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Research paper

# Doxorubicin-encapsulated thermosensitive liposomes modified with poly(*N*-isopropylacrylamide-*co*-acrylamide): Drug release behavior and stability in the presence of serum

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**Abstract**

In the field of the temperature sensitive drug delivery systems, we studied on the surface modification of liposomes by using poly(*N*-isopropylacrylamide-*co*-acrylamide) (PNIPAM-AAM) and polyethyleneglycol (PEG) to increase the release of doxorubicin (DOX) from liposomes and prolong the stability of liposomes in the presence of serum. The release of DOX from the PNIPAM-AAM/PEG modified liposomes is enhanced around the transition temperature of the polymer. In addition, the stability of the PNIPAM-AAM/PEG modified liposomes in serum shows a high level comparing with polymer unmodified liposomes. These results suggest that the modification on the surface of liposomes with both PNIPAM-AAM and PEG enhances the drug release from liposomes and reduces the protein adsorption in serum.


*Keywords:* Protein adsorption; Temperature-sensitive liposome; Poly(*N*-isopropylacrylamide); Surface modification

## 1. Introduction

Temperature-sensitive liposomes (TSL) were developed for the purpose of specific site targeting, in order to increase the antitumor efficacy of drugs and decrease the associated severe side effects [1]. In addition, liposomes having an enhanced permeation and retention effect for tumor vasculature related with hyperthermia treatment have been developed [2,3]. Hyperthermia treatment has been tried clinically to treat solid tumors, in combination with drug delivery systems, because it can synergistically induce tumor cytotoxicity in combination with chemotherapy and radiotherapy [3–5].

It is well known that liposomes made from naturally occurring lipids are biocompatible carriers, and their application to drug delivery systems reduces the drug toxicity and increases the therapeutic efficiency. Recently, a number of methods have been developed for the modification of the liposome surface with thermally responsive polymers, in order to increase drug release at hyperthermic temperatures. Kono et al. [6] and Hayashi et al. [7] designed liposomes modified with poly(*N*-isopropylacrylamide) (PNIPAM). It has been shown that the interaction of PNIPAM with liposomes imparts a temperature sensitive property to the liposomes.

In addition, Sadazuka et al. [8] and Woodle [9] reported the stability of PEG modified liposomes for protein adsorption in serum. PEG modified liposomes circulated for a long time in the bloodstream and drug accumulation in tumors enhanced. The PEG chains on the surface of the liposomes diminished the adsorption of opsonizing proteins with the increase of water-binding ability of the PEG chains [10].

Thus, we designed PNIPAM-AAM/PEG modified TSL as highly temperature-sensitive liposomes. In this study, we synthesized a copolymer of *N*-isopropylamide (NIPAM) and acrylamide (AAM) (Fig. 1A), as PNIPAM having hydrophobic isopropyl group. It can be fixed on liposome

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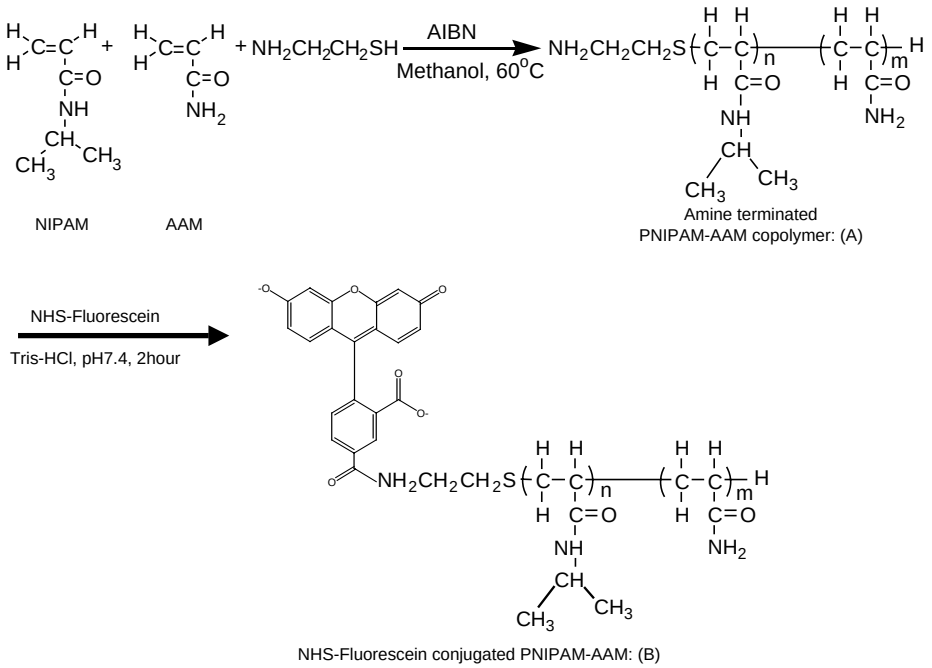


Fig. 1. Synthesis of (A) amine terminated PNIPAM-AAM and (B) NHS-fluorescein conjugated PNIPAM-AAM.

membranes through hydrophobic interactions between the hydrophobic groups and lipid membranes. Copolymer-modified TSL with varying compositions were prepared and the effect of modification on the surface of TSL for temperature-sensitive property of the liposomes has been studied.

## 2. Materials and methods

### 2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), L-a-phosphatidylcholine(soy-hydrogenated) (HSPC), cholesterol (CHOL) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG-2000) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Doxorubicin (DOX) as an anticancer drug was purchased from Sigma-Aldrich Co. (Louis, MO, USA). *N*-isopropylacrylamide (NIPAM) was purchased from Tokyo Kasei Kogyo (Japan), and recrystallized from a 60/40 (v/v) mixture of hexane/benzene. Acrylamide (AAM), 2-aminoethanethiol (AET) and 2, 2′-azobisbutyronitrile (AIBN) were purchased from Polysciences (Warrington, PA) and Aldrich Co. (Milwaukee, Wisconsin, USA), respectively. NHS-Fluorescein (5(&6)-carboxyfluorescein *N*-succinimydyl ester), albumin fluorescein isothiocyanate conjugate bovine (BSA-FITC) and aqueous fetal bovine serum were purchased from Sigma-Aldrich Co. (Louis, MO, USA). All other materials were of analytical grade and were used without further purification.

### 2.2. Synthesis of PNIPAM-AAM

The polymers, PNIPAM-AAM and PEG, were used as a liposome surface modifier to increase the drug release and to enhance the stability in serum. In the case of PEG, phospholipid of chemically synthesized DSPE-PEG-2000 was used as received from Avanti Polar Lipid, Inc. to enhance the stability of liposomes in serum without further purification.

An amine-terminated copolymer of NIPAM and AAM was prepared by previous report [5], which synthesized by free radical polymerization using a chain transfer agent as shown in Fig. 1A. The copolymers were synthesised with NIPAM:AAM ratios of 100:0, 90:10, 85:15, 83:17, 80:20 and 75:25 mol/mol (total 100 mmol). The monomers of NIPAAM:AAM, AIBN (1 mmol) as an initiator and AET (1 mmol) as a chain transfer agent were dissolved in methanol and the solution was heated at 60 °C for 20 h in a nitrogen atmosphere. The copolymer was recovered by precipitation with diethyl ether. The copolymer was redissolved in methanol, reprecipitated with diethyl ether and then dried under vacuum oven. The average molecular weight (MW) of the copolymers was determined by gel permeation chromatography (GPC, KF 804 column, RI930, PU980, Jasco). The lower critical solution temperature (LCST) of the PNIPAM-AAM was determined by differential scanning calorimetry (DSC, TA Instrument DSC2010).

### 2.3. Preparation of TSL

Table 1 shows the composition and formulation of various TSLs. The TSLs were prepared as follows [6,11];

Table 1
Composition and physical properties of various TSLs

| TS-liposomes | Composition | Molar ratio[a] (total 10 mmol) | Liposome size (nm) | Amount of polymer (mg/mg lipid) | | |
|---|---|---|---|---|---|---|
| | | | | Inner surface | Outer surface | Total |
| TSL1 | DPPC:HSPC:CHOL | 100:50:30 | 139.2±5 | – | – | – |
| TSL2 | DPPC:HSPC:CHOL:DSPE-PEG-2000 | 100:50:30:6 | 145.3±3 | – | – | – |
| TSL3 | DPPC:HSPC:CHOL:PNIPAM | 100:50:30:P[b] | 130.0±2 | 0.29±0.01 | 0.30±0.02 | 0.59±0.01 |
| TSL4 | DPPC:HSPC:CHOL:PNIPA-AAM17 | 100:50:30:P[b] | 140.3±2 | 0.25±0.01 | 0.20±0.02 | 0.45±0.01 |
| TSL5 | DPPC:HSPC:CHOL:PNIPAM-AAM25 | 100:50:30:P[b] | 128.3±3 | 0.30±0.01 | 0.31±0.02 | 0.61±0.02 |
| TSL6 | DPPC:HSPC:CHOL:DSPE-PEG-2000: PNIPAM-AAM17 | 100:50:30:6 P[b] | 125.3±4 | 0.23±0.01 | 0.23±0.02 | 0.46±0.02 |

[a] Mole concentrations of DPPC, HSPC, CHOL and DSPE-PEG-2000 are 5.376, 2.688, 1.613 and 0.323 mmol/ml, respectively.
[b] P is a PNIPAM-AAM and its initial concentration is 10 mg/ml.

the phospholipids (DPPC, HSPC, CHOL and DSPE-PEG-2000: total 10 mmol/ml) (Table 1) and the PNIPAM-AAM (10 mg/ml) were dissolved in chloroform solution and then the chloroform solution was removed by evaporator (Buchi Rotavapor R-200, Swizerland). The dried thin lipid/polymer film was dispersed in 1 ml of an aqueous DOX solution (1.73 mmol/ml, pH 7.4). After hydrating the lipid/polymer film with DOX solution, the freeze–thaw cycles were repeated five times to increase the drug loading. Freeze and thaw temperatures were −15 and 35 °C, respectively [12,13]. The TSL suspension was extruded through a polycarbonate membrane (pore size; 100 nm) using an extruder (Northern Lipids, Inc. USA) in an ice-cooled water bath. The free DOX and free polymer were removed by gel permeation chromatography (GPC, Retriever 500, Isco, Inc. USA) on a Sephacryl-400 column at 4 °C using 10 mmol Tris–HCl buffered solution. The separated TSL was stored at 4 °C before the experiment.

### 2.4. Amount of polymer bound on the surface of TSL

The amount of PNIPAM-AAM on the surface of the TSL was measured by fluorescence spectrophotometry (Barnstead, Apogent Tech, USA) after labeling NHS-Fluorescein on the terminal amine group of the polymer as shown in Fig. 1B. The emission and excitation wavelengths were 520 and 490 nm, respectively. Briefly, the TSL 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. After reaction of the mixed solution, the solution was dialyzed against distilled water for 48 h at 4 °C using a cellulose dialysis membrane (MWCO 10,000; Slide-A-Lyzer dialysis cassette, USA) to remove the free NHS-Fluorescein. The total amount of PNIPAM-AAM on the inner/outer surface of the TSL was measured by following the same procedure after disrupting the TSL structure with 0.2 ml Triton X-100 (10%, v/v) [14]. The amount of PNIPAM-AAM on the inner surface of the TSL was calculated by subtraction the amount of polymer on the outer surface from the total amount of polymer. The amount of NHS-Fluorescein labeled polymer was determined from the calibration curve of NHS-Fluorescein.

### 2.5. Release of DOX from TSL

The release of DOX from TSL was measured according to the previous report [6,7,15,16]. Briefly, freshly prepared TSL (1 ml) was added to 1 ml of serum (50%, v/v) at pH 7.4 in a quartz cell at a given temperature. The release of DOX from TSL was measured by fluorescence spectrophotometry. The emission and excitation wavelengths were 595 and 487 nm, respectively. The percentage of DOX release from TSL was defined as follows

$$\%\ \text{release} = (F_t - F_i)/(F_f - F_i) \times 100$$

where $F_i$ and $F_t$ mean the initial and intermediary fluorescence intensities of the TSL suspension, respectively. $F_f$ is the total fluorescence intensity of the TSL after the addition of Triton X-100 (10%, v/v).

### 2.6. Stability assay of TSL in the presence of serum

The adsorption of serum protein on the TSL in serum was estimated by measuring the change of the TSL size and turbidity in TSL suspension. The size of TSL was determined by light scattering with a particle size analyzer (Particle Analyzer, Otuska, Japan). The prepared TSL solution (1 ml) was added to 1 ml of serum (50%, v/v) and the samples were incubated at 37 °C with mild stirring before use.

To estimate the stability of liposomes, the turbidity change of TSL suspension in the serum was evaluated by the previous report [17]. A freshly prepared TSL solution (1 ml) was added to 1 ml of serum (50%, v/v) at pH 7.4 and the mixed solution was incubated at 37 °C with mild stirring. The turbidity change of the mixed solution was measured using a UV–visible spectrophotometer (UV mini 1240, Shimazu, Inc., Japan) at 450 nm.

### 2.7. Adsorption assay of serum protein on the TSL

The amount of the serum protein adsorption on the TSL surface was determined by fluorescence intensity of BSA-FITC using fluorescence spectrophotometry. The emission

and excitation wavelengths were 510 and 490 nm, respectively. Briefly, 1 ml of TSL solution and 0.5 ml of BSA-FITC solution (0.02 mg/ml) were mixed. It was incubated at 37 °C with mild stirring at given sampling time (1, 18, 24, 30, and 48 h) and then the samples were centrifuged at 18, 000 × *g* for 40 min and this step repeated three times to wash up the free BSA-FITC using distilled water. Finally, to indicate the amount of adsorbed protein on the surface of TSL, fluorescence intensity of TSL bounded BSA-FITC was measured.

## 3. Results and discussion

### 3.1. LCST of PNIPAM-AAM

The PNIPAM-AAM was used as a surface modifier of TSL to increase the thermosensitivity of liposome surface. Table 2 shows the changes in the LCST as a function of the concentration of AAM in the PNIPAM-AAM. The LCSTs of the polymers were determined by DSC. The LCST of the polymer increased with the increase of AAM content. The incorporation of AAM into the polymer chain increases the hydrophilicity of the polymer chain, resulting in the increase of LCST. Also, many studies have shown that PNIPAM can be fixed on liposome membranes through the hydrophobic interactions between the hydrophobic groups and the lipid membranes [6,7,15]. In this study, we chose the PNIPAM-AAM17 having 40 °C of LCST and MW of ca. 21,500 to increase the drug release at hyperthermic temperature.

### 3.2. Amount of PNIPAM-AAM on the surface of TSL

The amount of PNIPAM-AAM on the surface of TSL was determined with a fluorescence spectrophotometry after labeling NHS-Fluorescein on the terminal amine group of the polymer (Fig. 1B) [8]. The amount of fixed PNIPAM-AAM on the surface of TSL6 was 0.23 ± 0.01 mg/mg lipid (inner surface) and 0.23 ± 0.02 mg/mg lipid (outer surface), respectively (Table 1). From this result, we clearly predicted that the PNIPAM-AAM17 was well fixed on the surface of TSL. In addition, the size of TSLs was measured using light scattering with a particle size analyzer. As summarized in Table 1, the range of the TSLs size was approximately 120–140 nm. Moreover, aggregation or fusion was not observed for a period of 1 month in our prepared TSL in Tris-HCl buffered solution, indicating the TSLs had a similar physicochemical and pharmaceutical quality.

Table 2
Changes in the LCST as a function of the concentration of AAM

| Polymer | Molar ratio (NIPAM: AAM = mol:mol, total 100 mmol) | LCST (°C) | Molecular weight (MW) |
|---|---|---|---|
| PNIPAM | 100:0 | 33 ± 0.5 | 24,300 |
| PNIPAM/AAM10 | 90:10 | 35 ± 0.5 | 21,700 |
| PNIPAM/AAM15 | 85:15 | 37 ± 1.0 | 11,500 |
| PNIPAM/AAM17 | 83:17 | 40 ± 0.5 | 21,500 |
| PNIPAM/AAM20 | 80:20 | 43 ± 0.5 | 25,800 |
| PNIPAM/AAM25 | 75:25 | 47 ± 0.5 | 19,000 |

### 3.3. Release of DOX from TSL in serum

The release of DOX from TSL was measured to evaluate the effect of surface modification with thermosensitive polymer in serum. In order to clarify the effect of surface modification on the temperature dependent release from the PNIPAM-AAM modified TSLs, the release behavior of the PNIPAM-AAM modified TSLs as a function of different LCST was presented as shown in Fig. 2. The release of DOX from TSL6 was enhanced with incorporated PNIPAM-AAM, and especially at 39 °C where the percent release increased greatly with modification of PNIPAM-AAM17/PEG. In result, release of DOX from TSL6 was very slow below the transition temperature of the polymer. In contrast, the DOX release was enhanced around the transition temperature, indicating that incorporation of PNIPAM-AAM17/PEG into TSL6 membranes enhanced release greatly around the transition temperature of the polymer. It is indicate that temperature sensitivity of the liposome is considered to be given by alteration of the polymer chain from the hydrophilic state to the hydrophobic state in response to the ambient temperature, it reveal the response at a desired temperature are obtained by the modification with polymers having LCST at that temperature [15]. These results indicate that increase in hydrophobicity of the polymer with raising temperature induces stronger interaction of the polymer with lipid membranes, resulting in enhancement of DOX release from liposomes because the hydrophobicity of fixed polymer chains on the surface of

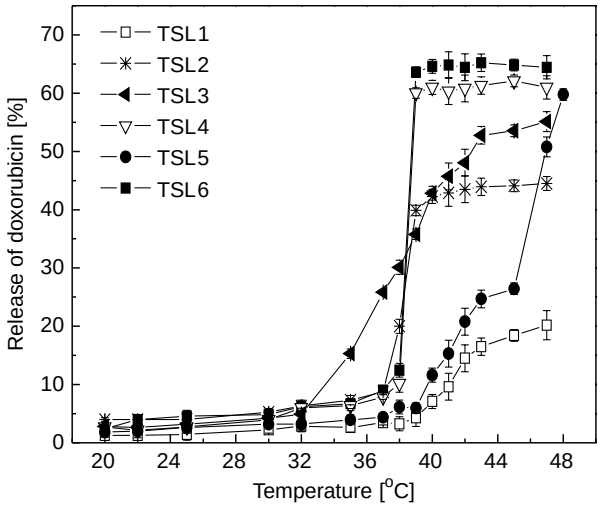


Fig. 2. Temperature-dependent DOX release from TSLs with different LCSTs of polymer. Data is shown as mean ± SD ($n = 3$).

liposome becoming increase with rising temperature around their LCST. Therefore, the liposome membrane was destabilized and the drug release from liposomes was enhanced. Consequently, the LCST of polymer clearly affect the drug release from liposomes at their transition temperature. These results are thought that the fixation of the polymer onto the inner/outer surface of TSL6 enhances the DOX release at the LCST of the polymer, as well as the leakage of DOX by thermally responsive liposomes during the thermal transition of PNIPAM-AAM. On the other hand, several studies have shown that the modification of liposomes with PEG prolongs their circulation in blood-stream due to the decrease of interaction between the liposomes and the serum protein [18–20]. Yamazaki et al. [21] reported on the interaction of poly(*N*-alkyl acrylamide)-modified liposomes with plasma proteins. They found that the polymer unmodified liposomes were fouled by plasminogen protein, whereas the polymer modified liposomes were not. As a result, the fixation of polymers such as PEG and PNIPAM-AAM on the surface of liposomes might help to protect the liposomes from serum proteins and enhance the release of DOX from the liposomes. Thus, a combination of two polymers on the TSL may act synergistically to increase the release of DOX and lower the adsorption of serum protein.

The time-dependent release of DOX from TSL with different temperatures was measured to evaluate the effect of surface modification with thermosensitive polymer in serum (50%, v/v) as shown in Fig. 3. For the TSL1, conventional TSL, the release was suppressed below 39 °C because of their inherent transition temperature to DPPC. While the release of DOX from the TSL1 was hardly enhanced at 43 °C, which is near the transition temperature of the lipid such as DPPC, acceleration of the release was observed above 43 °C (Fig. 3A). In the contrast, for the TSL6 (Fig. 3B), which was modified with PNIPAM-AAM17/PEG having 40 °C of LCST, the release of DOX was limited below 34 °C. However, a significant release (ca. 62% release) was induced at 39 °C, at which only very slow release was observed for the conventional TSL1 (Fig. 3A). Almost complete release was achieved above 39 °C. Apparently, the surface modified with PNIPAM-AAM17/PEG improved the temperature-sensitivity of the polymer-modified TSL6 at and above the transition temperature of the polymer. In result, we showed that release of DOX from conventional TSL modified with a PNIPAM-AAM17/PEG was facilitated above the transition temperature. Therefore, in this study, release of DOX from the PNIPAM-AAM17/PEG modified TSL6 was enhanced, presumably due to the PNIPAM chains were fixed on the surface of the TSL in this case. As a result, the release of DOX from polymer modified TSL is mainly induced by the thermally dehydrated transition of PNIPAM-AAM17/PEG and by the consequent reduction in its capacity to adsorb the serum protein of the PEG chain.

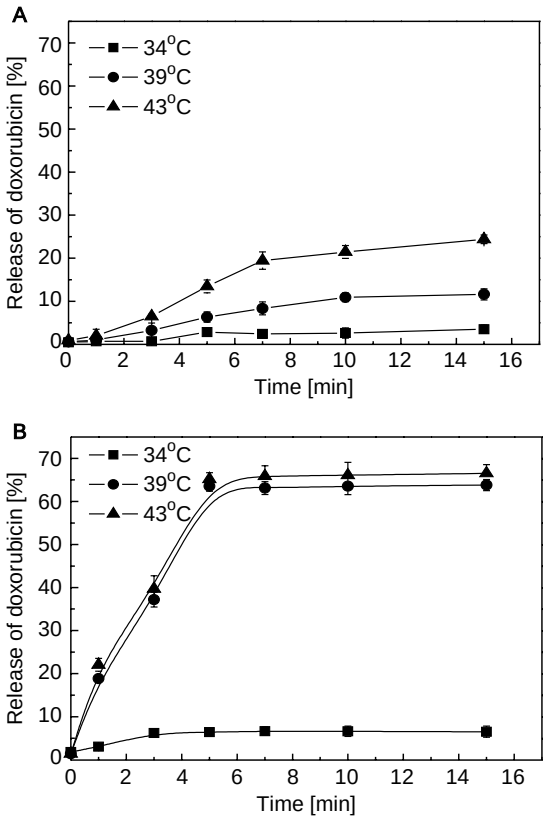


Fig. 3. Release profiles of DOX from TSLs modified with PNIPAM-AAM and/or PEG in serum at various temperatures; (A) TSL1 and (B) TSL6. Data is shown as mean ± SD ($n = 5$).

### 3.4. Stability of TSL in the presence of serum

The changes in TSL size in the presence of serum were clearly related to the adsorption of the serum protein on the liposomes [17]. The change in size of the liposomes with time at 37 °C in serum was shown in Fig. 4A. Average particle size of TSL1, polymer unmodified TSL, increased greatly from 154.5 to 500 nm after incubation for 48 h in serum at 37 °C. In the comparison of the particle size between PEG fixed TSL2 and PNIPAM-AAM17 fixed TSL3, the particle size of TSL2 was found to be smaller than that of TSL3 during incubation at 37 °C. For both the PNIPAM-AAM17/PEG fixed TSL6, no change in particle size was observed for 48 h. From this result, we can confirm that TSL1 shows high protein adsorption, but TSL2, TSL3 and TSL4 retain their initial particle sizes during incubation in serum. Also, the protein adsorption of PEG fixed TSL2 was lower than that of PNIPAM-AAM17 fixed TSL3. It is well known that conjugating substrates with PEG prevents interactions with proteins in the bloodstream and suppresses the recognition by the cells, due to its high flexibility and hydrophilicity in serum [22]. It is generally considered that the lower protein adsorption on the polymer fixed TSL6 is mainly caused by the repulsive interactions between the PEG chains and the protein, and that this adsorption is

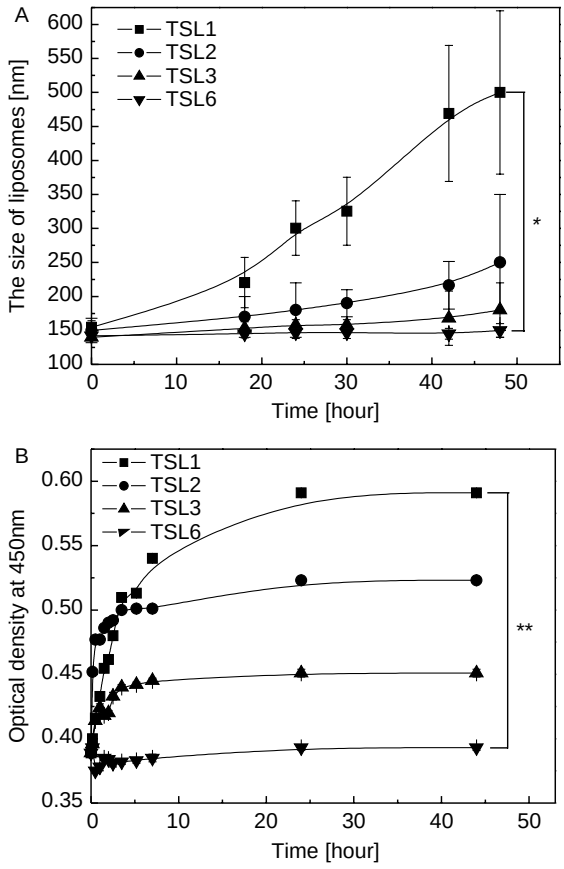


Fig. 4. (A) Particle size of the liposomes modified with PNIPAM-AAM and/or PEG after incubation at 37 °C in serum (*$P<0.007$, Student's $t$-test). (B) Turbidity changes of PNIPAM-AAM and/or PEG modified liposomes suspension in serum (**$P<0.001$, Student's $t$-test). Data is shown as mean ± SD ($n=3$).

additionally suppressed by PNIPAM-AAM with its hydrated and flexible chains.

Similarly, Fig. 4B shows the turbidity change of liposomal suspension in serum. The protein adsorption on the surface of TSL in serum was determined by measuring the turbidity using an UV-spectrophotometer at 450 nm [23]. The optical density of the polymer unmodified TSL1 rapidly increased, when compared with the polymer modified TSL2, TSL3 and TSL6. The optical density of TSL2 and TSL3 gradually increased over a period of 5 h at 37 °C. In the comparison of the PEG fixed TSL2 with the PNIPAM-AAM17 fixed TSL3, the optical density of TSL3 was higher than that of TSL2. Also, the optical density of the both PNIPAM-AAM17/PEG fixed TSL6 suspension at first slowly increased for 5 °h and then showed a constant density for 48 °h. We can infer from this that the PEG reduces the turbidity in serum containing TSL, because PEG chains prevent the protein adsorption between the serum protein and the TSL surface. This result shows that protein adsorption on the TSL surface is suppressed by the surface modification caused by PNIPAM-AAM and PEG.

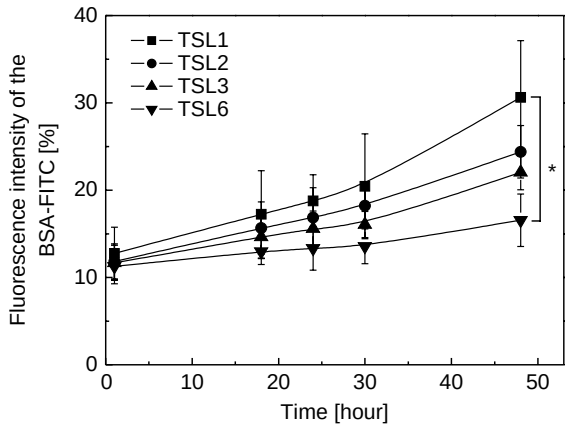


Fig. 5. Fluorescence intensity of the BSA-FITC adsorbed liposome suspension in Tris–HCl buffered solution (*$P<0.007$, Student's $t$-test). Data is shown as mean ± SD ($n=3$).

### 3.5. Fluorescence measurement of BSA-FITC for serum protein adsorption

Fig. 5 shows that the protein adsorption on the TSLs surface was determined by measuring the fluorescence intensity of BSA-FITC labeled TSL in 10 mmol Tris–HCl buffered solution at 37 °C. The fluorescence intensity of TSL1 linearly increased from 13 to 30% during incubation for 48 h. This means that serum proteins are adsorbed on the TSL1, because the increase in the fluorescence intensity of BSA-FITC is clearly due to the adsorption of serum protein on the TSL. In the comparison of the BSA-FITC fluorescence intensity between the PEG modified TSL2 and the PNIPAM-AAM17 modified TSL3, the fluorescence intensity of TSL3 was higher than that of TSL2. This implies that the PEG chains prevent the adsorption of serum proteins on the TSL surface. The fluorescence intensity of the PNIPAM-AAM17/PEG modified TSL6 shows only a small increase for 48 h. This implies that the combination of PNIPAM-AAM and PEG chains effectively prevent the adsorption of serum protein on the TSL surface.

## 4. Conclusions

Our experiments investigated the highest release of DOX at a hyperthermic temperature and the enhancement of the stability of the TSL in the presence of serum when the TSL was modified with PNIPAM-AAM and PEG. The PNIPAM-AAM modification on the surface of the TSL contributed to the release of DOX, because of the coil-globule transition of the thermosensitive polymer at their LCST and the bilayer transition of the liposome membranes. Also, the PEG modification on the surface of the TSL enhanced the stability of the latter, because the flexible PEG chains prevented the protein adsorption in the presence of serum. Thus, the combination of PNIPAM-AAM and PEG on TSL acted synergistically to increase the release of DOX and

prevent protein adsorption. Our results also provided valuable information which can be useful in designing TSL for use as temperature sensitive drug delivery systems, as well as in rendering the surface of the liposomes biocompatible, in order to increase the release of DOX and to prolong their stability in the presence of serum.

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