-PNIPAAm nanoparticles

The mean size and surface charge of CS-PNIPAAm nanoparticles were analyzed by DLS and electrophoretic light scattering technique in a solution of 0.01 M $NaCl_{(aq)}$ ($T$ = 25 °C, pH = 3.2). The results were listed in Table 2. It is shown that the mean diameter of nanoparticles was 295 ± 65 nm and the surface of nanoparticles carried positive charges and the average value of zeta potential was 16.1 mV. The positive surface charge came from the cationic nature of CS in the acidic environment due to the protonation of amino groups (Hu et al., 2002; Hu et al., 2004; Lin et al., 2007).

### 3.5. Thermo-response of CS-PNIPAAm nanoparticles

In addition, we studied the thermo-response of CS-PNIPAAm nanoparticles by observing the variation of particle size as a function of temperature. All the samples were prepared in 0.01 M $NaCl_{(aq)}$ at a pH value of 3.2. Fig. 5 shows that the mean diameter of nanoparticles varied with the temperature of medium. We could observe a decrease of particle size with the increase of the temperature of the medium. It was because that PNIPAAm was a thermo-sensitive material that would exhibit volume shrinkage above its LCST. Therefore, the CS-PNIPAAm nanoparticles became sensitive to temperature.

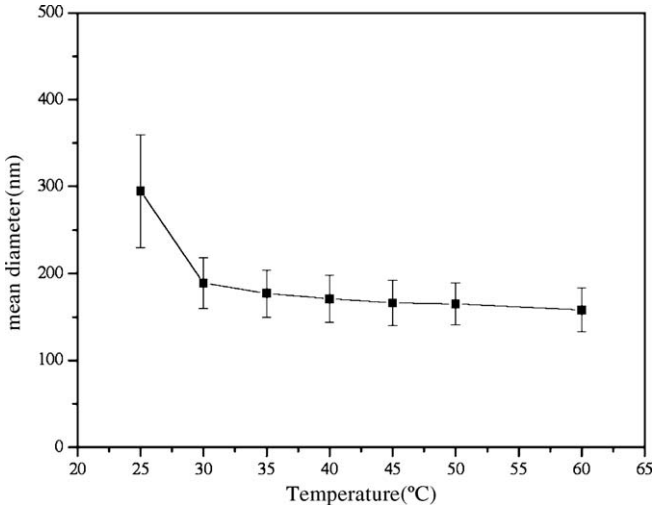


**Fig. 5.** The variation of mean diameter (based on intensity) of CS-PNIPAAm nanoparticles after being immersed in pH = 3.2 buffer solution at different temperatures.

## 4. Conclusion

In this study, CS-PNIPAAm nanoparticles were successfully prepared by using self-assembly method. At an appropriate concentration and temperature, CS-*g*-PNIPAAm self-assembled to form micelles. After crosslinking, the CS-PNIPAAm nanoparticles were obtained. Structure and morphology of nanoparticles were investigated. From TEM observation, we found a porous or hollow structure for nanoparticles of CS-PNIPAAm. The synthesized nanoparticles were found to be environmentally responsive, in which their particle size could be manipulated by changing the temperature of the medium. Because of their thermo- sensitive behavior, the CS-PNIPAAm nanoparticles have potential to be applied in hydrophilic drug delivery and long-time controlled release vehicle.

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