Drug Release from Thermo-Responsive Self-assembled Polymeric Micelles Composed of Cholic Acid and Poly(*N*-isopropylacrylamide)

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Cholic acid, conjugated with amine-terminated poly(*N*-isopropylacrylamide) (abbreviated as CA/ATPNIPAAm), was synthesized by a N, N'-dicyclohexyl carbodiimide (DCC)-mediated coupling reaction. Self-assembled CA/ATPNIPAAm micelles were prepared by a diafiltration method in aqueous media. The CA/ATPNIPAAm micelles exhibited a lower critical solution temperature (LCST) at 31.5°C. Micelle sizes measured by photon correlation spectroscopy (PCS) were approximately 31.6 ± 5.8 nm. The CA/ATPNIPAAm micelles were spherical and their thermal size transition was observed by transmission electron microscope (TEM). A fluorescence probe technique was used for determining the micelle formation behavior of CA/ATPNIPAAm in aqueous solutions using pyrene as a hydrophobic probe. The critical micelle concentration (CMC) was evaluated as 8.9×10^{-2} g/L. A drug release study was performed using indomethacin (IN) as a hydrophobic model drug. The release kinetics of IN from the CA/ATPNIPAAm micelles revealed a thermo-sensitivity by the unique character of poly(*N*-isopropylacrylamide) i.e. the release rate was higher at 25°C than at 37°C.

Key words: Drug release, Thermo-responsive, Self-assembled polymeric micelles, Poly(*N*-isopropylacrylamide)

INTRODUCTION

In recent decades, macromolecular self-assemblies have been intensively investigated and with particular attention to their nano-sized structure due to extensive applications in colloid science, electronics, environmental technology, biotechnology and biomedical engineering (Whitesides *et al.*, 1991; Lehn, 1993; Freedman, 1991; Akiyoshi and Sunamoto, 1996; Davis, 1981). Recently, amphiphilic polymers have become the focus of extensive research because of their ability to impart a greater degree of organization compared to homogeneous systems. Self-assemblies of block copolymer micelles (Xu *et al.*, 1991) or self-aggregates of hydrophobically modified polymers (Guenoun *et al.*, 1996) have been investigated on a theoretical basis concerning their formation or biotechnological and pharmaceutical applications. The self-assembly characteristics of amphiphilic polymers in aqueous solutions have attracted a great deal of attention that has focussed on the development of effective targetable drug carriers (Kataoka *et al.*, 1993; Akiyoshi *et al.*, 1993). Since the formation of self-assemblies from polymeric amphiphiles resembles that of low-molecular-weight amphiphiles, polymeric amphiphiles form micelles that consist of an inner core of hydrophobic segments and an outer shell of hydrophilic segments (Gao and Eisenberg, 1993). The hydrophobic inner core is surrounded and stabilized by the hydrophilic outer shell. The hydrophilic outer shell enhances dispersion, inhibits intermicellar aggregation and interactions with other hydrophobic components irrespective of the high inner core hydrophobicity.

Poly(*N*-isopropylacrylamide) (PNIPAAm) is well known to exhibit a thermo-reversible phase transition at 32°C. This transition temperature is called the lower critical solution temperature (LCST). This polymer is water-soluble thus hydrophilic, and exists in an extended chain form below its LCST. It undergoes a reversible phase transition to an insoluble and hydrophobic aggregate above the LCST. For this reason, this polymer has been widely investigated and applied to biomedical and pharmaceutical areas as well as other fields (Heskins and Guillet, 1968; Bae *et al.*, 1989; Hoffman, 1987; Feil *et al.*,

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1991; Yoshida *et al.*, 1995; Chen and Hoffman, 1995; Kohori *et al.*, 1998). Hydrophobically modified PNIPAAm shows thermo-responsive water-solubility and can form heterogeneous structures composed of hydrophilic micro-domains of PNIPAAm chains and hydrophobic micro-domains of any incorporated hydrophobic segments in aqueous solution. A heterogeneous structure is formed by the aggregation forces of the hydrophobic segments against the intramolecular hydrophilicity (Chung *et al.*, 1997). The synthetic self-assemblies act as host systems as a result of their heterogeneous structure for many hydrophobic molecules (Anton *et al.*, 1993). The self-assembled carriers are more useful, particularly in drug delivery systems, since most drugs have hydrophobic character.

In this study, cholic acid conjugated with amine-terminated PNIPAAm (abbreviated as CA/ATPNIPAAm) was synthesized, and self-assembled micelles were prepared by a diafiltration method. Cholic acid (CA) is one of the major bile acids. Bile acids are the main product of cholesterol metabolism and are biologically the most detergent-like molecules in the body. Since cholic acid can self-associate in water and form micelles, it can be expected that the CA/ATPNIPAAm can form core-shell type micelles in aqueous media. The inner core of the micelles acts as a microcontainer for hydrophobic drugs, while the outer shell has the ability to control the release of drugs through a temperature change. As a model hydrophobic drug, indomethacin (IN) was incorporated into the micelles and its release was studied *in vitro*. It is expected that CA/ATPNIPAAm has large potential for use as a drug carrier for thermo-sensitive drug delivery.

MATERIALS AND METHODS

Materials

N-isopropylacrylamide (NIPAAm, Tokyo Kasei) was purified by recrystallization in *n*-hexane and dried in vacuum at room temperature. 2,2'-Azobisisobutyronitrile (AIBN) was obtained from Polysciences Inc.. The 2-Aminoethanethiol hydrochloride (AETHCl) and KOHmethanol (potassium hydroxide volumetric standard, 1.003 M solution in methyl alcohol) were both obtained from the Aldrich Chemical Company. Cholic acid (CA) and indomethacin (IN) were purchased from the Sigma Chemical Company. *N,N'*-Dimethylformamide (DMF), dimethylsulfoxide (DMSO), and all other chemicals were of reagent grade and used without further purification.

Synthesis of ATPNIPAAm and CA/ATPNIPAAm conjugates

ATPNIPAAm, prepared according to the method reported by Chen et al. (Chen and Hoffman, 1995), was synthesized by the polymerization of NIPAAm (5.65 g, 50 mmol) in methanol (20 ml) at 60 for 22 h using AIBN (82.0 mg, 0.5 mmol) and AETHCl (113.0 mg, 1.0 mmol) as initiator and chain transfer reagent, respectively. KOH methanol was added to remove HCl from the AETHCl salt. The semitelechelic PNIPAAm with an amino end group was obtained by precipitating the reaction solution in diethyl ether. The reaction scheme is shown in Fig. 1.

CA/ATPNIPAAm conjugates were prepared by a coupling reaction using N,N'-dicyclohexyl carbodiimide (DCC) as a coupling agent, as shown in Fig. 2. CA (0.2 mmol), DCC (0.4 mmol), and ATPNIPAAm (0.2 mmol) were separately dissolved in DMSO. The DCC solution was added to the CA solution, and stirred for 30 min to activate the carboxyl group of CA. Subsequently, the ATPNIPAAm solution was added dropwise into the activated CA solution. The reactions were carried out at room temperature with stirring (Li, 1991). The reaction solution was filtered to remove the precipitated dicyclohexylurea (DCU). The polymer solution obtained was repeatedly precipitated in an excess of diethyl ether and dried under vacuum. The dried polymer was dissolved in DMSO and dialyzed against distilled water through a dialysis tube (MWCO: 12,000 g/mol) at 4°C for 1 day. The deionized water was exchanged at 2 hr intervals, and the resultant solution was then freeze-dried.

Preparation of micelles and drug loading

The formation of the micelles and the drug loading procedure were carried out by a diafiltration method (Yokoyama *et al.*, 1994; Cho *et al.*, 1997; Jeong *et al.*, 1998; Kwon *et al.*, 1995). Briefly, 20 mg of CA/ATPNIPAAm was dissolved in 10 ml DMF. To form core-shell structures, the

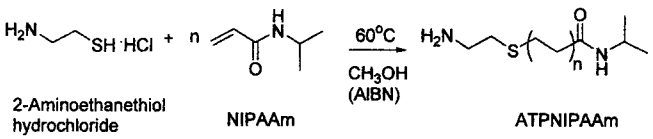

Fig. 1. Synthetic scheme of ATPNIPAAm

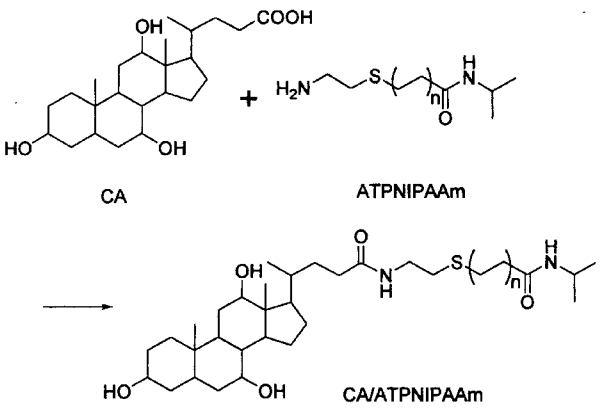

Fig. 2. Synthesis of CA/ATPNIPAAm

solution was dialyzed against 1.0 L × 3 of distilled water for 3~4 hr using a molecular weight cutoff (MWCO) 12,000 dialysis tube. The distilled water was then exchanged at 3~4 hr intervals during over a 24 hr period. Following this, the solution was freeze-dried. To form the IN-loaded micelles, 20 mg of CA/ATPNIPAAm was dissolved in 10 ml of DMF and subsequently 20 mg of IN was added. The solutes were dissolved entirely at room temperature, and dialyzed using a MWCO 12,000 dialysis tube against distilled water. The solution was then freeze-dried. CA/ATPNIPAAm empty micelles and IN-loaded micelles were stored at 4°C prior to use.

Measurement of drug loading content

A freeze-dried sample of IN-loaded micelles was suspended in methanol and vigorously stirred for 2 hr then sonicated for 15 min. The resulting solution was centrifuged at 3,000 rpm for 20 min and the supernatant removed for drug concentration measurements using a UV spectrophotometer (Shimadzu UV-1201, Japan) at 312 nm.

Transmittance measurement

The optical transmittance of the aqueous polymer solutions (1 g/L) at various temperatures was measured at 500 nm with a UV-VIS spectrometer. The samples were thermostated in a temperature controlled circular system. The temperature was gradually increased with a maximum heating rate of 0.5°C/min. The LCST value of the polymer solution was determined at a temperature indicating 50% optical transmittance.

Measurement of photon correlation spectroscopy (PCS)

The PCS spectrum was measured with a Zetasizer 3000 (Malvern instruments, England) using a He-Ne laser beam at a 633 nm wavelength (scattering angle of 90°). A micelle suspension was used for particle size measurements (concentration: 0.1 g/L) and was measured without filtering.

Measurement of fluorescence spectroscopy

To investigate the fluorescence spectroscopy characteristics, the drug free CA/ATPNIPAAm solutions were prepared as follows: 20 mg of CA/ATPNIPAAm was dissolved in 10 ml DMF and dialyzed up to 24 hr using the same method described above. The resultant solution concentration was adjusted to the various required CA/ATPNIPAAm concentrations.

The critical CA/ATPNIPAAm micelle concentration (CMC) was measured by fluorescence spectroscopy (Shimadzu *RF-5301 PC spectrofluorophotometer, Shimadzu Co.* Ltd., Japan) using pyrene as a hydrophobic probe (Wilhelm *et al.*, 1991; Kalyanasundaram and Thomas, 1977). To obtain sample solutions, a known amount of pyrene in acetone was added to each of a series of 20 ml vials and the acetone then evaporated. The final concentration of pyrene was 6.0×10^{-7} M. 10 ml of various concentrations of CA/ATPNIPAAm solutions were added to each vial and then heated for 3 hr at 65°C to equilibrate both pyrene and the micelles. The mixture was then left to cool overnight at room temperature. Fluorescence spectra were measured at an excitation wavelength of 339 nm. The emission wavelength was 390 nm for the excitation spectra. The excitation and emission bandwidths were both 1.5 nm.

Transmission electron microscope (TEM) observation

One drop of a CA/ATPNIPAAm solution containing 0.01 % of phosphotungstic acid was placed on a copper grid coated with carbon film, and dried at 20°C (below the LCST) or at 45°C (above the LCST). The observation was done at 80 kV in a JEOL, JEM-2000 FX II, Japan.

In vitro drug release studies

The release experiment was carried out *in vitro* as followed: 5 mg of the IN-loaded CA/ATPNIPAAm micelles and 1 ml phosphate buffered saline (PBS, 0.1 M and pH 7.4) were put into a dialysis tube (MWCO 12,000 g/mol). The dialysis tube was then introduced into a vial with 10 ml PBS, and stirred at 100 rpm at the desired temperature. At specific time intervals, whole medium was taken and replaced with fresh PBS. The amount of IN released was measured with a UV spectrophotometer (Shimadzu, UV-1201, Japan) at 312 nm.

RESULTS AND DISCUSSION

Synthesis and analysis of CA/ATPNIPAAm micelles

From the synthesis of ATPNIPAAm, amino end-groups were introduced to one terminal end of the PNIPAAm during telomerization with aminoethanethiol. The synthetic scheme is shown in Fig. 1. The number-average molecular weight of ATPNIPAAm was 7,880 as determined by gel permeation chromatography (GPC) (ratio of weight- to number-average molecular weights $\overline{M}_w/\overline{M}_n = 1.74$).

The CA/ATPNIPAAm was prepared by a coupling reaction using DCC as a coupling agent, as shown in Fig. 2.

The CA/ATPNIPAAm was dissolved in DMF and dialyzed against distilled water. The aqueous solution of CA/ATPNIPAAm, prepared by the dialysis method, became turbid in response to increasing temperature. Fig. 3 shows the optical transmittance of the aqueous polymer solutions at various temperatures. The lower critical solution temperature (LCST) was determined by the temperature showing 50% transmittance in the polymer solution. From the result, the LCST of CA/ATPNIPAAm was determined to be 31.5°C. This result exhibited an analogous thermo-sensitivity in comparison with the PNIPAAm homo-

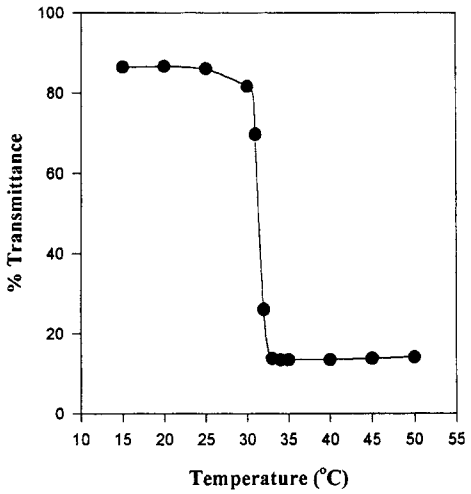

Fig. 3. Optical transmittance changes of aqueous solutions as a function of temperature. (Concentration: 1 g/L, absorbance at 500 nm)

polymer. Furthermore the temperature-responsive behavior was also observed in detail by TEM.

Fig. 4 shows the particle size distribution of the CA/ATPNIPAAm micelles. The CA/ATPNIPAAm micelle particle sizes were 31.6 ± 5.8 nm.

Fig. 5(a) shows TEM images of the CA/ATPNIPAm micelles dried at 20°C (below their LCST). The shape of the micelles was spherical with diameters ranging from 30~50 nm, which is analogous to the PCS data. The thermal transition of the micelles was observed with a sample dried at 45°C (above their LCST) in Fig. 5(b). In this figure, each particle retained their spherical shapes with no aggregation, but the sizes ranged from 10~20 nm. These results support the thermal sensitivity of the CA/ATPNIPAAm micelles, which is consistent with the PNIPAAm characteristics. It is thought that when the temperature is increased above the LCST of polymer, there is a change in the conformation of the micelles outer shell. The expanded form of PNIPAAm in the shell part changes into a compact one when the temperature is raised above the LCST.

The self-assembly behavior of CA/ATPNIPAAm micelle was investigated by fluorescence spectroscopy, and the critical micelle concentration (CMC) was determined. Wilhelm *et al.* reported the micelle formation of poly (styrene) (PS) and poly(ethylene oxide) (PEO) di- or triblock copolymers in water by a fluorescence technique using pyrene as a hydrophobic probe. Using this technique, they were able to determine the critical micelle concentration (CMC) from the fluorescence emission and excitation spectra as pyrene partitions between the aqueous and micellar

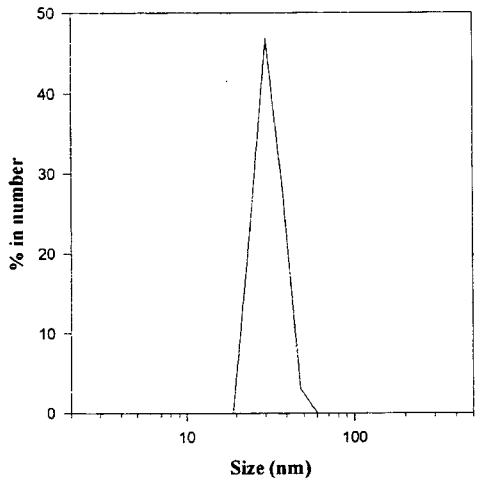

Fig. 4. Particle size distribution of CA/ATPNIPAAm micelles by PCS measurement.(Concentration: 0.1 g/L)

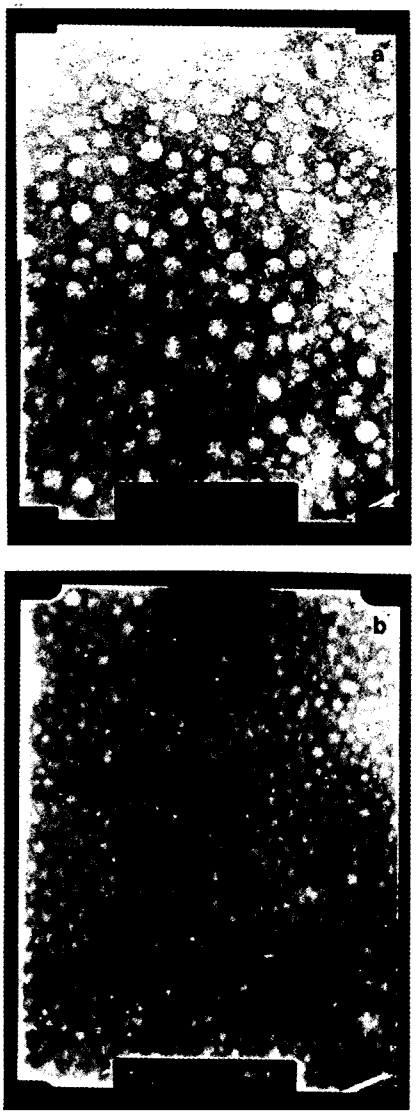

Fig. 5. TEM images of CA/ATPNIPAAm micelles prepared at 20°C (a) and 45°C (b). Micelles were negatively stained with 0.01% phosphotungstic acid

environment (Wilhelm *et al.*, 1991). This method was also used by Jeong *et al.* to probe the micelle formation of poly(γ-benzyl L-glutamate) (PBLG) and PEO block copolymer in water (Jeong *et al.*, 1998). If the CA/ATPNIPAAm can be self-assembled in water, formation of micelles should be proven by the same fluorescence probe technique as with block copolymer micelles.

Fig. 6 shows the fluorescence emission spectra of pyrene at a fixed excitation wavelength of 339 nm in the presence of various CA/ATPNIPAAm concentrations. The fluorescence intensity increased as the concentration of CA/ATPNIPAAm increased, which indicates the formation of CA/ATPNIPAAm self-assembly micelles in water such as block copolymeric micelles (Wilhelm *et al.*, 1991; Marctic and Nair, 1994). It is thought that pyrene was preferentially absorbed into the micelles composed of the core-shell structure when it was introduced into the aqueous phase using a suitable solvent (Xu *et al.*, 1991; Kwon *et al.*, 1993; Dowling and Thomas, 1990; Zhao *et al.*, 1990).

Fig. 7 (a) shows the excitation spectra of pyrene in the presence of various CA/ATPNIPAAm concentrations. The figure shows a red shift with increasing CA/ATPNIPAAm concentration. A red shift of pyrene in the excitation spectrum was also observed in micelle formation with PBLG/PEO block copolymers (Jeong *et al.*, 1998) and PS-PEO block copolymers (Wilhelm *et al.*, 1991). A plot of the intensity ratio of I_{338}/I_{334} vs. log C of CA/ATPNIPAAm in the pyrene excitation spectra is shown in Fig. 7 (b). The plot indicates that the ratio is almost flat at low concentrations, and rapidly increases at the high concentrations. The CMC was taken from the intersection of the tangent to the curve at the inflection point with the horizontal tangent through the points at low concentrations. The CMC value was estimated to be 8.9×10^{-2}

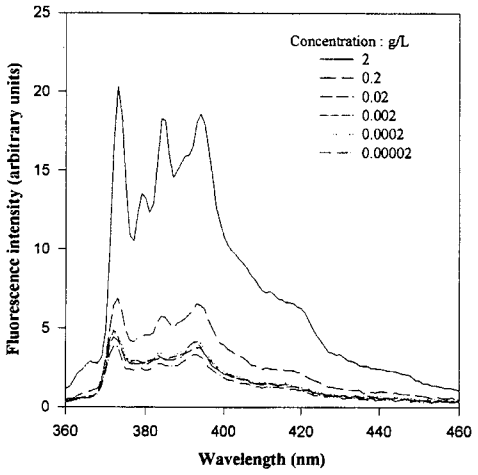

Fig. 6. Fluorescence spectra of pyrene (6.0×10^{-7} M) against the CA/ATPNIPAAm concentration in distilled water (excitation wavelength: 339 nm)

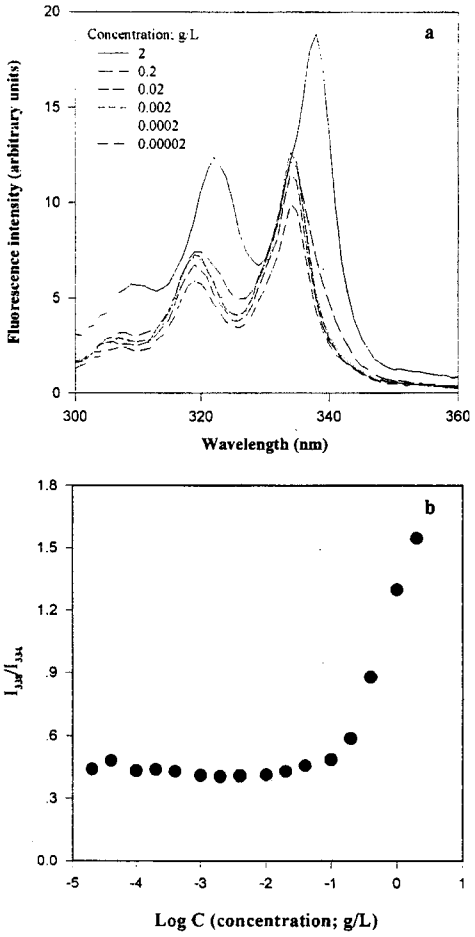

Fig. 7. Fluorescence spectra of pyrene (6.0×10^{-7} M) against the CA/ATPNIPAAm concentration in distilled water (emission wavelength: 390 nm) (a) and plots of the intensity ratio I_{338}/I_{334} from pyrene excitation spectra vs. log C of the CA/ATPNIPAAm in distilled water (b)

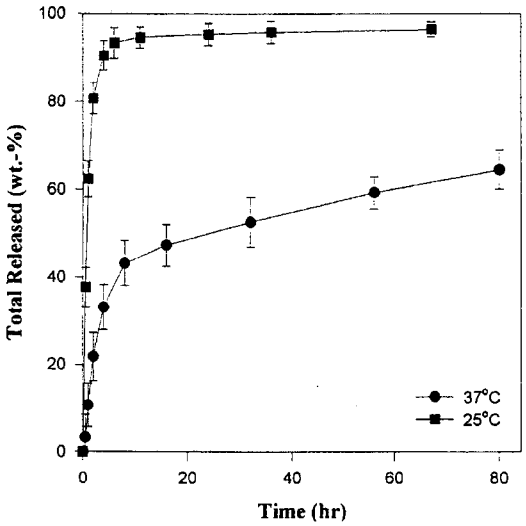

Fig. 8. Release of IN from CA/ATPNIPAAm micelles in PBS (0.1 M, pH 7.4) at 25°C and 37°C (n=3).

g/L. The CA/ATPNIPAAm micelles were formed at a higher concentration in contrast with the CMC of block copolymer micelles. It can be explained that the micelle is more readily formed when there is an increase in the amount of hydrophobic components (Jeong *et al.*, 1998). From fluorescence probe measurements, it is clear that upon a critical concentration (i.e. CMC), CA/ATPNIPAAm can form core-shell structure micelles in water with an amphiphilic nature in the same way as block copolymer micelles.

Drug release studies in vitro

To study the drug release behavior, IN-loaded CA/ATPNIPAAm micelles were simply redispersed in PBS (0.1 M, pH 7.4) without a surfactant. The IN loading content in the CA/ATPNIPAAm micelles was 39.3 wt.-%. Fig. 8 shows the kinetics of IN release from the micelles as a function of temperature. The figure shows that IN is slowly released as temperature increases. This result may have close relationship with the thermo-sensitivity of the CA/ATPNIPAAm micelles. It is thought that when the temperature is increased above the LCST of PNIPAAm, a conformational change of the outer shell occurs. The expanded form of PNIPAAm in the shell part is changed into a compact one when the temperature is raised above the LCST (31.5°C).

In conclusion, thermo-responsive self-assembled polymeric micelles composed of CA and ATPNIPAAm were synthesized and characterized. This micelle showed a thermal transition at 31.5°C; the LCST of CA/ATPNIPAAm. Particle size was measured by PCS, and the size range was 31.6 ± 5.8 nm. From the TEM observations, the micelles were spherically shaped with sizes that were temperature sensitive. The sizes of the micelles were 30~50 nm below the LCST, and about 10~20 nm above it. The CMC was estimated from fluorescence spectroscopy, which also showed that CA/ATPNIPAAm was self-associated in water to form core-shell structure micelles. IN release from these micelles was thermo-sensitive as expected. It is proposed that drug release from thermo-sensitive CA/ATPNIPAAm micelles might be applied to site-specific drug delivery systems by modulating the temperature of the target site.

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