Macromolecular Research, Vol. 13, No. 1, pp 54-61 (2005)

Release of Calcein from Temperature-Sensitive Liposomes in a Poly(*N*-isopropylacrylamide) Hydrogel

Hee Dong Han, Tae Woo Kim, and Byung Cheol Shin

Advanced Materials Division, Korea Research Institute of Chemical Technology, P.O. Box 107, Yuseong, Daejeon 305-606, Korea

Ho Suk Choi*

Department of Chemical Engineering, Chungnam National University, 220 Gung, Yuseong, Daejeon 305-764, Korea

Received October 19, 2004; Revised January 5, 2005

Abstract: We prepared temperature-sensitive liposomes (TS-liposomes) modified with a thermosensitive polymer, such as poly(*N*-isopropylacrylamide) (PNIPAAm), to increase the degree of drug release from liposomes at the hyperthermic temperature. A PNIPAAm hydrogel containing TS-liposomes was also prepared to obtain a hydrogel complex at body temperature. In addition, a depot system for local drug delivery using the polymer hydrogel was developed to enhance therapeutic efficacy and prevent severe side effects in the whole body. The PNIPAAm-modified TS-liposome was fixed into the PNIPAAm hydrogel having a high temperature-sensitivity. The release behavior of calcein, a model drug, from TS-liposomes in the PNIPAAm hydrogel was then initiated by external hyperthermia; the results indicated that sustained release as a function of temperature and time was caused by the thermosensitivity of the liposome surface and diffusion of the drug into the PNIPAAm hydrogel. Our results indicated that TS-liposomes in a PNIPAAm hydrogel represented a plausible system for local drug delivery.

Keywords: poly(*N*-isopropylacrylamide), hydrogel, liposome, calcein, depot system.

Introduction

The chemotherapy for cancer treatment have been developed using functional carriers system such as liposome, nanoparticle, and emulsion. These carriers systems were importantly managed and regulated for drug release and delivery to purposed tumor site. When a cancer is treated systemically with cancer drugs, only a small fraction of the administered dose of drug reached the tumor. As a result, the therapy for cancer patients has to be limited due to its severe side effects. To solve these problems associated with the application of chemotherapy for tumors, a number of liposomes with various functionalities have been designed. TS-liposome have been developed in order to increase the therapeutic efficiency of anti-cancer drugs at tumor site and decrease the associated serious side effects.[1] There are literatures reporting that the use of TS-liposome in a mouse model substantially improved the delivery of anti-cancer drugs to solid tumors when combined with hyperthermia.[2] Hyperthermia treatment has been tried clinically to treat the cancer, in combination with drug delivery systems, because it can synergistically induce tumor cytotoxicity in combination with chemotherapy and radiotherapy.[3,4] Furthermore, hyperthermia treatment was found to enhance the permeability of the tumor vasculature compared to the normal vasculature, which was further able to enhance the delivery efficacy of anti-cancer drugs to tumors.[5]

Recently, a number of methods have been developed for the modification of the liposome surface with thermally responsive polymers to enhance the temperature-sensitivity for increasing the release of anti-cancer drug at external hyperthermic temperatures.[1,4,5] Additionally, the surface modification of liposome using polymer such as PNIPAAm was to enhance the fixation efficiency when PNIPAAm-connected liposome was entrapped in the polymer hydrogel by hydrophobic interaction above their lower critical solution temperature (LCST).

On the other hand, the hydrogel system using PNIPAAm have been extensively studied because of their biomedical and engineering applications as well as pharmaceutical field.[6-10] Many researchers reported a lot of hyderogel systems showing a various temperature and/or pH sensitivity through various modifications such as an interpenetrating

*e-mail: hchoi@cnu.ac.kr

1598-5032/02/54-08©2005 Polymer Society of Korea

polymer networks and copolymer (random, graft or block) gels, which could be applied to local drug delivery for cancer treatment in the field of depot system.[6,10]

In this report, we prepared the PNIPAAm hydrogel containing TS-liposomes to investigate the sustained-release of drug at a local injection site compared with traditional intravenous injection. The release behavior of calcein from TS-liposomes in PNIPAAm hydrogel was evaluated by measuring the fluorescence intensity and showed sustained-release as a function of temperature and time compared with the traditional TS-liposomes system without containing hydrogel. This study suggested that the PNIPAAm hydrogel containing TS-liposomes represent a depot system for local drug delivery especially for clinical cancer treatment.

Experimental

Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), L-α-phosphatidylcholine(soy-hydrogenated) (HSPC) and cholesterol (CHOL) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Calcein as a model drug was purchased from Sigma-Aldrich Co. (Louis, MO, USA). *N*-isopropylacrylamide (NIPAAm) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan), and recrystallized from a 60/40 (v/v) mixture of hexane/benzene. 2-Aminoethanethiol (AET) and 2, 2-azobisobutyronitrile (AIBN) were purchased from Aldrich Co. (Milwaukee, Wisconsin, USA). NHS-fluorescein (5(&6)-carboxyfluorescein *N*-succinimydyl ester) was purchased from Sigma-Aldrich Co. (Louis, MO, USA). Ammonium persulfate (APS) was purchased from Kanto Chemical Co. (Tokyo, Japan). *N,N,N',N'*-tetramethylethylenediamine (TEMED) was purchased Sigma-Aldrich Co. (Louis, Mo, USA). All other materials were of analytical grade and were used without further purification.

Synthesis of Amine-terminated PNIPAAm and PNIPAAm. The amine-terminated PNIPAAm was synthesized by free radical polymerization using a chain transfer agent (Figure 1(A)) to use as a surface modifier of liposome and to evaluate the surface fixation efficiency on the liposome surface of PNIPAAm because terminal amine group of PNIPAAm was well conjugated with the NHS-fluorescein. The Synthesis of the polymer was carried out as follows; the monomer of NIPAAm (100 mmol) was dissolved in 40 mL of methanol containing AIBN (1 mmol) as an initiator and AET (1 mmol) as a chain transfer agent. And then, dried nitrogen was bubbled through the monomer solution for 20 min. After polymerization at 60 °C for 20 h, the polymer was precipitated by pouring the reaction solution into diethyl ether. The precipitated polymer was redissolved in methanol. This procedure was repeated twice, and the polymer was then separated by size using solvent fractionation.[11]

The PNIPAAm was synthesized by aqueous redox polymerization to use as a matrix of hydrogel system. Synthesis

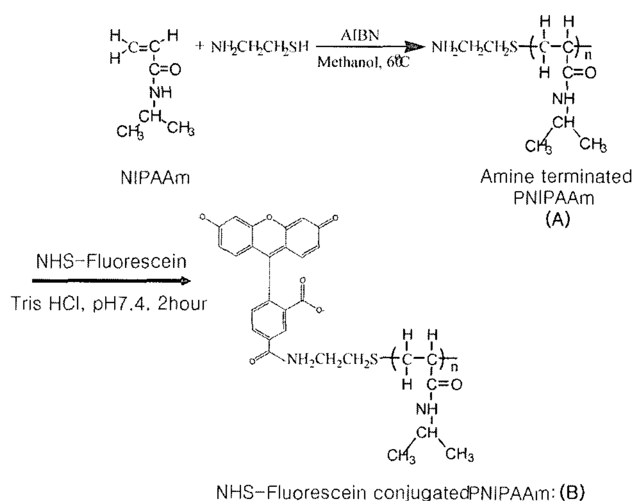

Figure 1. Synthesis of amine-terminated PNIPAAm (A) and NHS-fluorescein conjutated amine-terminated PNIPAAm (B).

of polymer with NIPAAm was carried out as follows; the NIPAAm of 170 mmol was dissolved in distilled water of 100 mL, to which 1.7 mmol APS and 0.12 mL TEMED were added at room temperature as an initiator and an accelerator, respectively. Dried nitrogen gas was bubbled through the monomer solution for 20 min. After polymerization at room temperature for 1 h, the polymer was obtained by the aggregation of PNIPAAm into distilled water with increasing temperature above their LCST. The aggregated polymer was collected, and then was vacuum-dried.

In both cases of amine-terminated PNIPAAm as a surface modifier and PNIPAAm as a hydrogel matrix, the average molecular weights (M.W.) of the polymers were determined by a gel permeation chromatography (GPC, KF 804 column, intelligent refractive index detector (RI930), intelligent HPLC pump (PU980), Jasco). The LCST of the polymers was determined using a differential scanning calorimetry (DSC, TA Instrument DSC2010).

Preparation of Liposome. The surface modified TS-liposome using PNIPAAm, traditional TS-liposome, and non-TS-liposome were prepared.[12-14] The surface modified TS-liposome was composed of the phospholipids (DPPC: HSPC:CHOL=100:50:30) of total 10 mM and amine terminated PNIPAAm of 10 mg/mL. The traditional TS-liposome was composed of the phospholipids (DPPC:HSPC: CHOL=100:50:30) of total 10 mM. The non-TS-liposome was composed of the phospholipids (DSPC:HSPC:CHOL =100:50:30) of total 10 mM. Among these liposomes were prepared as follow; each phospholipids and polymer, respectively, were dissolved in chloroform solution in a round bottom flask and then the chloroform solution was removed by an evaporator (Buchi Rotavapor R-200, Swizerland). Dried thin lipid film was dispersed in 1 mL of calcein (10 mmol) solution at pH 7.4. After hydration of the lipid film with

calcein solution, the freeze-thaw cycles for high drug encapsulation were repeated five times.[15,16] Freeze and thaw tem-peratures were -15 and 32 °C, respectively. Free calcein and free polymer were removed by gel permeation chromatography (GPC, Retriever 500, Isco, Inc. USA) using a Sephacryl-400 column at 4 °C in 10 mM Tris-HCl buffered solution. The separated liposomes were stored at 4 °C before experiment.

Measurement of Amount of the Amine-terminated PNIPAAm on the Surface of TS-liposomes. The amine-terminated PNIPAAm was used as a surface modifier of TS-liposomes to enhance the thermosensitivity of TS-liposome surface and the structure was shown in Figure 1(A). The amount of amine-terminated PNIPAAm on the surface of TS-liposomes was measured by fluorescence spectrophotometry (Barnstead, Apogent Tech, USA) using NHS-fluorescein, which is well fixed on the terminal amine group of amine-terminated PNIPAAm (Figure 1(B)). The emission and excitation wavelengths were 520 and 490 nm, respectively. Briefly, the prepared liposomal solution (2 mL) was stirred in the presence of 0.1 mL NHS-fluorescein (0.1 mg/mL DMSO) for 2 h at 4 °C. The mixed liposomal solution was dialyzed against distilled water for 4 8h at 4 °C using a cellulose dialysis membrane (MWCO 10,000; Slide-A-Lyzer dialysis cassette, USA) to remove free NHS-fluorescein. The total amount of amine-terminated PNIPAAm on the inner or outer surface of liposomes was measured by following the same procedure after disrupting the liposomes surface with 0.2 mL Triton X-100 (10% v/v).[17] The amount of polymer on the inner surface of the liposomes was calculated by subtracting the amount of polymer on the outer surface from the total amount of polymer. The amount of NHS-fluorescein labeled amine-terminated PNIPAAm was evaluated from the calibration curve of NHS-fluorescein.

Measurement of Water Content in PNIPAAm Hydrogel. Temperature-dependent water content of PNIPAAm hydrogel was measured to indicate the drug release pattern by diffusion mechanism. The PNIPAAm solution (1, 2 and 4% w/v) was deswellen in Tris-HCl buffered solution for 90 min at 37 °C. After the deswelling was reached for 20 min, the excess Tris-HCl buffered solution was rapidly removed from the deswelling medium, and the volume of excess Tris-HCl buffered solution from PNIPAAm hydrogel was calculated. The percentage deswelling ratio, R, which means water content in PNIPAAm hydrogel, is defined as;

$$R\,(\%) = (V_t - V_e) / V_t \times 100 \qquad (1)$$

where V_e and V_t are the water volume of excess Tris-HCl buffered solution from PNIPAAm hydrogel and the initial water volume of PNIPAAm solution, respectively.

Preparation of PNIPAAm Hydrogel Containing TS-liposomes. The preparation of PNIPAAm hydrogel containing surface modified TS-liposomes was carried out as follows; the PNIPAAm of 0.04, 0.08 and 0.16 g was dissolved in 2 mL Tris-HCl buffered solution and then the 2 mL of liposomal solution was added. The total mixed volume was 4 mL and final concentrations of the PNIPAAm were 1, 2 and 4% w/v, respectively. These solutions were heated above their LCST and resultantly we made the PNIPAAm hydrogel containing TS-liposomes.

Determination of Entrapped TS-liposomes in PNIPAAm Hydorgel. The determination of entrapped TS-liposomes in PNIPAAm hydrogel was carried out as follows; to confirm the entrapped TS-liposomes in PNIPAAm hydrogel, the fluorescence intensity of calcein was measured after separating the PNIPAAm hydrogel and the supernatant solution from the mixture. First, the fluorescence intensity of calcein in separated supernatant solution from the mixture was measured. Then, the fluorescence intensity of calcein in redissolved hydrogel in buffered solution was measured at room temperature. The percentage release was calculated by eq. (2).

$$\%\ \text{release} = F_t / F_f \times 100 \qquad (2)$$

where F_t is the intermediate fluorescence intensity of calcein in the liposome and F_f is the total fluorescence intensity of calcein in disrupted liposome structure after adding Triton X-100 (10% v/v).[17]

Release of Calcein from Surface Modified TS-liposomes in PNIPAAm Hydrogel. The release of calcein from TS-liposomes was evaluated by measuring the fluorescence intensity and the release mechanism was shown in Figure 2.[12,14,18,19] Briefly, the prepared liposomal solution (2 mL) was mixed in the 2 mL of PNIPAAm solution at a given temperature. The release of calcein from TS-liposomes was measured as a function of temperatures using fluorescence spectrophotometry. The emission and excitation wavelengths were 510 and 480 nm, respectively. The percentage release of calcein from TS-liposomes was calculated from eq. (2).

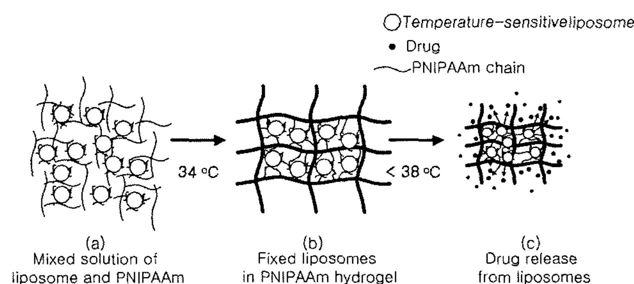

Figure 2. Schematic illustration of the calcein release from surface modified TS-liposomes in PNIPAAm hydrogel. (a) a mixed solution of PNIPAAm and surface modified TS-liposomes at 4 °C, (b) the PNIPAAm hydrogel containing surface modified TS-liposomes above their LCST, and (c) shows the release of calcein from surface modified TS-liposomes in PNIPAAm hydrogel at the transition temperature of liposome membrane.

Results and Discussion

Physical Properties of Amine-terminated PNIPAAm and PNIPAAm. The physical properties of amine-terminated PNIPAAm and PNIPAAm were summarized in Table I. The amine-terminated PNIPAAm has the LCST of 33 °C and the M.W. of ca. 21,500 Da. It was used as a modifier on the surface of TS-liposomes to increase the thermosensitivity of liposome surface. It has been well known that hydrophobic side groups of the amine-terminated PNIPAAm act as anchors, which was well fixed onto the liposome surface.[20,21]

The PNIPAAm as a hydrogel matrix has the LCST of 32 °C and the M.W. of ca. 17,400 Da. The sol phase of PNIPAAm in aqueous solution were changed to gel phase above body temperature. Notably, the LCST of PNIPAAm was lower than body temperature. Therefore, PNIPAAm can be used as a hydrogel matrix due to it formed a physically cross-linked hydrogel above their LCST. We used it as a model polymer to investigate the formation of hydrogel containing TS-liposome because they have a superior thermosensitivity which has been well characterized in aqueous solution, even though they are not good for clinical application due to their inherent toxicity.[3,4,12,14]

Amount of Amine-terminated PNIPAAm on the Surface of TS-liposomes. Amine-terminated PNIPAAm was used as a surface modifier of TS-liposome and was considered as follows; firstly, amine-terminated PNIPAAm induced to enhance the thermosensitivity of liposome, resultantly the drug release was increased at their transition temperature. Secondly, the PNIPAAm chain was well fixed with liposomes surface when form the hydrogel caused by their hydrophobic interaction. Finally, it was easy to detect amount of amine-terminated PNIPAAm on the surface of TS-liposome via the amine conjugated NHS-fluoresein intensity, which could be indicated the polymer fixation on the liposome surface, indicating that NHS-fluorescein was well conjugated because succinimidyl ester group was fixed with terminal amine group of amine-terminated PNIPAAm.[22] Therefore, NHS-fluorescein was used to measure the amount of polymer on the surface of TS-liposome. The amount of the fixed polymer on the surface of TS-liposome was determined by measuring the fluorescence intensity of NHS-fluorescein labeled polymer. The chemical structure of NHS-fluorescein labeled polymer was shown in Figure 1(B) and the amount of the fixed polymer on the inner or outer surface of liposome was summarized in Table II. The polymer of surface modified TS-liposomes contains 0.23 ± 0.01 mg/mg lipid and 0.23 ± 0.02 mg/mg lipid on the inner and outer surface, respectively. This result clearly shows that the PNIPAAm was fixed on the surface of TS-liposomes. Additionally, Polozova *et al.*[23] investigated the effect of binding to liposomal membranes by measuring the fluorescence intensity of liposome modified with pyrene labeled PNIPAAm. They found that both types of anchor-bearing polymer could associate with phospholipids liposomes,[12] which was similar to our observation. Thus, we could confirm that the amine-terminated PNIPAAm was successfully fixed on the surface of TS-liposomes via the measurement of amount of fixed PNIPAAm.

Table I. Physical Properties of the PNIPAAm and Amine-terminated PNIPAAm

	PNIPAAm	Amine-terminated PNIPAAm
LCST (°C)	32	33
M_n (Da)	5,500	7,560
M_w (Da)	17,400	21,500
Polydispersity (Dp)	3.03	2.84

Table II. Amount of the Amine-terminated PNIPAAm on Surface of TS-liposomes

	Polymer Added (mg/mg lipid)	Outer Surface[b] (mg/mg lipid)	Inner Surface[b] (mg/mg lipid)
Liposome[a]	1.33	0.23±0.01	0.23±0.02

[a]Temperature-sensitive liposome modified with PNIPAAm.
[b]Results are expressed as means±S.D. (n=3).

Water Content in PNIPAAm Hydrogel. The water content in PNIPAAm hydrogel was measured to estimate the release pattern of water from hydrogel, because decreasing of water content in hydrogel may be related with release of drug. Moreover, this result could be predicted how much time needs for formation of a physically cross-linked PNIPAAm hydrogel at designed temperature. The water content in PNIPAAm hydrogel was shown in Figure 3. The

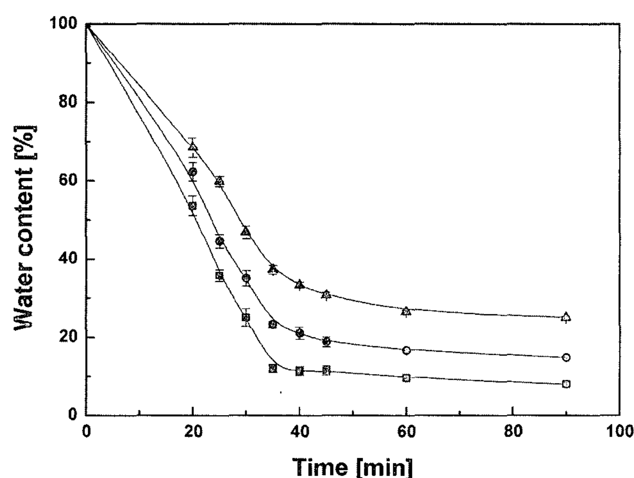

Figure 3. Water content in PNIPAAm hydrogel in 10 mM Tris-HCl buffered solution as a function of time at different concentrations: (■) 1, (●) 2 and (▲) 4% w/v. The constant temperature was 37 °C. Data is shown as mean ± S.D. (n=3).

water content in 1, 2 and 4% w/v PNIPAAm hydrogel was rapidly decreased within 35, 40 and 45 min at 37 °C, respectively. The shrinkage tendency as a function of time was similar PNIPAAm hydrogel systems having different polymer concentration. Deswelling rate of 4% w/v PNIPAAm hydrogel was much lower and slower than that of the 1 and 2% w/v PNIPAAm hydrogel because the rate of water diffusion was depended on the concentration of polymer. The 4% w/v PNIPAAm hydrogel loses 70% water in 45 min, but the 1 and 2% w/v PNIPAAm hydrogel lose 80 and 90% water in 35 min, respectively. Thus, as the concentration of polymer in hydrogel system increased higher, deswelling rate became more low and slow. PNIPAAm networks undergo hydrophobic interactions between alkyl side groups, which induced polymer network aggregation. This thermally induced dehydration of the PNIPAAm chains promotes rapid shrinking of the crosslinked network.[24] Thus, the rate of water diffusion from PNIPAAm hydrogel was decreased and was clearly depended on the concentration of polymer.

Determination of Entrapped Liposomes in PNIPAAm Hydrogel. In order to determine the entrapping efficiency of surface modified TS-liposomes in PNIPAAm hydrogel, the calcein intensity was measured. The fluorescence intensity of calcein indicates the loading of liposome in hydrogel. The fluorescence intensity of calcein for surface modified TS-liposomes was summarized in Table III. The each hydrogel containing TS-liposome samples shrinked and released aqueous solution from the hydrogel via the incubation of hydrogel for 20 min at 35 °C. In case of the traditional TS-liposomes, the fluorescence intensities of calcein of released solution and redissolved solution of shrinked hydrogel with buffered solution were 80.1 ± 2.0 and 20.0 ± 1.5%, respectively. This implies that the released solution also contained a lot of traditional TS-liposomes. However shrinked hydrogel little contained the liposomes. In case of surface modified TS-liposomes, the fluorescence intensities of calcein of released solution and redissolved solution of shrinked hydrogel were 24.3 ± 2.2 and 70.0 ± 1.5%, respectively. This implies that the released solution just contains a small amount of the TS-liposomes whereas shrinked hydrogel contained a lot of the TS-liposomes. These results imply the two points.

Table III. Calcein Intensity of Traditional TS-liposomes or PNIPAAm Modified TS-liposomes in Hydrogel. The Fluorescence Intensity of Calcein was Calculated by Eq. (2)

		Calcein Intensity[a] (%)
Traditional TS-liposomes	Supernatant	80.1±2.0
	PNIPAAm solution	20.0±1.5
Surface modified TS-liposomes	Supernatant	24.3±2.2
	PNIPAAm solution	70.0±1.5

[a]Results are expressed as means±S.D. (n=3).

First, the fluorescence intensity of calcein was clearly related with entrapping efficiency of TS-liposomes. Thus, we confirmed that PNIPAAm hydrogel contained a lot of surface modified TS-liposomes. Second, these results indicated that the surface modification of TS-liposomes was clearly related with physical bonding by hydrophobic interaction between liposome surface and PNIPAAm hydrogel. As a result, the surface modified TS-liposomes were successfully entrapped in PNIPAAm hydrogel due to the enhancement of hydrophobic interaction between the polymer chains during the gelation above their LCST. From these results, the traditional TS-liposomes were not used in the drug release test from hydrogel matrix since amount of TS-liposomes in hydrogel was too small to be measured. Moreover, this is not satisfied with dose concentration in vivo system.

Release of Calcein from TS-liposomes in PNIPAAm Hydrogel. The schematic illustration for the release behavior of calcein from the surface modified TS-liposomes in PNIPAAm hydrogel was shown in Figure 2. Briefly, the drug release was depended on the external hyperthermic themperature and the diffusion function of drug from PNIPAAm hydrogel matrix. The release system of drug from hydrogel matrix was represented (a) a mixed solution of PNIPAAm and surface modified TS-liposomes at 4 °C, (b) the PNIPAAm hydrogel containing surface modified TS-liposomes above their LCST and (c) the release of calcein from TS-liposomes in PNIPAAm hydrogel by external hyperthermia.

The release of calcein was measured to evaluate the effect of surface modification using amine-terminated PNIPAAm. In this experiment, we observed the release rate of calcein from surface modified TS-liposomes was higher than traditional TS-liposomes and the drug release was sharply and rapidly increased around 35-40 °C as shown in Figure 4. The drug release was dramatically increased occurring above LCST of PNIPAAm, where the PNIPAAm chain undergoes conformational transition from a hydrated coil to a dehydrated globule, resulting in disintegration of the TS-liposomes due to the bilayer-to-hexagonal transition of the TS-liposome membrane.[1] Therefore, the PNIPAAm modified TS-liposomes, complete release was achieved from the TS-liposomes in a short period of temperature and time. Thus, we indicated that the drug release from PNIPAAm modified TS-liposomes was governed by external hyperthermic temperature. On the other hand, the traditional TS-liposomes were not used in this study because of small amount of entrapped liposomes in PNIPAAm hydrogel (see above section).

The release of calcein from PNIPAAm modified TS-liposomes in PNIPAAm hydrogel was measured to investigate the possibility of usage for a sustained drug delivery system. The release profiles of calcein from TS-liposomes in PNIPAAm hydrogel at various concentrations were shown in Figure 5. The release of calcein from TS-liposomes as a control condition without PNIPAAm hydrogel drastically increased within 40 min at 37 °C and a very slow release

continued for a prolonged period after 40 min which is typical release pattern of surface modified TS-liposomes.[14] On the other hand, the release of calcein from surface modified TS-liposomes linearly increased and was 55, 50 and 42% in 1, 2 and 4% w/v PNIPAAm hydrogel until 90 min at 37 °C, respectively. Suisha *et al.*[25] reported that the release of drug from xyloglucan gels was diffusionally controlled and the system had the potential for the sustained drug release and

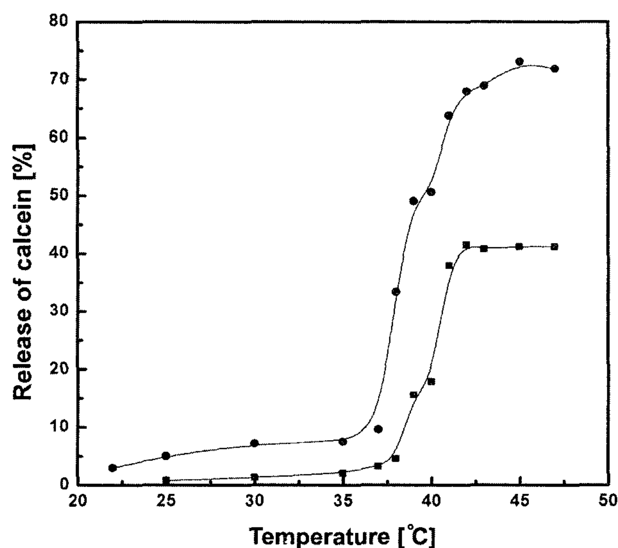

Figure 4. Release profile of calcein from TS-liposomes in Tris-HCl buffered solution as a function of temperature. The incubation time for individual samples was 5 min: traditional TS-liposomes (■) and PNIPAAm modified TS-liposomes (●). Data is shown as mean ± S.D. (n=3).

the release become slower as the concentration of polymer. Thus, this result shows the sustained release of calcein from surface modified TS-liposomes in PNIPAAm hydrogel compared with the surface modified TS-liposomes without PNIPAAm hydrogel and it was mainly controlled by concentration of polymer and thermally dehydrated transition of PNIPAAm hydrogel. Additionally, the release of drug in hydrogel was affected by thermally responsive of liposome membrane. Therefore, the release kinetics of drug could be depended and modulated by changing the polymer concentration and the transition temperature of liposome membrane. Notably, for the control TS-liposome without PNIPAAm hydrogel, the release of drug was rapidly increased. In this case, we indicated that the healthy cell was attacked by drug release from TS-liposomes via conventional intravenous injection when injected in body and occurring complete release by body temperature. However, for the PNIPAAm modified TS-liposome by local injection, the drug release from TS-liposome could be sustained-release and continuously supplied the dose to treat the solid tumor.

The temperature-dependent release of calcein from surface modified TS-liposomes in PNIPAAm hydrogel was shown in Figure 6. In the case of surface modified TS-liposomes as a control without PNIPAAm hydrogel, the release of calcein was rapidly increased and showed the highest release of calcein at 38 °C, meaning that the surface property for temperature of TS-liposomes depends on temperature. In addition, the surface modified TS-liposomes can enhance the release of calcein from TS-liposomes above LCST of polymer. Also, the PNIPAAm hydrogels contribute to the gradual increase of calcein release. In cases of 1, 2 and 4% w/v

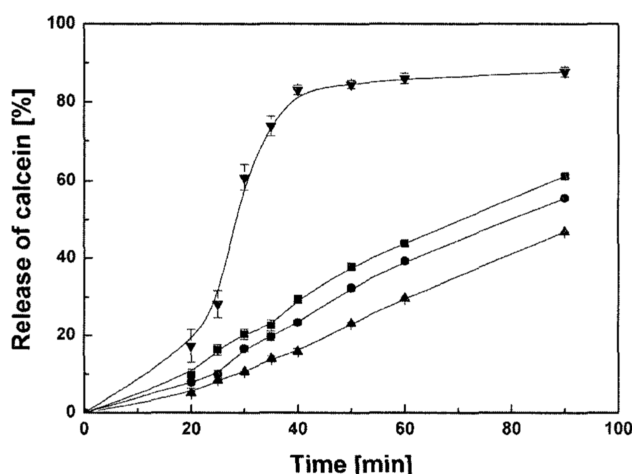

Figure 5. Release profile of calcein from surface modified TS-liposomes in PNIPAAm hydrogel as a function of time at various concentrations: (■) 1, (●) 2 and (▲) 4% w/v. Surface modified TS-liposomes without PNIPAAm as a control (▼). The constant temperature was 37 °C. Data is shown as mean ± S.D. (n=5).

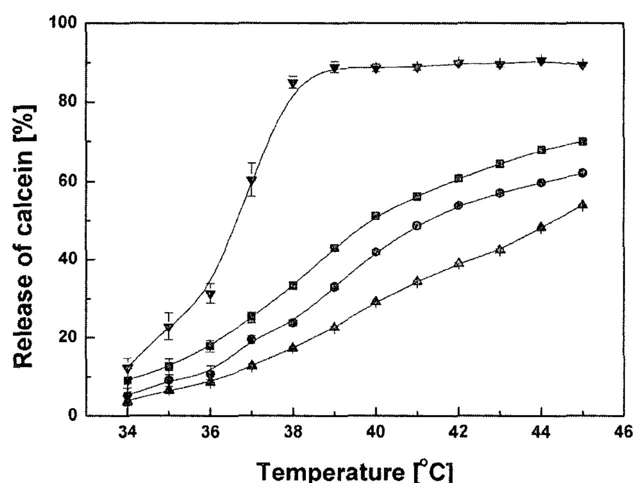

Figure 6. Release profile of calcein from surface modified TS-liposomes in PNIPAAm hydrogel as a function of temperature at various concentrations: (■) 1, (●) 2 and (▲) 4% w/v. Surface modified TS-liposomes without PNIPAAm as a control (▼). The incubation time for the individual samples was 5 min. Data is shown as mean ± S.D. (n=5).

PNIPAAm hydrogel containing TS-liposomes, the release of calcein from TS-liposomes in PNIPAAm hydrogel linearly increased. This implies that the PNIPAAm hydrogel prolongs the release of calcein from liposomes, because the PNIPAAm hydrogel prevent the excess release of drug from liposomes and triggers the leakage of the contents from the disintegration of the liposome membrane.[14] The hydrophobic gel containing hydrophilic drug significantly prolonged drug release when it compared with the liposomal solution and the control gel.[26] The hydrophobic groups of gel have a tendency to adsorb on the hydrophobic surface, while the hydrophilic groups of gel protrude out from the surface. It has been suggested that the hydrophobic groups of gel possibly penetrates into the lipid bilayer of liposomes, resulting the release of hydrophilic drugs into liposome was delayed and their retention and diffusion time were increased. Thus, 4% w/v PNIPAAm hydrogel containing surface modified TS-liposomes showed longer and smaller release of calcein than 1 and 2% w/v PNIPAAm hydrogel.

The release profile of calcein in 2% w/v PNIPAAm hydrogel as a function of temperature was shown in Figure 7. The release of calcein was rapidly increased from surface modified TS-liposomes without PNIPAAm hydrogel as a control condition. It also showed the highest release of calcein at 38 °C because the TS-liposomes membrane also depends on temperature. On the other hand, the release of calcein from non-TS-liposomes showed the transient leakage. It was obvious that the non-TS-liposomes were not temperature-dependent for the release of calcein. The release of calcein from PNIPAAm hydogel without TS-liposomes gradually increased at 38 °C and started to decrease for a prolonged period after 38 °C. In addition, the release of calcein from PNIPAAm modified TS-liposomes in PNIPAAm hydrogel showed sustained-release compared with control TS-liposomes without PNIPAAm hydrogel. This result showed that the release of calcein from TS-liposomes was mainly induced by the bilayer transition of the liposome membrane caused by thermally dehydrated transition of PNIPAAm.

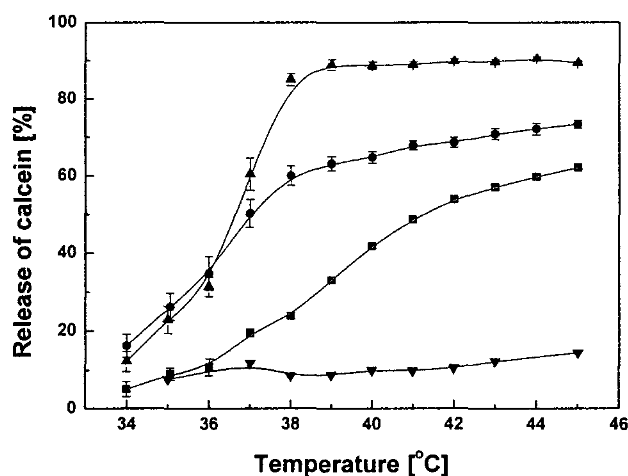

Figure 7. Temperature-dependent calcein release from PNIPAAm hydrogel as a function of temperature; PNIPAAm hydrogel containing calcein without surface modified TS-liposomes (●), PNIPAAm hydrogel containing surface modified TS-liposomes (■), surface modified TS-liposomes in Tris-HCl buffered solution without PNIPAAm hydrogel as a control (▲) and non-TS-liposomes (▼). The PNIPAAm concentration was 2% w/v. The incubation time for the individual samples was 5 min. Data is shown as mea n± S.D. (n=3).

Conclusions

Our experiments confirmed the sustained-release of calcein from surface modified TS-liposomes in PNIPAAm hydrogel. The thermosensitive polymer modified TS-liposomes enhance thermally dependent release of calcein from TS-liposomes due to thermally responsive transition of liposomes membrane and PNIPAAm chain. In addition, the PNIPAAm hydrogel sustained the leakage release of drug from TS-liposomes. Therefore, the surface modified TS-liposomes in PNIPAAm hydrogel contribute to control the sustained-release of drug for a promising site-specific administration. These results implied that the combination of surface modified TS-liposomes and PNIPAAm hydrogel had a benefit from several synergistic factors favorable to sustain drug release at local tumor site. This study provides useful information in designing local drug delivery system for specific site targeting, as well as sustained drug release or less side effect of tumor treatment.

Acknowldgements. This study was supported by a grant of the International Mobile Telecommunications 2000 R&D project, Ministry of Information & Communication, and the Strategic National R&D Program, Ministry of Science & Technology, Republic of Korea.

References

(1) K. Kono, *Adv. Drug. Deliv. Rev.*, **53**, 307 (2001).
(2) D. Needham and M. W. Dewhirst, *Adv. Drug. Deliv. Rev.*, **53**, 285 (2001).
(3) G. Kong, G. Anyarambhatla, W. P. Petros, R. D. Braun, O. M. Colvin, D. Needham, and M. W. Dewhirst, *Cancer Res.*, **60**, 6950 (2000).
(4) O. Ishida, K. Maruyama, H. Yanagie, M. Eriguchi, and M. Iwastru, *Jpn. J. Cancer Res.*, **91**, 118 (2000).
(5) D. E. Mayer, B. C. Shin, G. A. Kong, M. W. Dewhirst, and A. Chilkoti, *J. Control. Release,* **74**, 213 (2001).
(6) J. W. Lee, D. S. Lee, and S. W. Kim, *Macromol. Res.*, **11**, 189 (2003).
(7) S. B. Lee, S. M. Seo, Y. M. Lim, S. K. Cho, Y. M. Lee, and Y. C. Nho, *Macromol. Res.*, **12**, 269 (2004).
(8) K. H. Kim and Y. J. Shin, *Polymer(Korea),* **18**, 412 (1994).

(9) Y. S. Park, H. D. Han, S. U. Hong, S. S. Kim, and B. C. Shin, *Polymer(Korea)*, **28**, 59 (2004).
(10) B. C. Shin, S. S. Kim, J. K. Ko, J. Jegal, and B. M. Lee, *Eur. Polym. J.*, **39**, 579 (2003).
(11) L. H. Tung, *Fractionation of Synthetic Polymers*, Marcel Dekker, New York, 1977, p. 103.
(12) K. Kono, R. NAakai, K. Morimoto, and T. Takagishi, *Biochim. Biophys. Acta*, **1416**, 239 (1999).
(13) D. Needham, G. Anyarambhatla, G. Kong, and M. W. Dewhirst, *Cancer Res.*, **60**, 1197 (2000).
(14) H. Hayashi, K. Kono, and T. Takagishi, *Bioconjugate Chem.*, **10**, 412 (1999).
(15) L. D. Mayer, M. J. Hope, P. R. Cullis, and A. S. Janoff, *Biochim. Biophys. Acta*, **817**, 193 (1985).
(16) M. J. Hope, M. B. Bally, G. Webb, and P. R. Cullis, *Biochim. Biophys. Acta*, **812**, 55 (1985).
(17) O. Lopez, A. de la Maza, L. Coderch, C. Lopez-Iglesias, E. Wehrli, and J. L. Parra, *FEBS Lett.*, **426**, 314 (1998).
(18) H. Hayashi, K. Kono, and T. Takagishi, *Biochem. Biophys. Acta*, **1280**, 127 (1996).
(19) J. C. Leroux, E. Garrec, D. L. Hong, and D. C. Drummond, *J. Control. Release*, **72**, 71 (2001).
(20) H. Ringsdorf, J. Venzmer, and F. M. Winnik, *Chem. Int. Ed. Engl.*, **30**, 315 (1991).
(21) H. Ringsdorf, E. Sackmann, J. Simon, and F. M. Winnik, *Biochim. Biophys. Acta*, **1153**, 335 (1993).
(22) G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, San Diego, 1996, pp 305-307.
(23) A. Polozova, A. Yamazaki, J. L. Brash, and F. M. Winnik, *Colloid Surf. A Physicochem. Eng. Aspects*, **147**, 17 (1999).
(24) R. Yoshida, K. Uchida, Y. Kaneko, K. Sakai, A. Kikuchi, Y. Sakurai, and T. Okano, *Nature*, **374**, 240 (1995).
(25) F. Suisha, N. Kawasaki, S. Miyazaki, M. Shirakawa, K. Yamatoya, M. Sasaki, and D. Attwood, *Int. J. Pharm.*, **172**, 27 (1998).
(26) A. Paavola, I. Kilpelainen, J. Yliruusi, and P. Rosenberg, *Int. J. Pharm.*, **199**, 85 (2000).