ELSEVIER

Journal of Controlled Release 62 (1999) 115–127

journal of controlled release

www.elsevier.com/locate/jconrel

Thermo-responsive drug delivery from polymeric micelles constructed using block copolymers of poly(*N*-isopropylacrylamide) and poly(butylmethacrylate)

J.E. Chung, M. Yokoyama, M. Yamato, T. Aoyagi, Y. Sakurai, T. Okano*

Institute of Biomedical Engineering, Tokyo Women's Medical University, Kawada-cho 8-1, Shinjuku-ku, Tokyo 162-8666, Japan

Abstract

To achieve a combination of spatial specificity in a passive manner with a stimuli-responsive targeting mechanism, a temperature-responsive polymeric micelle is prepared using block copolymers of (poly(*N*-isopropylacrylamide-*b*-butylmethacrylate) (PIPAAm-PBMA)). The micelle inner core formed by self-aggregates of PBMA segments successfully loaded with a drug (adriamycin), and the outer shell of PIPAAm chains played a role of stabilization and initiation of micellar thermo-response. Optimum conditions were investigated for the micelle formation and drug loading into the inner cores in a view of micellar stability and function as drug carriers. Outer shell hydrophilicity that prevents inner core interaction with biocomponents and other micelles can be suddenly switched to hydrophobic at a specific site by local temperature increase beyond the LCST (lower critical solution temperature) (32.5°C). These micelles showed reversible structural changes allowing drug release upon heating/cooling thermal fluctuations through the LCST. Polymeric micelles incorporated with adriamycin showed a dramatic thermo-responsive on/off switching behavior for both drug release and *in vitro* cytotoxicity according to the temperature responsive structural changes of a micellar shell structure. The reversible and sensitive thermo-response of the micelle opens up opportunities to construct a novel drug delivery system in conjunction with localized hyperthermia.

Keywords: Poly(*N*-isopropylacrylamide); Poly(butylmethacrylate); Polymeric micelle; Thermo-response; Drug delivery

1. Introduction

Poly(*N*-isopropylacrylamide) (PIPAAm) in aqueous solution is well-known to exhibit a thermo-responsive phase transition at 32°C [1]. This transition temperature is called a lower critical solution temperature (LCST). This polymer, water-soluble and hydrophilic, shows an extended chain conformation below its LCST, undergoing a phase transition to an insoluble and hydrophobic aggregate above the LCST. This phase transition remarkably occurs with narrow temperature changes through the LCST and is reversible corresponding to temperature changes. We have utilized the thermo-responsive properties of PIPAAm and its gels as on–off switches for drug release [2], attachment/detachment of cells and so on.

Hydrophobic chains of collapsed PIPAAm above the LCST actively interact with biocomponents such as cells and proteins, or other hydrophobic components, while below the LCST hydrated flexible

*Corresponding author. Tel.: +81-3-3353-8111, ext. 30232; fax: +81-3-3359-6046.

0168-3659/99/$ – see front matter
PII: S0168-3659(99)00029-2

chains do not interact with them. We have already reported thermo-responsive surfaces grafted with PIPAAm chains for novel hydrophobic liquid chromatography matrices modulating separation and solute–surface partitioning by temperature control [3–5]. We have also reported that polystyrene surfaces grafted with PIPAAm chains creating hydrophilic/hydrophobic switchable surfaces built a novel cell recovery system to control cell attachment and detachment without any cell damage by thermal modulation [6–8]. Cells attached and grew, maintaining their active metabolism on the hydrophobic PIPAAm surface above the LCST. As cooling below the LCST, the cells spontaneously detached from the hydrated PIPAAm surface not only as a result from decreased interaction between the cells and the PIPAAm surface but also by active cell morphological changes. Utilization of the PIPAAm thermo-response is able to construct systems to modulate interaction with cells including cellular morphology and cellar functions.

A-B type block copolymer of PIPAAm and a hydrophobic segment shows thermo-responsive water solubility and can form heterogeneous microstructures, that is, micellar structures composed of hydrophilic microdomains of soluble PIPAAm segments together with hydrophobic aggregated microdomains of incorporated hydrophobic segments in aqueous solution below the LCST. The hydrophobic inner core of the micelle contains drugs, while the PIPAAm outer shell plays a role of stabilization and temperature-response. The outer shell hydrophilicity that prevents inner core interaction with biocomponents and other micelles can be suddenly switched to hydrophobicity at a specific site by temperature increasing through the LCST.

We have already shown that a polymeric micelle constructed by block copolymers of poly(ethylene oxide) (PEG) and poly(L-asparate) containing an anticancer drug (adriamycin (ADR)) was selectively accumulated at a solid tumor by a passive targeting mechanism due to the hydrophilicity of PEG chains and its size ($>$100 nm) [9,10]. Polymeric micelle size ranges able, to be tailored at the polymer synthesis steps, can inhibit non-selective scavenging by the reticuloendothelial system (RES) and can utilize the enhanced permeability and retention (EPR) effects at tumor tissues [11,12].

From this perspective, thermo-responsive micelles are able not only to utilize spatial specificity in a passive manner due to their sizes but also to increase the spatial specificity in combination with a physical targeting mechanism which is achieved by the introduction of a thermo-responsive polymer segment such as PIPAAm. The thermo-response is expected to accomplish multiple functions for a double targeting system in both passive and stimuli-responsive manners enhancing vascular transport and drug release, and/or embolization induced by local temperature change. Selective accumulation of micelles at a malignant tissue site could be increased as a result of micelle adsorption enhancement to cells mediated by hydrophobic interactions between polymeric micelles and cells. Simultaneously, this strategy can achieve temporal drug delivery control: drug expresses its activity only for a time period defined by local heating and cooling.

In order to design and facilitate a reversibly temperature-responsive micelle for a drug delivery system, we have researched the formation mechanism, the structural stability and the temperature-response relating to intra- or intermolecular hydrophilic/hydrophobic interactions and molecular architectures [13–15]. We now report temperature-responsive micelle formation using block copolymers of PIPAAm and poly(butylmethacrylate) (PBMA) (PIPAAm-PBMA), and *in vitro* drug release and cytotoxicity of such micelles containing ADR modulated by temperature control.

2. Experimental

2.1. Materials

N-Isopropylacrylamide (IPAAm, kindly provided by Kohjin, Tokyo, Japan) was purified by recrystallization in hexane and dried *in vacuo* at room temperature. 2-Mercaptoethanol (ME), styrene (St), *N*,*N*′-dimethylformamide (DMF) and tetrahydrofuran (THF) were obtained from Wako Pure Chemicals (Tokyo, Japan) and purified by standard methods. Butylmethacrylate (Tokyo Kasei) and 3-mercaptopropionic acid (MPA, Aldrich, Milwaukee, WI, USA) were distilled under reduced pressure. *N*-Ethylacetamide was purchased from Tokyo Kasei.

Benzoylperoxide (BPO) and triethylamine (TEA) were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Pyrene and 1,3-bis(1-pyrenyl)propane were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

2.2. Polymerization of semitelechelic poly(N-isopropylacrylamide) (PIPAAm) and poly(butylmethacrylate) (PBMA)

Hydroxy-terminated PIPAAm (PIPAAm-OH) was prepared by radical polymerization using ME as a chain transfer agent [2,16,17]. In order to obtain PIPAAm-OH, IPAAm (3 M), ME and BPO (5×10^{3} M) were dissolved in THF (100 ml). Each solution was repeatedly degassed under reduced pressure in freeze–thaw cycles and sealed in an ampule. Polymerization was carried out at 70°C and stopped by freezing after 7 h. After evaporation of most of the THF, the polymer was precipitated three times in an excess of diethyl ether and dried *in vacuo*. The dried polymer was dissolved in cold water and dialyzed against water using a dialysis membrane Spectra/Por® CE, MWCO=500 at 4°C for 3 days. The product was obtained as a white powder by lyophilization. Molecular weight of PIPAAm-OH were determined by gel permeation chromatography (GPC, TOSOH, SC-8020, polystyrene standards) in DMF containing LiCl (10 mM) (elution rate: 1 ml/min) at 40°C.

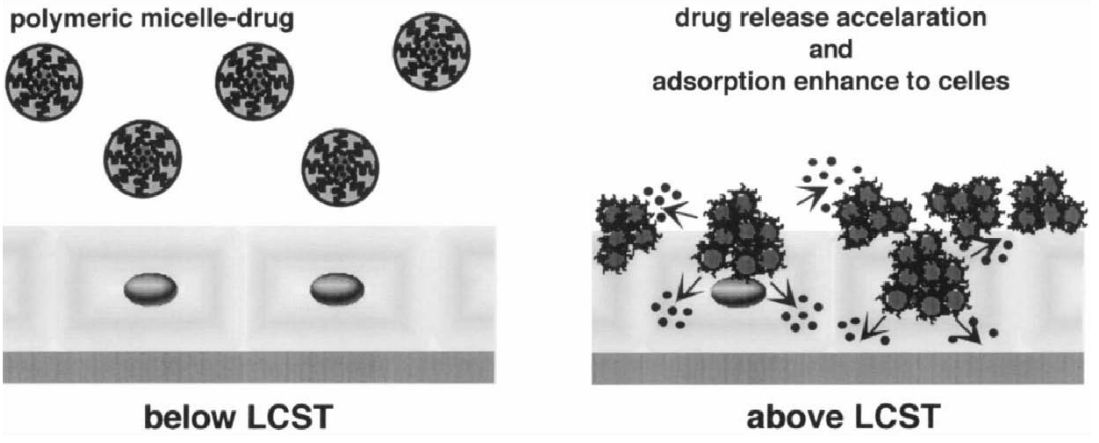
Carboxyl-terminated PBMA (PBMA-COOH) was prepared by radical polymerization using MPA as a chain transfer agent and purified. The terminal carboxy group concentration was determined by nonaqueous potentiometric titration using 0.01 N CH_3NaO dissolved in a mixture of methanol and dioxane.

2.3. Preparation of block copolymer of PIPAAm and PBMA (PIPAAm-PBMA) (Scheme 1)

BMA-COOH (5.7×10^{-5} mol) was reacted with $SOC1_2$ (2.7×10^{-3} mol) at 60°C for 24 h. The lyophilized product, triethylamine (2.9×10^{-4} mol) and pyridine (2.9×10^{-4} mol) were dissolved in THF (5 ml). The solution was added dropwise to a large excess amount of PIPAAm-OH solution (PIPAAm-OH=1.1×10^{-4} mol/THF 5 ml) at room temperature under an N_2 atmosphere. After 24 h reaction at room temperature, the product was precipitated twice in a large excess of diethyl ether and then precipitated again in warm water (30°C) in order to obtain pure block copolymers of PIPAAm-PBMA.

2.4. Micelle formation

A solution of PIPAAm-PBMA block copolymer (15 mg) in *N*-ethylacetamide (3 ml) was put into a dialysis bag (MWCO=12 000–14 000) and dialyzed against water at 20°C for 24 h.



Scheme 1. Interactions between PIPAAm-PBMA micelles and cells modulated by temperature control.

2.5. Optical transmittance measurements

Optical transmittance of aqueous polymer solutions (5000 mg/l) at various temperatures was measured at 542 nm with a UV spectrometer (Ubest-30, Japan Spectroscopic Co., Ltd., Tokyo, Japan). Sample and reference cells were thermostated with a circular water jacket from 10 to 40°C. LCSTs of polymer solutions were determined as the temperature bringing about a 50% decrease in optical transmittance.

2.6. Fluorescence measurements

Fluorescence spectra were recorded using a spectrofluorometer (FP-770, Japan Spectroscopic Co., Ltd., Tokyo, Japan). The temperature of a water-jacketed cell holder was controlled with a thermostated circulating bath. Pyrene and 1,3-bis(1-pyrenyl) propane (PC_3P) were used as hydrophobic fluorescent probes. Pyrene solution in acetone (4.8×10^{-4} M, 5 μl) or PC_3P solution in acetone (1.3×10^{-4} M, 5 μl) was added to aqueous polymer solutions (20 000 mg/l, 4 and 3 ml, respectively). These samples containing pyrene (*ca.* 6×10^{-7} M) or PC_3P (*ca.* 2.2×10^{-7} M) were kept for 24 h at 20°C before measurements. Excitation was carried out at 333 nm (PC_3P) and 340 nm (pyrene). Emission spectra were recorded ranging from 350 to 600 nm. Excitation and emission band widths were 10 and 3 nm, respectively. From pyrene emission spectra, the intensity (peak height) ratios (I_1/I_3) of the first band (374 nm) to the third band (385 nm) were analyzed as a function of concentrations of the polymer solution and temperature (heating or cooling rate=1°C/min). Values for cmc were determined from the mid-points of the plots for I_1/I_3 changes. The PC_3P excimer emission to monomer emission ratios (I_E/I_M) were calculated from excimer intensity (I_E) at 474 nm and monomer intensity (I_M) at 378 nm.

2.7. Incorporation of adriamycin (ADR)

PIPAAm-PBMA block copolymer (19 mg) and ADR hydrochloride (19 mg) were dissolved separately in 1.5 ml of *N*-ethylacetamide. The ADR solution was added to the block copolymer solution after triethylamine (6.0 μl) was added dropwise. The solution was dialyzed against water at 20°C for 48 h. The obtained red solution was ultrafiltered three times. The UV absorbance of loaded and unloaded ADR was measured at 500 and 485 nm, respectively.

2.8. In vitro drug release

ADR release from micelles was measured at various temperatures through the micelle LCST using UV absorbance at 485 nm in a time-course.

2.9. In vitro cytotoxicity measurement of micelles containing ADR

In vitro cytotoxic activity of free ADR or the micelles loaded with ADR was measured using bovine aorta endothelial cells. Bovine aortic endothelial cells were obtained as previously reported using dispase for cell dissociation from freshly harvested bovine aorta. The primary cultures were plated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL fungizone, and incubated at 37°C in a humidified atmosphere with 5% CO_2. Fungizone was discontinued on the seventh day of culture. Cells were routinely split at a ratio 1:4 and carried in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were plated at a density of 3×10^4 cells/well. The cells were exposed with free ADR or micelles loaded with ADR at below and above the LCST for 5 days. In order to assay cytotoxicity of free ADR or micelles loaded with ADR, culture medium was replaced with 10% FBS-supplemented phenol red-free DMEM containing 10% alamar Blue, a dye which is subjected to reduction by cytochrome c activity and changes the color from blue to red. After 4-hour incubation, reduction of the dye was estimated by absorbance at 560 and 600 nm.

3. Results and discussion

3.1. Synthesis of PIPAAm-PBMA

Hydroxyl-semitelechelic PIPAAm and carboxylic-

semitelechelic PBMA were synthesized by telomerization using ME and MPA as telogens, respectively. Telomerization was used to regulate quantitative incorporation of functional groups to one end of each PIPAAm chain [2,16,17]. The molecular weight of PIPAAm-OH determined from GPC data was 6100. The molecular weight of PBMA-COOH determined by the end-group assay was 8900. End groups of PBMA-COOH were changed to acid chloride groups by reaction with $SOCl_2$. Block copolymers of PIPAAm-PBMA were obtained by reaction of the hydroxyl groups of PIPAAm with activated terminal groups of PBMA. The yield was *ca.* 13%. Achievement of block coupling was confirmed by molecular weight enhancement determined from GPC data (Fig. 1). In addition, the obtained block copolymers showed different solubility from both homopolymers of PIPAAm and PBMA. Water at 30°C and diethyl ether are good solvents for PIPAAm and PBMA, respectively. However, the block copolymer was insoluble in both of these solvents.

3.2. Micelle formation

In general, incorporation of hydrophilic or hydrophobic groups into PIPAAm chains changes the LCST [18–22]. PIPAAms with hydrophilic groups raised the LCST to higher temperatures than that of the corresponding pure PIPAAm and slowed down phase transition phenomena by stabilizing polymer dissolution by strengthening interactions between polymer chains and water, resulting in an increase in enthalpy of hydrogen bonding between the polymer and water [18,19]. Particularly, the hydrophilic contribution to the LCST transition is distinctly great for terminal-located hydrophilic groups [15]. By contrast, hydrophobic groups also altered the hydrophilic/hydrophobic balance in PIPAAm and promoted a PIPAAm phase transition at lower solution temperatures than the LCST of the corresponding pure PIPAAm: a polymer reaching a critical concentration of these moieties is no longer water-soluble [20–22]. In the same way that the hydrophilic contribution alters the PIPAAm LCST, the hydrophobic contribution also depends on the locations of the hydrophobic group. A terminal hydrophobic group had a very significant impact on LCST reduction.

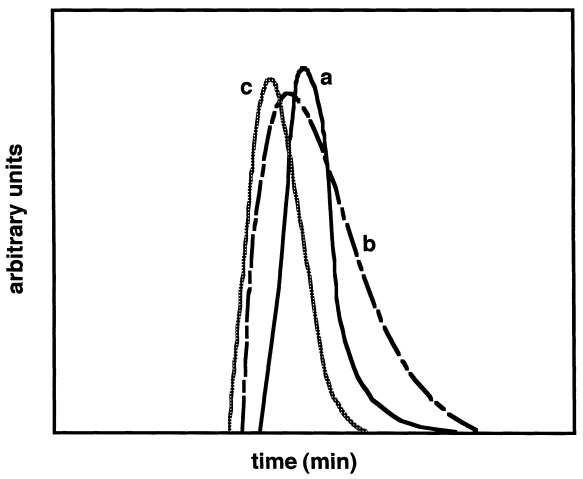


Fig. 1. GPC data of PIPAAm-OH (a), PBMA-COOH (b) and block copolymer of PIPAAm and PBMA (PIPAAm-PBMA) (c), DMF containing LiCl (10 mM), elution rate; 1 ml/min at 40°C.

However, supramolecular structures comprising hydrophobically-modified PIPAAm at terminal positions showed the same LCST and same phase transition kinetics as freely mobile, linear PIPAAm chains [13,15]. When terminally hydrophobic groups self-aggregate by hydrophobic interactions, water clusters immobilized around the hydrophobic segments are excluded from the hydrophobically aggregated inner core, and the isolated hydrophobic micellar core does not directly interfere with PIPAAm chain dynamics in aqueous media. The PIPAAm chains of the micellar outer shell remain as mobile linear chains in this core-shell micellar structure. As a result, the thermo-responsive properties of PIPAAm in the outer PIPAAm chains of this structure are unaltered [13,15,23]. We have shown previously that strongly hydrophobic terminally-modified PIPAAm formed clearly phase-separated micellar structures and preserved the stable structures during thermo-responsive structural changes when responding to thermal stimuli [13–15].

The obtained PIPAAm-OH macromonomer showed higher LCST of 34.5°C than pure PIPAAm LCST (32.5°C). We have previously reported that the hydrophilic contributions were dramatically enhanced by incorporation of hydroxyl groups onto the PIPAAm chain terminal as their increasing mole

fractions due to stronger hydrogen bonding with water [15]. However, micelles formed by block copolymers showed the same LCST as that of pure PIPAAm irrespective of hydrophobic PBMA segments corporation (Fig. 2). It proved two confirmations: one is that hydroxyl groups of the PIPAAm ends were completely reacted with PBMA end groups, and the other one is that the block copolymers formed core-shell micellar structures with completely isolated hydrophobic inner cores from an aqueous phase [13,15].

The hydrophobic microenvironment of PIPAAm-PBMA micelle aqueous solutions was characterized by fluorescence spectroscopy using pyrene and PC_3P as fluorescence probes. The fluorescence spectrum of pyrene at the low concentration possesses a vibrational band structure which exhibits a strong sensitivity to polarity of the pyrene environment [24]. The ratio (I_1/I_3) of intensity of the first band (I_1) to that of the third band (I_3) was monitored as a function of each PIPAAm-PBMA micelle concentration [25] (Fig. 3). As concentrations of PIPAAm-PBMA increased, a large decrease in I_1/I_3 was observed. This indicates partitioning of the hydrophobic probe into a hydrophobic environment. From these plots, it is possible to estimate a concentration corresponding to the onset of hydrophobic aggregation of PBMA segments. This value determined from the mid-points of the plots for I_1/I_3 changes was a low concentration of 20 mg/l, providing evidence for an apparent stability of the micelles and allowed their use in very dilute aqueous milieu such as body fluids.

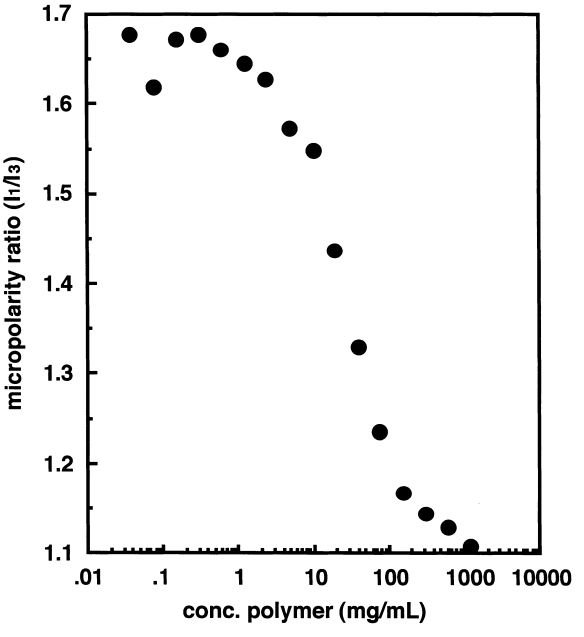


Fig. 3. Plot of the ratio of intensities (I_1/I_3) of the pyrene fluorescence spectrum as a function of polymer concentration of PIPAAm-PBMA solutions. λ_{ex}=340 nm, [pyrene]=1.6×10^{-7} M at 20°C.

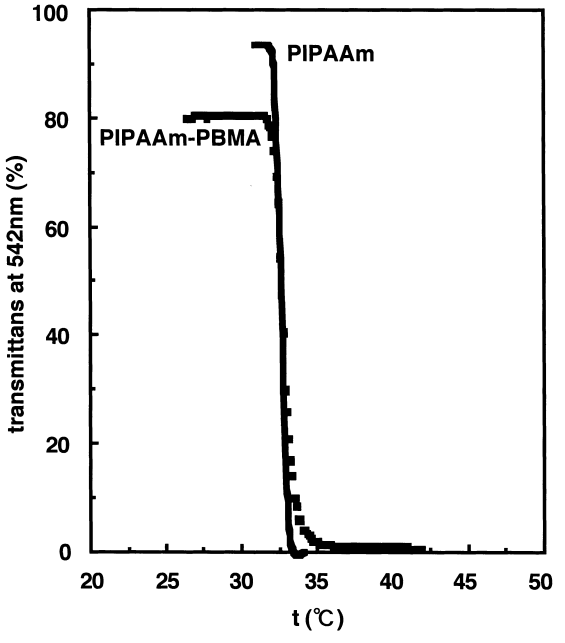


Fig. 2. LCST profiles for PIPAAm and PIPAAm-PBMA determined by transmittance at 542 nm, [polymer]=5000 mg/l.

3.3. Thermo-responsive structural changes of PIPAAm-PBMA micelles

Fig. 4 shows micropolarity changes sensed by pyrene molecules in solutions of PIPAAm-PBMA as a function of temperature. The solution showed increased polarity of the pyrene environment with increasing temperature through the LCST, while the solution showed a constant micropolarity with less polar below the LCST. Pure PIPAAm solutions showed an abrupt decrease in polarity when the temperature was raised through its LCST, indicating

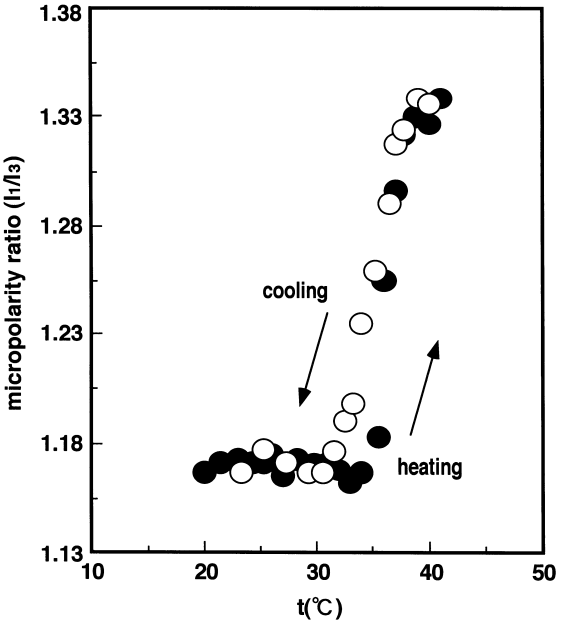


Fig. 4. Plot of the ratio of intensities (I_1/I_3) of the vibrational bands in the pyrene fluorescence spectrum as a function of temperature for PIPAAm-PBMA, λ_{ex}=340 nm, [pyrene]=1.6×10^{-7} M, 1°C/min, [polymer]=5000 mg/l.

the transfer of pyrene into the precipitated polymer-rich phase [13]. On the other hand, a micelle solution showed lower polarity than that of pure PIPAAm solution for the entire temperature region due to a presence of hydrophobic PBMA cores of the micelles. Aggregation of collapsed PIPAAm outer shells could have induced micelle structural deformation which changed in the pyrene microenvironment into more polar, resulting in the increased pyrene polarity above the LCST. The structural deformation which allowed the change in pyrene partitioning recovered back to the initial micelle structure upon increasing rehydration of the PIPAAm chains below the LCST when cooling the turbid solution it was back to transparency below the LCST. A small hysteresis around the LCST was observed to be caused by the delayed hydration of the PIPAAm chains upon cooling [13].

PC_3P is a sensitive probe for local viscosity measurements [26,27] by forming an intramolecular excimer. The extent of excimer emission depends upon the rate of conformational change of the chain linking the two pyrenyl groups, leading to a stable 'sandwich' conformation between excited and unexcited aromatic moieties [28]. This motion is resisted by local viscosity. As a consequence, the excimer to monomer intensity ratio (I_E/I_M) provides information on the microviscosity of the PC_3P local environment. Fig. 5 shows representative emission spectra for PC_3P in PIPAAm (Fig. 5(a)) and PIPAAm-PBMA (Fig. 5(b)) solutions above their cmc as a function of temperature. PIPAAm solutions showed a continuous reduction in I_E/I_M as the temperature increased below the LCST, since hydrophobic polymer-rich phases solubilizing PC_3P probes were getting rigid as the polymer chain dehydrated. However, the value of I_E/I_M discontinuously decreased by a temperature increase through the LCST, implying the phase transition of PIPAAm chains. Above the LCST, it remains essentially unaffected by further temperature increase. It implied that the motion of PC_3P is suppressed by the microviscosity created by hydrophobic, contracted polymer chain aggregation. On the other hand, the ratios (I_E/I_M) of PC_3P dissolved in PIPAAm-PBMA micelle solutions were markedly lower than those of PIPAAm solutions over the entire temperature region due to highly compact cores of aggregated PBMA chains (Fig. 5(b)). Interestingly, the micelle solutions showed an increase in I_E/I_M as the temperature increased through the LCST irrespective of the PIPAAm phase transition. It showed evidence for a decrease in rigidity of inner cores above the LCST. Therefore, conformational changes of aggregated and collapsed outer shell chains could induce some deformation of the inner core structure, resulting in both its micropolarity increase and microrigidity decrease. These results suggest potential for the polymeric micelle structural changes selectively to modulate drug release from the inner cores upon heating.

3.4. ADR incorporation into PIPAAm-PBMA micelles

PIPAAm-PBMA block copolymers formed a micellar structure by self-association of the hydrophobic PBMA segments in water, a good solvent for PIPAAm chains below the LCST but a non-solvent for the PBMA chains. It was effective to prepare stable and monodispersed micelles from polymer/*N*-

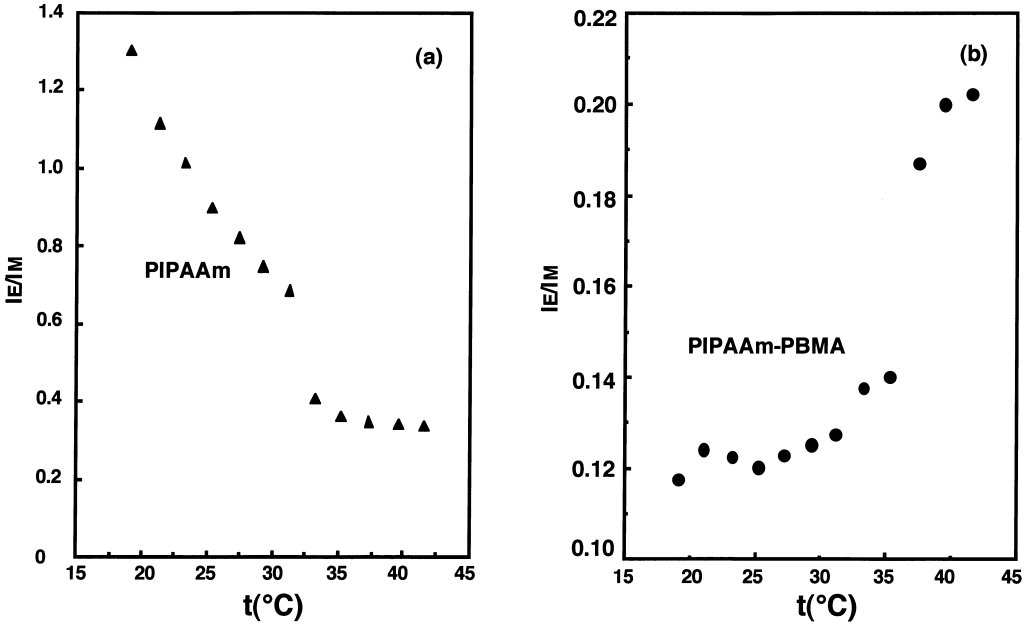


Fig. 5. Plot of the ratio of intensities (I_E/I_M) of the vibrational bands in the PC_3P fluorescence spectrum as a function of temperature for PIPAAm (a) and PIPAAm-PBMA (b), λ_{ex}=333 nm, [PC_3P]=2.2×10^{-7} M, 1°C/min, [polymer]=20000 mg/l.

ethylacetamide (good solvent for both polymer blocks) solution dialyzed against water. Hydrophobic drugs can be physically incorporated into the inner cores comprising PBMA chains by hydrophobic interaction between hydrophobic segments and drugs.

PIPAAm-PBMA micelle formation and drug loading resulting from solvent exchange during dialysis was significantly affected by interaction of the solvents with both polymers and drugs, solvent exchange speed and solution temperature. In the gradual decrease of organic solvent composition during dialysis, spontaneous hydrophobic association of PBMA segments both with themselves and with hydrophobic drugs is a driving force for micelle formation and drug loading into micelle cores. Therefore, drug choice and drug loading are defined by interactions of drugs with both the hydrophobic segments and solvents. Micelle formation, loaded with high drug concentration as much as possible, simultaneously without precipitation was able to be provided at an optimum hydrophobic interaction among the polymer, the drug and the solvent. Control of the optimum conditions should provide important information to generalize optimum drug loading conditions in polymeric micelles. Optimum hydrophobic interactions to form PIPAAm-PBMA micelles containing ADR was regulated by modulating such as hydrophilic/hydrophobic block lengths of the polymer, concentration of both the polymer and ADR in the dialysis bag, TEA amounts and dialysis temperature. PIPAAm(6100)-PBMA(8900) was most successful for both micelle formation and drug (ADR) load (9.6 wt. %) by choosing *N*-ethylacetamide as a good solvent for both the polymers and the ADR and other selected conditions. The polymer-ADR solution of *N*,*N*-dimethylformaldehyde or dimethylsulfoxide precipitated during dialysis against water. A dialysis membrane (Spectra/Por) with a pore size of MWCO=12 000–14 000 provided optimum solvent exchange rates for micelle formation of this PIPAAm-PBMA/ADR combination. The micelle-ADR solution was obtained as a transparent red solution at *ca.* 20°C (dialysis temperature) relating to polymer solubility, especially for PIPAAm segments in water. Other higher or lower dialysis temperatures than 20°C led to precipitates.

In order to remove hydrochloride salt groups from

ADR, 1.3 molar equivalent TEA was added to the ADR solution dropwise prior to mixing with the polymer solution. A larger amount of TEA addition resulted in precipitation during dialysis, while adding a less TEA failed to improve ADR loading due to weak hydrophobic interactions. In addition, higher polymer concentrations and higher ratios of ADR/polymer in the dialysis solution increased ADR loading. However, a polymer concentration higher than 1.3 wt. % and a ratio of ADR/polymer higher than 1:1 led to precipitation. Supernatants of the solutions including precipitates showed large size distributions (>500 nm) and even showed high drug loading. The ADR loaded into PIPAAm-PBMA micelles under selected optimum conditions showed monodispersed size distribution (Fig. 6).

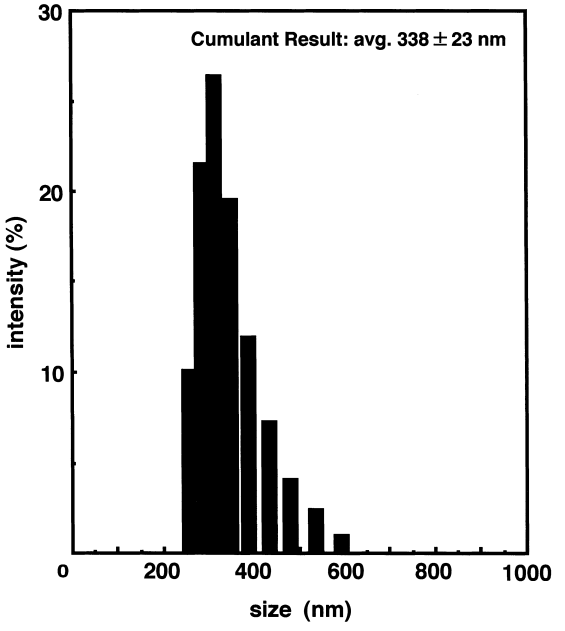


Fig. 6. Average diameter observed by DLS measurements for PIPAAm-PBMA containing ADR (9.6 wt.%) micelle solutions. 10 mW He-Ne laser (632 nm), fixed angle=90°, [polymer]=5000 mg/l.

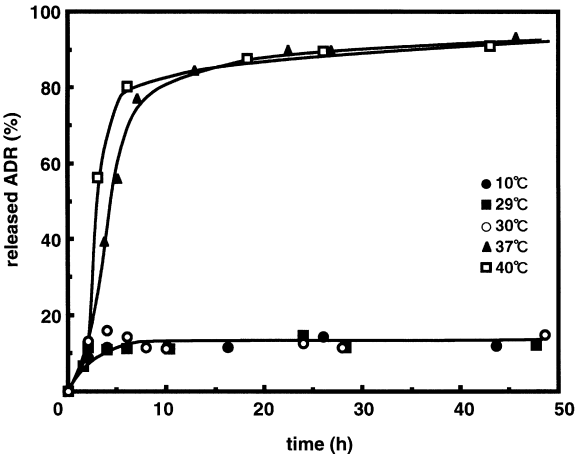


Fig. 7. Drug (ADR) release from thermo-responsive PIPAAm-PBMA micelles containing ADR.

3.5. Thermo-responsive ADR release

ADR release profiles from polymeric micelles showed drastic changes with temperature alterations through the LCST (Fig. 7). ADR release was selectively accelerated upon heating through the LCST, while ADR release was well suppressed below the LCST. Temperature-accelerated ADR release was consistent with temperature-induced structural change of the polymeric micelle (Figs. 4 and 5(b)). Structural deformation of the polymeric micelles upon heating would lead to ADR release. Fig. 8 shows that ADR release from polymer micelles is thermo-responsively on/off switched corresponding to reversible structural changes of micelles by temperature changes through the LCST. The ADR release initiated upon heating above the LCST was accelerated as increasing ADR concentration surrounding the micelles. Moreover, it was quit by just cooling below the LCST and accelerated again upon another heating.

3.6. Thermo-responsive cytotoxicity

Fig. 9 showed *in vitro* cytotoxic activity of PIPAAm-PBMA micelles containing ADR at 29 and 37°C compared with that of free ADR. Polymeric micelles showed higher cytotoxic activity than that of free ADR at 37°C (above the LCST), while it showed lower cytotoxic activity than that of free ADR at 29°C (below the LCST). Cytotoxicity was remarkably well enhanced corresponding to both the micelle structural change (Fig. 4) and resulted in ADR release (Fig. 7) which were selectively initiated

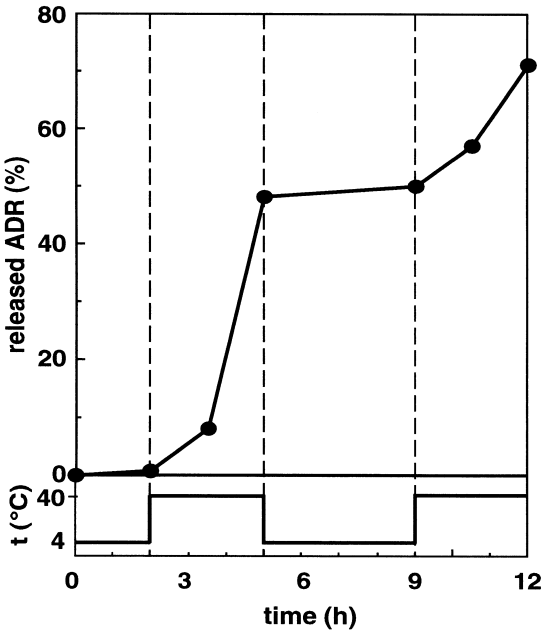


Fig. 8. On/off switched drug (ADR) release from PIPAAm-PBMA micelles containing ADR responding temperature changes.

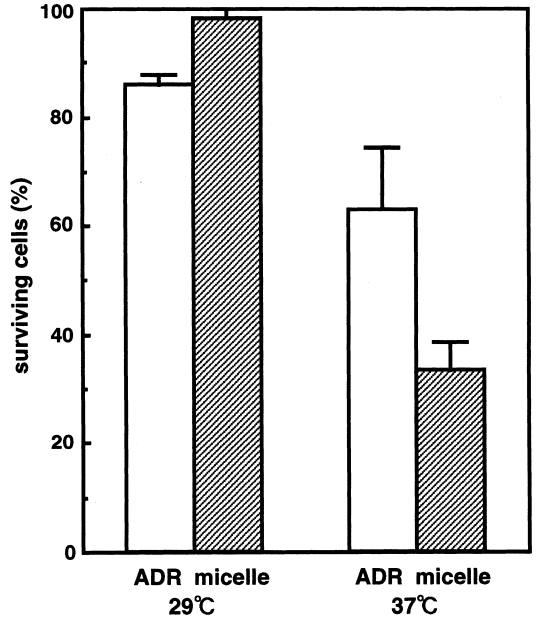


Fig. 9. *In vitro* cytotoxicity of free ADR (0.1 μg/ml) and thermo-responsive PIPAAm-PBMA micelles containing ADR (0.1 μg/ml) against bovine aorta endothelial cells at 29°C (below the LCST) and 37°C (above the LCST). Incubation time: 4 days.

by heating through the LCST. In Fig. 10, photographs of the cells treated with either free ADR or ADR loaded PIPAAm-PBMA for 5 days at 30°C (below LCST) and 40°C (above LCST) are shown together with control experiments at each temperature. Cells exposed to free ADR (2 μg/ml) were almost entirely killed due to ADR cytotoxicity at 30°C (Fig. 10(a)). While the cells with the same amount of ADR in PIPAAm-PBMA micelles (Fig. 10(b)) were observed so vividly like a control experiment (Fig. 10(c)), demonstrating the relative non-cytotocixity of the PIPAAm-PBMA micelles even containing the same amount of ADR, below the LCST. However, scarcely any survived cells were observed when the micelles-ADR were added to cells at 40°C (Fig. 10(e)). These photographs also showed well that the ADR cytotoxicity against endothelial cells was able to be reversibly switched using the PIPAAm-PBMA micelle carriers combined with temperature modulation. Higher cytotoxicity of polymeric micelles-ADR than that of the same amount of free ADR suggests different routes for drug uptake action by cells caused by the carrier properties. Fig. 11 shows photographs of cells treated by PIPAAm-PBMA micelles containing 20 μg/ml of ADR (Fig. 11(a)) and by the same amount of free ADR (Fig. 11(b)) at 40°C. Cells treated by micelles-ADR were observed to be red and aggregates surrounding the cells. Such a phenomenon was observed only for cells treated by micelles-ADR. We have previously reported that hydrophobic PIPAAm chains collapsed above its LCST actively interacted with cells, while hydrated PIPAAm chains below the LCST did not [6–8]. These micelles underwent physical property changes from hydrophilic to hydrophobic as well as structural changes, switching on drug release upon heating through the LCST. Active interaction between the polymeric micelles and cells was supposed to provide high drug uptake by the cells through more effective routes, which has been focused in our current research. Observed results indicate that thermo-responsive polymeric micelles can express specific drug toxicity elicited by local heating as a result of changes in the physical properties of the polymeric micelles, inducing drug release and/or enhanced adsorption to cells mediated by hydrophobic interactions between cells and polymeric micelles (Scheme 1).

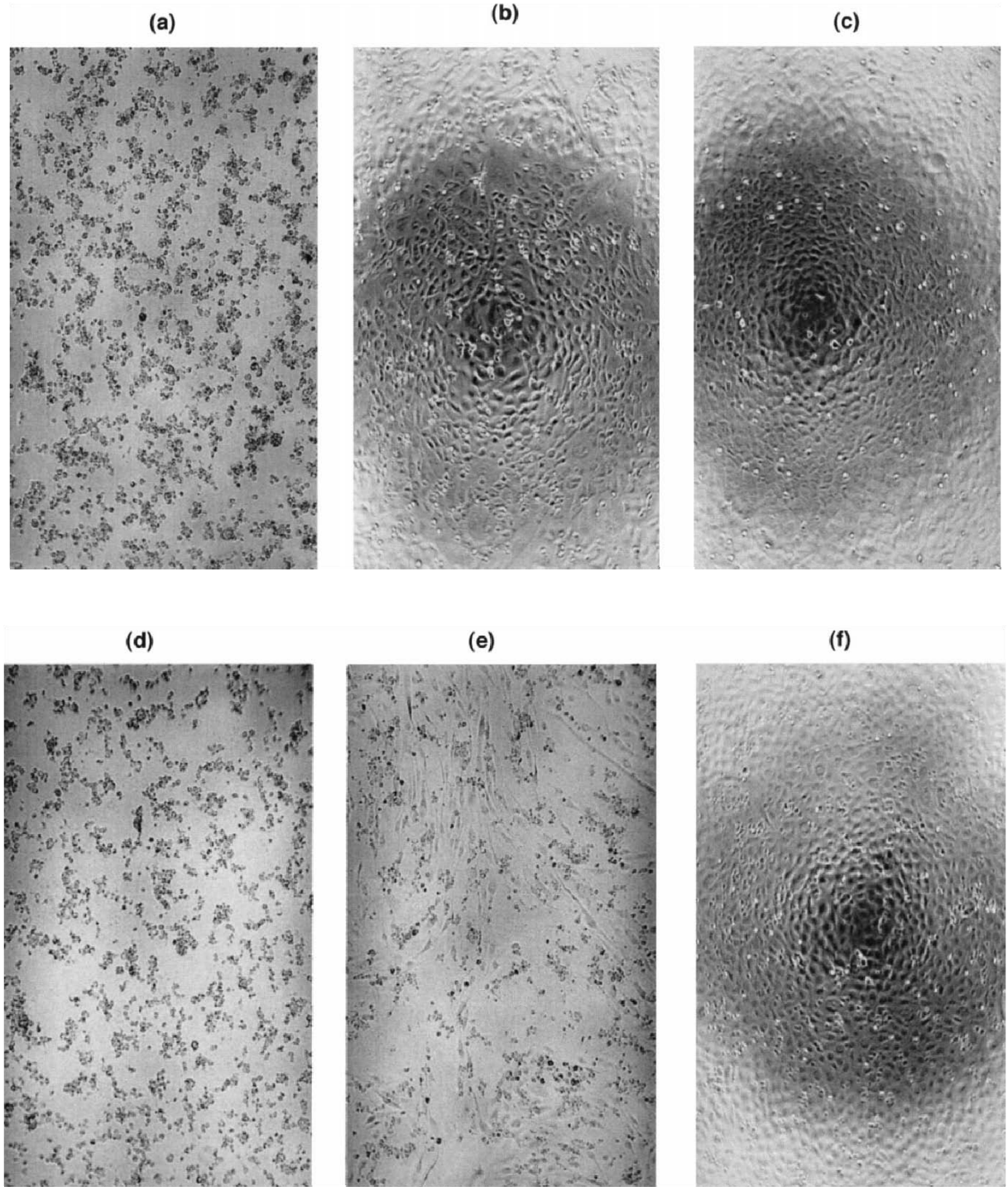


Fig. 10. Photographs of cells exposed to free ADR (2 μg/ml) (a), PIPAAm-PBMA micelles containing ADR (2 μg/ml) (b) and a control experiment without ADR (c) at 30°C (below the LCST), and with free ADR (2 μg/ml) (d), PIPAAm-PBMA micelles containing ADR (2 μg/ml) (e) and a control experiment without ADR (f) at 40°C (above the LCST) for 5 days.

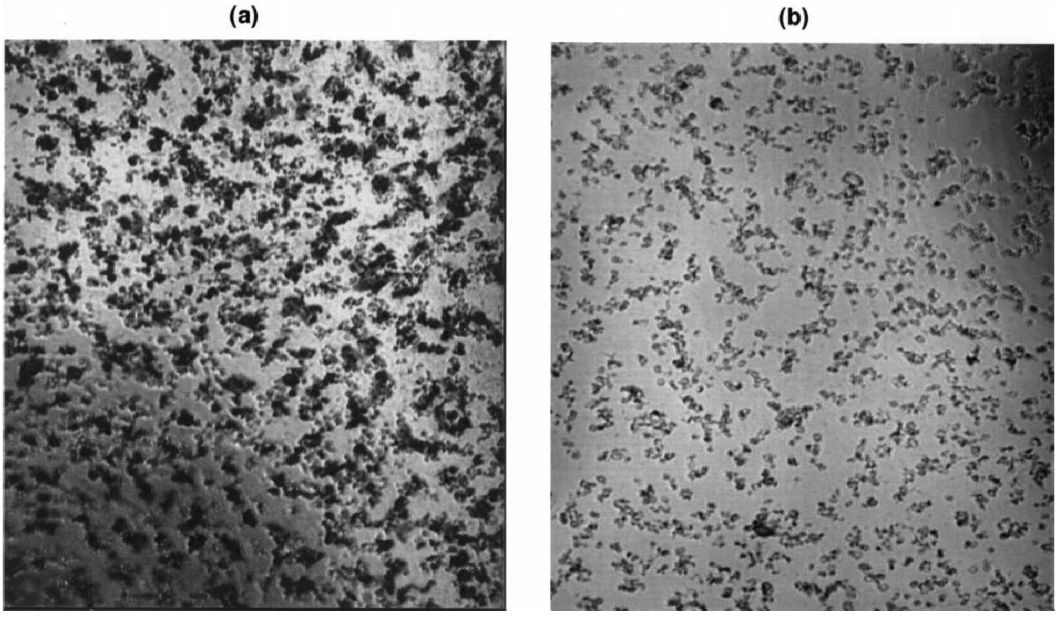


Fig. 11. Photographs of cells exposed to PIPAAm-PBMA micelles containing ADR (20 μg/ml) (a), free ADR (20 μg/ml) (b) and a control experiment without ADR (c) at 40°C for 5 days.

4. Conclusion

Thermo-responsive polymeric micelles were constructed using block copolymers (PIPAAm-PBMA) in order to combine spatial specificity in a passive manner with stimuli-responsive targeting mechanism. The micelle inner core formed by self-aggregates of PBMA segments were loaded with an anticancer drug, ADR, by optimizing the hydrophobic interactions between ADR and PBMA segments. While the outer shell of PIPAAm chains played a role of not only stabilization below a LCST due to their hydrophilicity but also initiation of drug release upon heating through the LCST resulting from PIPAAm structural deformation. Polymeric micelles incorporated with ADR exhibited reversibly thermo-responsive drug release modulated by temperature switching through the LCST. Micelle-ADR effectively suppressed drug cytotoxicity in culture below the LCST, however, selectively showed higher cytotoxicity than the same amount of free ADR maybe due to both enhanced drug release and increased interaction with cells resulting from changes in PIPAAm structure. Namely, the micelle-ADR system showed *in vitro* cytotoxicity only when heated above the LCST. Reversible and sensitive thermo-responsive drug delivery from polymeric micelle carriers will construct novel multifunctional drug delivery system achieving both spatial and temporal control in conjunction with localized hyperthermia.

Acknowledgements

This research was supported in part by a Grant-in-Aid for Scientific Research (No. 10145104) on Priority Areas (No. 296, Bio-molecular Design for Biotargeting) from the Ministry of Education, Science, Sports and Culture.

References

[1] M. Heskins, J.E. Guillet, Solution properties of poly(*N*-isopropylacrylamide), J. Macromol. Sci. Chem. A2 (1968) 1441–1455.

[2] R. Yoshida, K. Uchida, Y. Kaneko, K. Sakai, A. Kikuchi, Y. Sakurai, T. Okano, Comb-type grafted hydrogels with rapid de-swelling response to temperature changes, Nature 374 (1995) 240–242.

[3] H. Kanazawa, K. Yamamoto, Y. Matsushima, N. Takai, A. Kikuchi, Y. Sakurai, T. Okano, Temperature responsive chromatography using poly(*N*-isopropylacrylamide)-modified silica, Anal. Chem. 68 (1996) 100–105.
[4] H. Kanazawa, Y. Kashiwase, K. Yamamoto, Y. Matsushima, A. Kikuchi, Y. Sakurai, T. Okano, Temperature responsive liquid chromatography 2. Effect of hydrophobic groups in *N*-isopropylacrylamide copolymer-modified silica, Anal. Chem. 69 (1997) 823–830.
[5] T. Yakushiji, K. Sakai, A. Kikuchi, T. Aoyagi, Y. Sakurai, T. Okano, Graft architectural effects on themo-responsive wettability changes of poly(*N*-isopropylacrylamide)-modified surfaces, Langmuir 14 (1998) 4657–4662.
[6] N. Yamada, T. Okano, H. Sakai, F. Karikusa, Y. Sawasaki, Y. Sakurai, Thermoresponsive polymeric surfaces; Control of attachment and detachment of cultured cells, Makromol. Chem., Rapid Commun. 11 (1990) 571–576.
[7] T. Okano, N. Yamada, H. Sakai, Y. Sakurai, A novel recovery system for cultured cells using plasma-treated polystyrene dishes graphed with poly(*N*-isopropylacrylamide), J. Biomed. Mater. Res. 27 (1993) 1243–1251.
[8] T. Okano, N. Yamada, M. Okuhara, H. Sakai, Y. Sakurai, Mechanism of cell detachment from temperature-modulated, hydrophilic–hydrophobic polymer surfaces, Biomaterials 16 (1995) 297–303.
[9] M. Yokoyama, M. Miyauchi, N. Yamada, T. Okano, Y. Sakurai, K. Kataoka, S. Ioue, Characterization and anticancer activity of the micelle-forming polymeric anticancer drug adriamycin-conjugated poly(ethylene glycol)-poly(aspartic acid) block copolymer, Cancer Res. 50 (1990) 1693–1700.
[10] M. Yokoyama, T. Okano, Y. Sakurai, H. Ekimoto, C. Shibazaki, K. Kataoka, Toxicity and antitumor activity against solid tumors of micelle-forming polymeric anticancer drug and its extremely long circulation in blood, Cancer Res. 51 (1991) 3229–3236.
[11] H. Maeda, L.W. Seymour, Y. Miyamoto, Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics in vivo, Bioconjugate Chem. 3 (1992) 351–361.
[12] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy: Mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs, Cancer Res. 46 (1986) 6387–6392.
[13] J.E. Chung, M. Yokoyama, K. Suzuki, T. Aoyagi, Y. Sakurai, T. Okano, Reversibly thermo-responsive alkyl-terminated poly(*N*-isopropylacrylamide) core-shell micellar structures, Colloids Surfaces (B: Biointerfaces) 9 (1997) 37–48.
[14] S. Cammas, K. Suzuki, Y. Sone, Y. Sakurai, K. Kataoka, T. Okano, Thermoresponsive polymer nanoparticles with a core-shell micelle structure as site-specific drug carriers, J. Control. Rel. 48 (1997) 157–164.
[15] J.E. Chung, M. Yokoyama, T. Aoyagi, Y. Sakurai, T. Okano, Effect of molecular architecture of hydrophobically modified poly(*N*-isopropylacrylamide) on the formation of thermo-responsive core-shell micellar drug carriers, J. Control. Rel. 53 (1997) 119–130.
[16] S. Takeuchi, M. Oike, C. Kowitz, C. Shimasaki, K. Hasegawa, H. Kitano, Microsphere prepared with a temperature-responsive macromonomer, Makromol. Chem. 194 (1993) 551–558.
[17] Y. Kaneko, K. Sakai, A. Kikuchi, R. Yoshida, Y. Sakurai, T. Okano, Influence of freely mobile grafted chain length on dynamic properties of comb-type grafted poly (*N*-isopropylacrylamide) hydrogels, Macromolecules 28 (1995) 7717–7723.
[18] L.-C. Dong, A.S. Hoffman, A novel approach for preparation of pH-sensitive hydrogels for enteric drug delivery, J. Control. Rel. 15 (1991) 141–152.
[19] G. Chen, A.S. Hoffman, Grafted copolymers that exhibit temperature-induced phased transitions over a wide range of pH, Nature 373 (1995) 49–52.
[20] L.D. Taylor, L.D. Cerankowski, Preparation of films exhibiting a balanced temperature dependence to permeation by aqueous solutions, J. Polym. Sci. Poly. Chem. 13 (1975) 2551–2570.
[21] Y.G. Takei, T. Aoki, K. Sanui, N. Ogata, T. Okano, Y. Sakurai, Temperature-responsive bioconjugates. 2. Molecular design for temperature-modulated bioseparations, Bioconjugated Chem. 4 (1993) 341–346.
[22] R. Yoshida, K. Sakai, T. Okano, Y. Sakurai, Modulating the phase transition temperature and thermo-sensitivity in *N*-isopropylacrylamide copolymer gels, J. Biomater. Sci. Polymer Ed. 6 (1994) 585–598.
[23] F.M. Winnik, A.R. Davidson, G.K. Hamer, H. Kitano, Amphiphilic poly(*N*-isopropylacrylamide) prepared by using a lipophilic radical initiator: synthesis and solution properties in water, Macromolecules 25 (1992) 1876–1880.
[24] D.C. Dong, M.A. Winnik, The Py scale of solvent polarities, Can. J. Chem. 62 (1984) 2560.
[25] K. Kalyanasundaram, J.K. Thomas, Environmental effects on vibronic band intensities in pyrene monomer fluorescence and their application in studies of micellar system, J. Am. Chem. Soc. 99 (1977) 2039.
[26] L.M. Almeida, W.L.C. Vaz, K.A. Zachariasse, V.M.C. Madeira, Fluidity of sarcoplasmic reticulum membranes investigated with dipyrenylpropane, an intramolecular excimer probe, Biochemistry 21 (1982) 5972–5977.
[27] K.A. Zachariasse, W.L.C. Vaz, C. Sotomayor, W. Kuhnle, Investigation of human erythrocyte ghost membrane with intramolecular excimer probes, Biochim. Biophys. Acta 688 (1982) 323–332.
[28] R.L. Melnick, H.C. Haspel, M. Goldenberg, M. Greenbaum, S. Weinstein, Use of fluorescent probes that from intramolecular excimers to monitor structural changes in model and biological membranes, Biophys. J. 34 (1981) 499–515.