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International Journal of Pharmaceutics 205 (2000) 165–172

international journal of pharmaceutics

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Thermo-responsive self-assembled polymeric micelles for drug delivery in vitro

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Received 17 November 1999; received in revised form 21 June 2000; accepted 28 June 2000

Abstract

Cholic acid (CA) was coupled with amine-terminated poly (*N*-isopropylacrylamide) (ATPNIPAAm) using *N*,*N*′-dicyclohexyl carbodiimide as a coupling agent. Self-assembled CA/PNIPAAm conjugate (abbreviated as CN) micelles were prepared by diafiltration method in water. The CN micelles exhibited the lower critical solution temperature (LCST) at 31.5°C. The CN micelles were observed as spherical shapes and their dried sizes were ranged between 30 and 50 nm by the transmission electron microscope (TEM) images. Hydrated micelle sizes measured by photon correlation spectroscopy (PCS) were ranged 337.5 ± 67.8 nm. And reversible size changes of CN micelles were observed with two point temperature 10 and 40°C, respectively. From the fluorescence spectra, fluorescence intensity of pyrene in the CN micelles was increased and red-shifted as the concentration of CN increased, indicating the formation of self-assembled polymeric micelles in water. The critical micelle concentration (CMC) was evaluated as 8.9×10^{-2} g/l. Much more indomethacin (IN) was released from the CN micelles at 10 than at 40°C due to the thermo-sensitivity of the PNIPAAm in the CN polymer.

Keywords: Thermo-responsive; Polymeric micelle; Drug delivery; In vitro

1. Introduction

In recent decades, self-assembled polymeric micelles were intensively investigated and their structure considerably attracted due to their extensive applications such as colloid science, electronics, environmental technology, biotechnology and biomedical engineering (Davis, 1981; Freedman, 1991; Whitesides et al., 1991; Lehn, 1993; Akiyoshi and Sunamoto, 1996). Recently, amphiphilic polymers have become the focus of broad research for their ability to form the self-assembled polymeric micelles. Self-assemblies of block copolymer micelles (Xu et al., 1991) or self-aggregates of hydrophobically modified polymers (Guenoun et al., 1996) have been investigated with respect to theoretical approaches for their formation or their biotechnological and

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0378-5173/00/$ - see front matter
PII: S0378-5173(00)00501-9

pharmaceutical applications. The self-assembled characteristic of amphiphilic polymers in aqueous solution has been explosively attended for development of effective targetable drug carriers (Akiyoshi et al., 1993; Kataoka et al., 1993). Since the formation of self-assemblies from polymeric amphiphiles resembles that of low-molecular-weight amphiphiles, polymeric amphiphiles form micelles consisting of the inner core of hydrophobic segments and the 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 high inner core hydrophobicity.

Poly(*N*-isopropylacrylamide) (PNIPAAm) is well known to exhibit thermo-reversible phase transition at 32°C. This transition temperature is called as a lower critical solution temperature (LCST). This polymer is water-soluble and hydrophilic, and exists in an extended chain form below its LCST, undergoing 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 and other fields (Heskins and Guillet, 1968; Hoffman, 1987; Bae et al., 1989; Feil et al., 1991; Chen and Hoffman, 1995; Yoshida et al., 1995; Kohori et al., 1998). Hydrophobically modified PNIPAAm shows thermo-responsive water-solubility and can form heterogeneous structures composed of hydrophilic microdomains of PNIPAAm chains and hydrophobic microdomains of incorporated hydrophobic segments in aqueous solution. A heterogeneous structure is formed by the aggregation forces of hydrophobic segments against intramolecular hydrophilicity (Chung et al., 1997). The synthetic self-assemblies act as host systems by the heterogeneous structure for many hydrophobic molecules (Anton et al., 1993). Especially, in drug delivery systems, the self-assembled carriers are very useful since most drugs have a hydrophobic character.

In this study, we synthesized CA/PNIPAAm conjugates, and prepared self-assembled polymeric micelles by diafiltration method. CA is one of the major bile acids. Bile acids are the main product of cholesterol metabolism and biologically the most detergent-like molecules in the body. Since CA can self-associate in water and form micelles, it can be expected that the CN can form core-shell type micelles in aqueous media. The inner core of the polymeric micelles acts as a microcontainer of hydrophobic drugs, while the outer shell has the ability to control the release of drugs from the CN micelles by temperature change. Indomethacin (IN) was incorporated into the micelles as a hydrophobic model drug, and the release from the CN micelles was performed in vitro.

2. Materials and methods

2.1. Materials

N-isopropylacrylamide (NIPAAm, obtained from Tokyo Kasei, Tokyo, Japan) was purified by recrystallization in *n*-hexane and dried in vacuum at room temperature. 2,2′-Azoisobutyronitrile (AIBN) was obtained from Polysciences Inc. (Warrington, PA). 2-Aminoethanethiol hydrochloride (AET·HCl), KOH·methanol (potassium hydroxide volumetric standard, 1.003 M solution in methyl alcohol), and *N*,*N*′-dicyclohexyl carbodiimide were obtained from Aldrich Chemical Company (Milwaukee, WI). Cholic acid (CA) and indomethacin (IN) were purchased from Sigma (St. Louis, MO). *N*,*N*′-Dimethylformamide (DMF), dimethylsulfoxide (DMSO), and all other chemicals were of reagent grade and used without further purification.

2.2. Synthesis of CA/PNIPAAm conjugate

Amine-terminated PNIPAAm (ATPNIPAAm) was prepared according to the method reported by Chen and Hoffman (1995). Briefly, ATPNIPAAm was synthesized by polymerization of NIPAAm (5.65 g, 50 mmol) in methanol (20 ml) at 60°C for 22 h using AIBN (82.0 mg, 0.5 mmol) and AET·HCl (113.0 mg, 1.0 mmol) as initiator and chain transfer reagent, respectively. After re-

action, KOH·methanol was added to remove HCl from the AET·HCl salt. The semitelechelic PNIPAAm with amino end group was obtained by precipitating the reaction solution into diethyl ether. The reaction scheme is shown in Fig. 1. The number–average molecular weight of ATPNIPAAm obtained by GPC was 7880 and molecular weight distribution of the obtained polymer was 1.74.

CA/PNIPAAm conjugate (abbreviated as CN) was prepared by coupling reaction of CA with ATPNIPAAm 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. And then, the ATPNIPAAm solution was dropped into the activated CA solution. The reactions were carried out at room temperature for 10 days (Li, 1991). The reaction solution was filtered to remove the precipitated dicyclohexylurea (DCU). The solution was put into dialysis membrane (molecular weight cutoff (MWCO) 12 000 g/mol) and left in deionized water for 1 day. The deionized water was exchanged at 2-h intervals, and the resultant solution was freeze-dried.

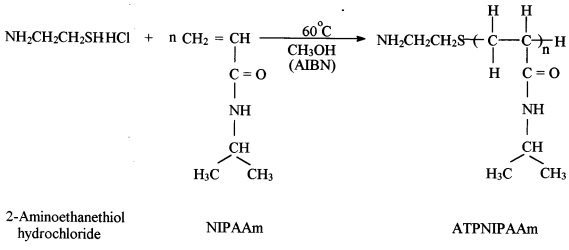

Fig. 1. Synthetic scheme of the ATPNIPAAm.

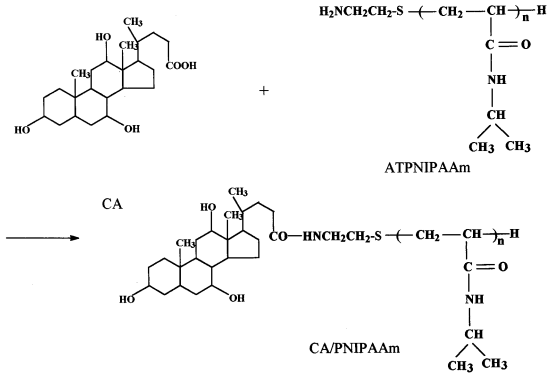

Fig. 2. Synthesis of CA/PNIPAAm conjugate.

2.3. Preparation of polymeric micelles and IN loading

The CN micelles were formed using the diafiltration method (Yokoyama et al., 1994; Kwon et al., 1995; Cho et al., 1997; Jeong et al., 1998). A total of 20 mg of CN was dissolved in 10 ml of DMF. To form the polymeric micelles, the solution was dialyzed using MWCO 12 000 g/mol dialysis membrane against distilled water. The medium was replaced every hour for the first 3 h and every 3 h for 1 day. Subsequently, the solution was freeze-dried. To prepare IN-loaded micelles, 20 mg of CN was dissolved in 10 ml of DMF and subsequently 20 mg of IN was added. The solution was solubilized entirely at room temperature, and dialyzed using MWCO 12 000 g/mol dialysis membrane against distilled water. And then the solution was freeze-dried. The prepared micelles were kept in refrigerator at 4°C until use.

2.4. Transmittance measurement

Optical transmittance of the polymer in aqueous solutions (concentration: 1 g/l) at various temperature was measured at 500 nm with a UV–VIS spectrometer. The samples were thermostated with a temperature controlled circular system. The temperature was gradually increased with a maximum heating rate of 0.5°C/min. Values for the LCST of polymer solutions were determined at a temperature indicating 50% optical transmittance. To observe the reversible thermo-responsibility, optical transmittance was evaluated by temperature fluctuation between 10 and 40°C.

2.5. Transmission electron microscopy (TEM) observation

A drop of CN micelle solution containing 0.01% phosphotungstic acid was placed on a copper grid coated with carbon film, and dried at 20°C (below the LCST). The observation was carried out at 80 kV with JEM-2000 FX II (Jeol, Japan).

2.6. Photon correlation spectroscopy (PCS) measurement

PCS was measured with a Zetasizer 3000 (Malvern instruments, UK) with He–Ne laser beam at a wavelength of 633 nm (scattering angle of 90°). A micelle solution (concentration: 1 g/l) was used for particle size measurement without filtering. Reversible size changes of CN micelles were examined to confirm the thermo-responsibility.

2.7. Measurement of fluorescence spectroscopy

To investigate the fluorescence spectroscopy characteristics, CN solutions without drug were prepared as follows: 20 mg of CN was dissolved in 10 ml DMF and dialyzed up to 24 h as the same method described above. Resultant solution was adjusted to the various concentrations of CN. Critical micelle concentration (CMC) of the CN was obtained by measuring fluorescence spectroscopy (Shimadzu RF-5301 PC spectrofluorophotometer, Shimadzu, Japan) using pyrene as a hydrophobic probe (Kalyanasundaram and Thomas, 1977; Wilhelm et al., 1991). 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 was evaporated. The final concentration of pyrene was 6.0×10^{-7} M. A total of 10 ml of various concentrations of CN solution were added to each vial and then heated for 3 h at 65°C to equilibrate the pyrene and the micelles, and left to cool overnight at room temperature. Fluorescence emission spectra were measured at excitation wavelength of 339 nm. Emission wavelength was 390 nm for excitation spectra. Excitation and emission bandwidths were 1.5 and 1.5 nm, respectively.

2.8. Measurement of drug loading content

A freeze-dried sample of IN-loaded polymeric micelles was suspended into methanol and vigorously stirred for 2 h and sonicated for 15 min. The resulting solution was centrifuged with 3000 rpm for 20 min and the supernatant was taken for measuring the drug concentration using UV spectrophotometer (Shimadzu UV-1201) at 312 nm.

2.9. In vitro drug release studies

The release experiment in vitro was carried out as follows: 5 mg of IN-loaded CN micelles and 1 ml phosphate buffer solution (PBS, 0.1 M and pH 7.4) were put into a dialysis membrane (MWCO 12 000 g/mol). Then the dialysis membrane was introduced into vial with 10 ml PBS (0.1 M, pH 7.4), and the media was stirred at 100 rpm at 10 and 40°C. At specific time intervals, the whole medium was replaced with fresh PBS. The concentration of IN released from CN micelle was determined by UV spectrophotometer (Shimadzu, UV-1201, Japan) at 312 nm.

3. Results and discussion

3.1. Analysis of CN micelles

Fig. 3 shows optical transmittance of aqueous solutions of CN at various temperature, and the LCST was determined by the temperature showing 50% transmittance in the polymer solution. The results indicated that the transition point of the CN was 31.5°C, which was almost similar to the LCST of PNIPAAm. Reversible changes of optical transmittance were represented in Fig. 4. The optical transmittance of CN micelles was modified by temperature fluctuation. This figure showed that the CN micelles can change the structure quickly from hydrated state below the LCST to dehydrated state above the LCST.

Fig. 5 shows transmission electron micrographs of the CN micelles dried at 20°C. The shapes of the CN micelles was observed as approximately spherical shapes, and the diameters of these micelles were ranged 30–50 nm as a dehydrated state.

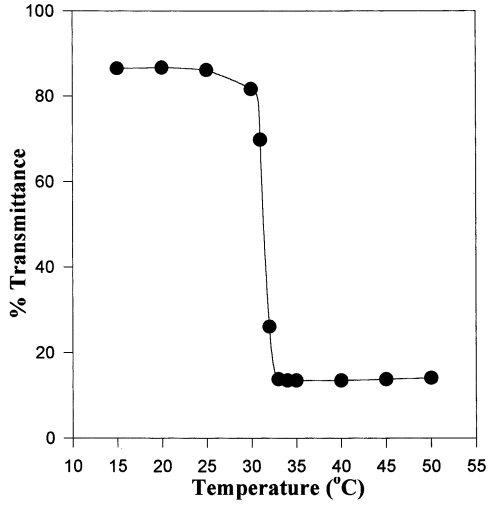

Fig. 3. Transmittance changes of aqueous solution as a function of temperature (concentration: 1 g/l, absorbance at 500 nm).

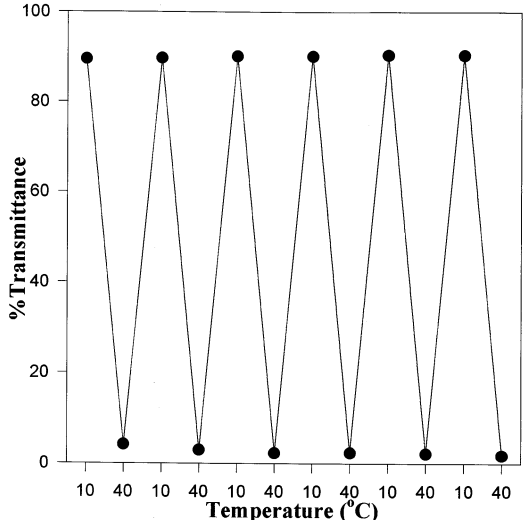

Fig. 4. Reversible changes of optical transmittance against temperature fluctuation.

Comparatively, the sizes of hydrated state were reversibly changed by temperature fluctuation as shown in Fig. 6. This result may be caused by the thermal sensitivity of the PNIPAAm outer shell of CN micelles. It is thought that, when the temperature is low, PNIPAAm chains of outer shell exist as an expanded form, and shrank one at high temperature. However, particle sizes were unexpectedly increased above the LCST. This indicates that intermicellar aggregates formed upon heating. And then, the aggregated micelles reversibly

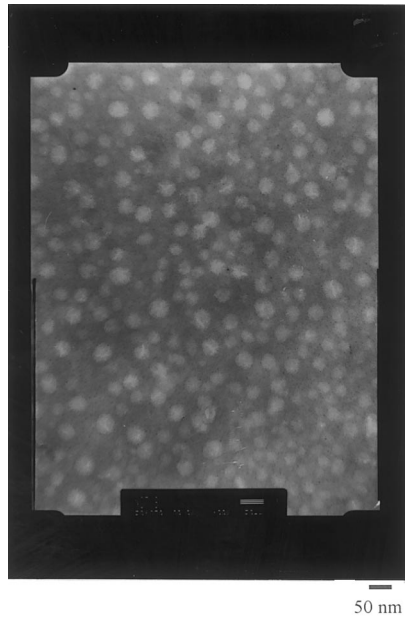

Fig. 5. Transmission electron micrograph of CN micelles dried at 20°C. Samples were negatively stained with 0.01% phosphotungstic acid.

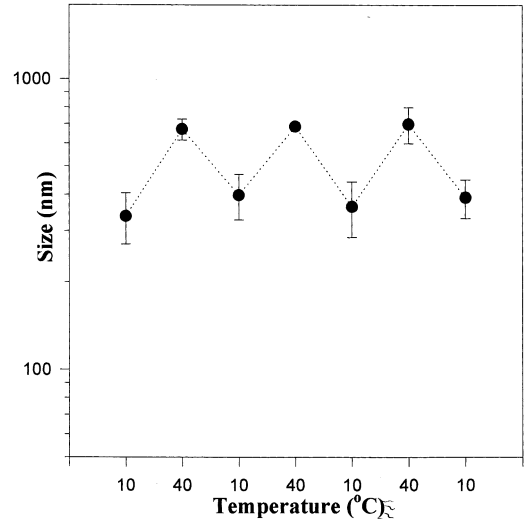

Fig. 6. Particle size changes by reversible temperature fluctuation.

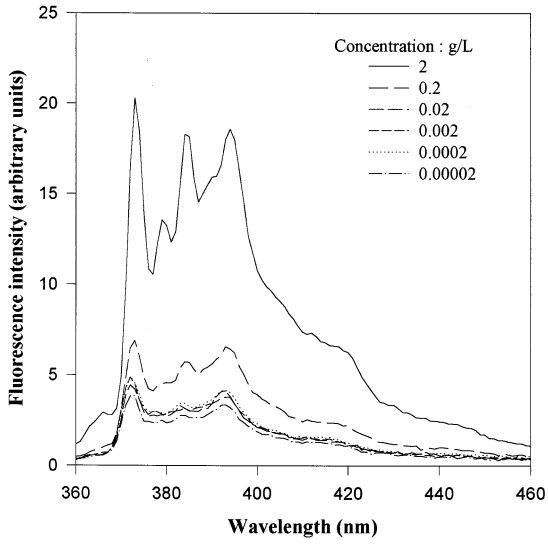

Fig. 7. Fluorescence spectra of pyrene (6.0×10^{-7} M) against CN concentration in distilled water (excitation wavelength: 339 nm).

redispersed to the initial micellar structure upon cooling below the LCST.

The behavior of self-assembled micelle formation was investigated by fluorescence spectroscopy study, and the CMC was determined. Wilhelm et al. already reported the micelle formation of polystyrene (PS)/poly(ethylene oxide) (PEO) di- or triblock copolymers in water by fluorescence technique using pyrene as a hydrophobic probe and determined CMC from the fluorescence emission and excitation spectra as pyrene partitions between aqueous and micellar environments (Wilhelm et al., 1991). This method was also used by Jeong et al. (1998) to confirm the polymeric micelle formation of poly(γ-benzyl L-glutamate) (PBLG) and PEO block copolymer in water. If the CN can be self-assembled in water, formation of micelles should be proved by the fluorescence probe technique with the same as block copolymer micelles. Also, the CMC can be estimated to prove the potential of self-assembly formation upon critical concentrations using pyrene as a hydrophobic probe.

Fig. 7 shows the fluorescence emission spectra of pyrene at a fixed excitation wavelength of 339 nm against various concentrations of CN. The fluorescence intensity was increased with increasing the concentration of CN, which indicated the formation of self-assembled polymeric micelles of CN in water such as block copolymeric micelles (Wilhelm et al., 1991; Marctic and Nair, 1994). It is thought that pyrene was preferentially solubilized into the micelles composed of core-shell structure when pyrene was introduced into aqueous phase from a good solvent (Dowling and Thomas, 1990; Zhao et al., 1990; Xu et al., 1991; Kwon et al., 1993).

Fig. 8 (a) shows the excitation spectra of pyrene in the various concentrations of CN. Red shift of pyrene in the excitation spectra was observed with increasing concentration of CN with similar tendency of PBLG/PEO block copolymers (Jeong et al., 1998) and PS/PEO block copolymers (Wilhelm et al., 1991). The intensity ratio of I_{338}/I_{334} versus log C of CN in the pyrene excitation spectra was plotted in Fig. 8 (b). This result represented that the ratio was almost flat at the low concentration, and rapidly increased at the high concentration. The CMC was taken from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentrations. The estimated CMC value was 8.9×10^{-2} g/l. The CN micelles were formed at a higher concentration in contrast with the CMC of block copolymer micelles. It can be explained that the micelle can be easily formed with an increase of hydrophobic components (Jeong et al., 1998). From the study of fluorescence probe measurements, it can be said that the CN can form core-shell type micelles in water upon critical concentrations (i.e. CMC) and has amphiphilic nature such as block copolymer micelles.

3.2. Drug release study

The calculated IN loading content was 27.7 wt.%. To study the drug release behavior, IN-loaded micelles of CN was simply redispersed in PBS (0.1 M, pH 7.4) without surfactant. Fig. 9 shows the release of IN from the micelles at 10 and 40°C. Higher contents of IN were released at 10 than 40°C after 8 h, as expected. After 2 h, IN was slowly released over time at 10°C, whereas it

became a plateau with time after 2 h at 40°C. This result may have close relationship with the thermal sensitivity of the CN micelles. It is thought that, when the temperature is increased above the LCST of PNIPAAm, conformational change of the outer shell occurred in the micelles. The expanded form of PNIPAAm in the shell part is changed into the compact one when the temperature is raised above the LCST of the polymer (31.5°C).

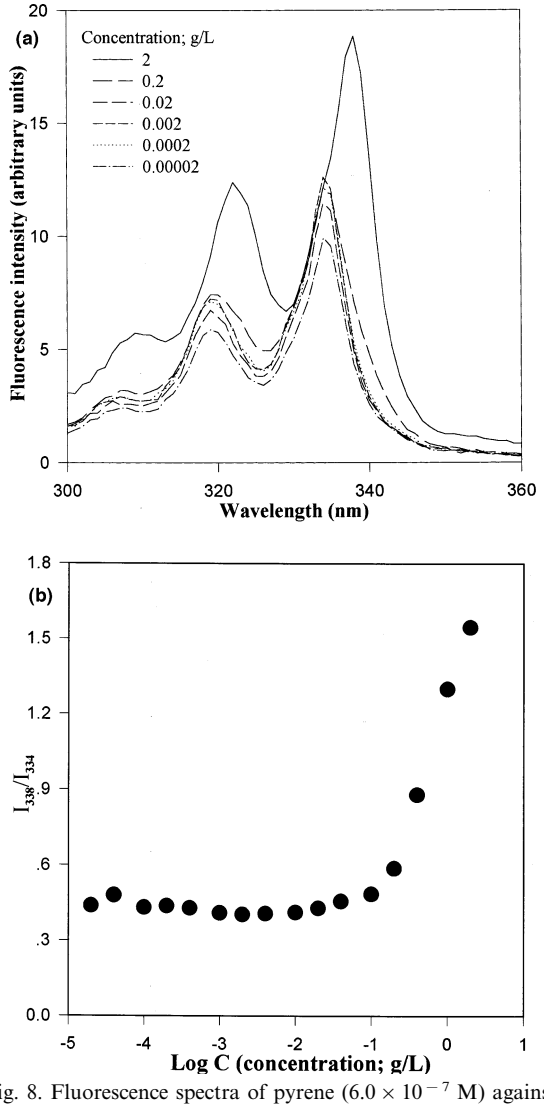

Fig. 8. Fluorescence spectra of pyrene (6.0×10^{-7} M) against CN concentration in distilled water (emission wavelength: 390 nm) (a) and plots of the intensity ratio I_{338}/I_{334} from pyrene excitation spectra vs. log C of the CN in distilled water (b).

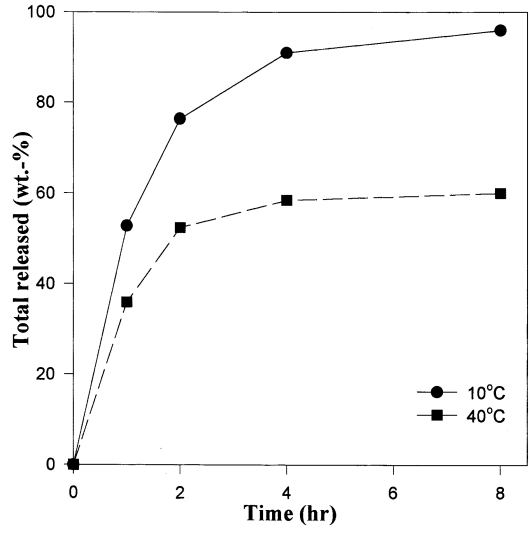

Fig. 9. Release of IN from micelles of CN in PBS (0.1 M, pH 7.4) at 10 and 40°C, respectively ($n = 3$).

In conclusion, it can be expected that drug release from the thermo-responsive CN micelles will be applicable to the site-specific drug delivery by controlling the temperature of the target site.

Acknowledgements

This study was supported by the 1999 research grant from Chosun University.

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