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**Preparation of dual-stimuli-responsive liposomes using  
methacrylate-based copolymers with pH and temperature sensitivities  
for precisely controlled release**

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## **Abstract**

Dual-signal-sensitive copolymers were synthesized by copolymerization of methoxy diethylene glycol methacrylate, methacrylic acid, and lauroxy tetraethylene glycol methacrylate, which respectively provide temperature sensitivity, pH sensitivity, and anchoring to liposome surfaces. These novel copolymers, with water solubility that differs depending on temperature and pH, are soluble in water under neutral pH and low-temperature conditions, but they become water-insoluble and form aggregates under acidic pH and high-temperature conditions. Liposomes modified with these copolymers exhibited enhanced content release at weakly acidic pH with increasing temperature, although no temperature-dependent content release was observed in neutral conditions. Interaction between the copolymers and the lipid monolayer at the air–water interface revealed that the copolymer chains penetrate more deeply into the monolayer with increasing temperature at acidic pH than at neutral pH, where the penetration of copolymer chains was moderate and temperature-independent at neutral pH. Interaction of the copolymer-modified liposomes with HeLa cells demonstrated that the copolymer-modified liposomes were adsorbed quickly and efficiently onto the cell surface and that they were internalized more gradually than the unmodified liposomes through endocytosis. Furthermore, the copolymer-modified liposomes enhanced the

37 content release in endosomes with increasing temperature, but no such  
38 temperature-dependent enhancement of content release was observed for unmodified  
39 liposomes.

40

41 **Keywords:** temperature-sensitive / pH-sensitive / liposome / drug delivery system

42

## 1. Introduction

Producing drug carriers that deliver drugs accurately to target sites of target cells in target tissues is a desirable goal of modern medical research. For progress to higher-precision drug delivery, construction has been attempted of stimulus-responsive carrier systems that control the generation of biofunctions such as membrane fusion and drug release in response to a chemical or physical stimulus [1-5]. Some intensively studied stimulus-responsive carriers are liposomes exhibiting response to mildly acidic environments or elevated temperature, designated respectively as pH-sensitive liposomes [6-8] and temperature-sensitive liposomes [9-11]. Indeed, liposomes have been regarded as an ideal drug carrier because of their biodegradability, body safety, and excellent capability to encapsulate drugs [12,13].

Actually, pH-sensitive liposomes have been designed to become destabilized and to release drugs specifically at sites of diseased tissues with mildly acidic atmospheres such as tumors [14,15]. In addition, these liposomes have been shown to introduce their contents into the cytosol of a cell after their internalization by cells through endocytosis and subsequent membrane fusion with endosomes, which have a weakly acidic interior [16-20]. Temperature-sensitive liposomes, another type of stimulus-responsive liposome, are also important drug carriers because of their ability to deliver drugs at a

desired site of the body where the temperature is slightly elevated, above the physiological temperature [9-11,21,22].

Conventionally, these functions of liposomes have been provided using a transition behavior of liposome membranes between gel and liquid crystalline phases or lamellar and hexagonal phases [6,9,11]. For example, dipalmitoyl phosphatidylcholine-based temperature-sensitive liposomes with a gel-to-liquid crystalline phase transition temperature of 42 °C exhibit drug-release enhancement around that temperature, where the liposomal membrane becomes leaky because of the coexistence of gel and liquid crystalline phases [23,24]. In addition, pH-sensitive liposomes have been prepared from phosphatidylethanolamines with unsaturated acyl chains, which tend to take an inverted hexagonal phase, using pH-sensitive amphiphiles as stabilizers [6,25,26].

Another efficient approach to prepare stimulus-responsive liposomes is surface modification of liposomes with stimulus-sensitive polymers [8,20,27–29]. Stimulus-responsive properties of liposomes are based on interaction between the liposome membranes and the polymer chains fixed on the liposome surface. Therefore, a combination of polymers that exhibit a sharp response to stimuli and stable liposomes can produce stimulus-responsive liposomes with excellent performance [30–34].

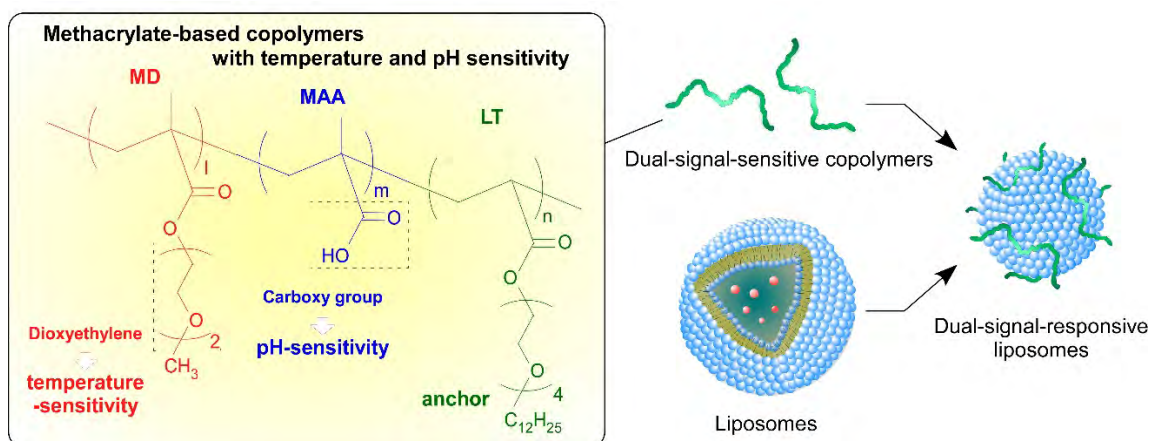
According to this approach, we have developed temperature-responsive and pH-responsive liposomes of various types by surface modification of stable liposomes with pH-sensitive polymers or temperature-sensitive polymers [20,29,34–36]. For example, liposomes modified with temperature-sensitive polymers such as copolymers of vinyl ethers and copolymers of N-isopropylacrylamide were found to have rapid drug release in response to mild heating because the polymer chains become hydrophobic; they then strongly destabilize liposome membranes [35]. As another example, liposomes modified with pH-sensitive polymers such as carboxylated poly(glycidol) derivatives and dextran derivatives become destabilized in mildly acidic environments, resulting in drastic release of their contents and membrane fusion [20,28,37,38].

For use in a recent study, we developed a new type of stimulus-responsive liposomes, with functions controlled dually by ambient temperature and pH using polymers with pH-sensitivity and temperature-sensitivity [39,40]. Such a dual-signal-responsive property of liposomes might be useful to improve the accuracy of drug delivery into target cells of diseased tissues of the body because the liposomes release contents only in acidic compartments, such as cell endosome and lysosome of the target tissues that are locally heated [39]. For use in earlier studies, we prepared dual-stimuli-sensitive polymers using hyperbranched poly(glycidol)s as base polymers.

We then examined their modification by succinylation and subsequent introduction of N-isopropylamide [39] or oligoethylene glycol [40]. The obtained polymers exhibited sensitivity to both pH and temperature, but precise control of their polymer structures was difficult because of the inefficient reactivity of hydroxyl groups on the bulky polymer chain structure.

As another type of dual-stimuli-sensitive polymer that exhibits response to both pH and temperature, we designed copolymers in this study using random copolymerization of methoxy diethylene glycol methacrylate (MD), methacrylic acid (MAA), and lauroxy tetraethylene glycol methacrylate (LT), which act respectively as a temperature-sensitive unit, a pH-sensitive unit, and an anchoring unit (Figure 1). These dual-stimuli-responsive liposomes prepared using MD-MAA-LT copolymers are expected to be useful for accurate drug delivery to target cells of target sites in the body. Therefore, we prepared MD-MAA-LT copolymer-modified liposomes using egg yolk phosphatidylcholine (EYPC), which is frequently used as a liposomal lipid component for anticancer drug delivery. We investigated the dual-stimuli-responsive properties of the copolymer-modified liposomes, their mechanism of dual-stimuli response, and their interaction with cancer-derived cells.





**Figure 1.**

## 2. Experimental

### 2.1. Materials

Methoxy diethylene glycol methacrylate (MD, BLEMME<sup>®</sup> PME-100), lauroxy tetraethylene glycol methacrylate (LT, BLEMME<sup>®</sup> PLE-200) and EYPC were obtained from NOF Corp. (Tokyo, Japan). Methacrylic acid (MAA) was obtained from Kuraray Co. Ltd. (Tokyo, Japan). Calcein was purchased from Sigma-Aldrich Corp. (St. Louis, USA). Lissamine rhodamine B-sulfonylphosphatidylethanolamine (Rh-PE) was purchased from Avanti Polar Lipids Inc. (Birmingham, AL, USA). Triton X was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Phospholipid Test-Wako-C was obtained from Wako Pure Chemical Inds. Ltd. (Osaka, Japan). Fetal

bovine serum (FBS) was obtained from MP Biomedicals (Santa Ana, California, U.S.A.).

## 2.2. Synthesis of copoly(MD-MAA- LT)s

Copoly(MD-MAA-LT)s were synthesized by radical copolymerization of MD, MAA and LT using dipropyl peroxydicarbonate as an initiator. In brief, a mixture of monomers (100 g) and dipropyl peroxydicarbonate (2.2 g) were added into freshly distilled isopropanol (100 g) under N<sub>2</sub> atmosphere and were kept at 60 °C for 2 h. Then the solution was heated at 80 °C for 1 h under N<sub>2</sub> atmosphere. The obtained polymers were recovered by removal of solvents under the vacuum. For example, copoly(MD-MAA<sub>48</sub>-LT) was synthesized as follows. A mixture of MD (55.9 g), MAA (28.8 g), LT (15.3 g) and dipropyl peroxydicarbonate (2.2 g) were added dropwise into freshly distilled isopropanol (100 g) under N<sub>2</sub> atmosphere and were kept at 60 °C for 2 h. The solution was further heated at 80 °C for 1 h under N<sub>2</sub> atmosphere. Then, the solvent was completely removed under vacuum. The yield was 99.3 g (97.2%). Other copolymers were synthesized via the same procedure using varying monomer ratios in feed, as shown in Table 1. The number-average molecular weight ( $M_n$ ), the weight-average molecular weight ( $M_w$ ), and polydispersity index ( $M_w/M_n$ ) of the copolymers were evaluated using gel-permeation chromatography on a system equipped

with a column (Shodex LF-804; Showa Denko K.K.) with THF as an eluent. Polystyrene was used as a standard. Compositions of the copolymers were estimated using  $^1\text{H}$  NMR (JNM-AL-400, JEOL) and acid–base titration according to test methods described in JIS K 0070-1992.

### 2.3. *Cloud Point Determination*

Optical density (500 nm) of copolymer solution (10 mg/mL) containing 10 mM phosphate and 140 mM NaCl with varying pHs was followed as a function of temperature using a V-560 spectrophotometer (Jasco Corp., Tokyo, Japan) equipped with a Peltier type thermostatic cell holder, coupled with an ETC-505T controller. Temperature was raised at 2 °C/min. Cloud point was taken as the initial break point in the resulting transmittance versus temperature curve.

### 2.4. *DSC measurements*

Thermograms of copolymer solutions (10 mg/mL) containing 10 mM phosphate and 140 mM NaCl with varying pHs were recorded using NANO DSC (TA Instruments, New Castle, DE, USA). The copolymer solutions were placed in a sample cell and analyzed at a heating rate of 1.0 °C/min.

### 2.5. *Acid-base titration for estimation of ionization of copolymers*

An aqueous solution of copolymer containing about  $1.1 \times 10^{-4}$  mol of carboxyl

groups (50 mL) was prepared and was adjusted as pH 11. The solution was titrated with HCl (0.01 M) under N<sub>2</sub> atmosphere at room temperature.

## *2.6. Preparation of pyranine-loaded liposomes*

A dry thin membrane of mixture of egg yolk phosphatidylcholine (EYPC) (10 mg) and copolymer (0-3 mg) was dispersed in 1 mL of aqueous 35 mM pyranine, 50 mM DPX, and 25 mM phosphate solution (pH 7.4) by brief sonication using a bath type sonicator at 4 °C and the liposomes was further hydrated by freeze-thawing. Then, the liposome suspension was extruded 51 times through a polycarbonate membrane with a pore size of 100 nm. Liposomes were purified using a Sepharose 4B column with Dulbecco's phosphate-buffered saline (PBS). Lipid concentration was determined using Phospholipid Test-Wako-C. Rh-PE-labeled liposomes were also prepared according to the same procedure except that the lipid membranes containing 0.1 mol% Rh-PE was used for liposome preparation.

## *2.7. Pyranine release from liposomes*

A small aliquot of liposome suspension was added to PBS (total volume 2.5 mL) at various temperatures and pHs (lipid concentration: 0.02 mM). Pyranine fluorescence at 512 nm with excitation at 416 nm was measured by using a FP-8500 spectrofluorometer (JASCO, Japan). Pyranine fluorescence was quenched by DPX

inside of the liposomes, but it exhibited intense fluorescence when released from the liposome. 100% release of pyranine was achieved by adding 10% Triton X-100 (25  $\mu$ L) to the liposome suspension. Percent release of pyranine from liposomes was defined as

$$\text{Release (\%)} = (F^t - F^i) / (F^f - F^i) \times 100$$

where  $F^i$  and  $F^t$  mean the initial and intermediary fluorescence intensities of liposome suspension, respectively.  $F^f$  is the fluorescence intensity of the liposome suspension after addition of Triton X-100.

## 2.8. Surface pressure measurements

Surface pressures were measured with a potentiometer USI-3-22 (USI System, Fukuoka, Japan). A small aliquot (4  $\mu$ L) of a chloroform solution of EYPC (10 mg/mL) with or without copolymer at the weight ratio of 1/1 was spread onto PBS of pH 7.4 or 6.0 (100 x 400 mm<sup>2</sup>), which was used as the subphase, in the trough. The monolayer was compressed at a rate of 20 mm/s after the initial delay period of 20 min for evaporation of organic solvent. The subphase temperature was controlled to be 10, 20, 30 or 38 °C during the measurement.

## 2.9. Liposome-cell interaction

199 HeLa cells ( $2.0 \times 10^5$ ) were seeded in a 35 mm dish and grown in DMEM  
200 supplemented with 10% FBS for 24 h in humidified atmosphere containing 5% CO<sub>2</sub> at  
201 37 °C. The cells were washed with PBS and covered with DMEM supplemented with  
202 10% FBS (2 mL). Then, a small aliquot of the suspension of Rh-PE-labeled and  
203 pyranine-loaded copolymer-modified or plain liposomes was added to the cells (final  
204 lipid concentration: 0.2 mM) and incubated at 37 °C for 2 h. Then, the cells were  
205 washed with PBS and observed immediately or after further culture in the medium for 8  
206 h using a LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss). For flow  
207 cytometric analysis, HeLa cells ( $5.0 \times 10^4$ ) were seeded in a 24-well plate and grown in  
208 DMEM supplemented with 10% FBS for 24 h in humidified atmosphere containing 5%  
209 CO<sub>2</sub> at 37 °C. The cells were washed with PBS and covered with DMEM supplemented  
210 with 10% FBS. Then, a small aliquot of the suspension of Rh-PE-labeled and  
211 pyranine-loaded copolymer-modified or plain liposomes was added to the cells (final  
212 lipid concentration: 0.5 mM) and incubated at 20, 30, or 37 °C for 2 h. For  
213 liposome-treated cells at 37 °C, cells were additionally heated at 45 °C for 10 min using  
214 water-bath. Then, the cells were washed with PBS and fluorescence intensity for the  
215 liposome-treated cells was evaluated using a CytoFLEX S flow cytometer (Beckman  
216 Coulter, Tokyo, Japan).

### 3. Results and discussion

#### 3.1. Characteristic evaluation of copolymers

Table 1 shows compositions and molecular weights of copolymers used for this study. Temperature-sensitive polymers such as poly(N-isopropylacrylamide) are well known to dissolve in an aqueous solution, with water-solubility that changes at a specific temperature, and are known to undergo phase separation into a polymer-rich phase and a polymer-poor phase [41]. At the molecular level, the polymer chains change their characteristics from hydrophilic to hydrophobic and undergo a conformational transition from a hydrated coil to a dehydrated globule at that specific temperature [41-43]. Such temperature-responsive properties of temperature-sensitive polymers have been characterized by their turbidity increase, which is designated as the cloud point [44]. Therefore, we examined the effects of copolymer compositions on temperature sensitivity by detecting the cloud point of their aqueous solutions around neutral and mildly acidic pHs related to physiological pH, endosomal pH, and lysosomal pH, which are, respectively, around pH 7.4, pH 6.0, and pH 5.0 (Figure S1).

**Table 1. Synthesis and Characterization of Copolymers**

Copolymer	In feed MD/MAA/LT (mol%)	Composition MD/MAA/LT (unit mol%)	Mn	Mw	Mw/Mn
MD	100/0/0	100/0/0	20,570	85,610	4.16
MD-MAA <sub>20</sub>	80.0/20.0/0	80.9/19.1/0	18,870	74,400	3.94
MD-MAA <sub>20</sub> -LT	74.7/20.0/5.3	75.3/19.5/5.2	23,680	72,770	3.07
MD-MAA <sub>50</sub> -LT	44.7/50/5.3	48.0/47.6/4.4	20,480	67,680	3.30

The MD homopolymer solution exhibited a cloud point at about 20 °C irrespective of the solution pH, indicating that this polymer is temperature-sensitive but not pH-sensitive. In contrast, the MD-MAA<sub>20</sub> copolymer exhibited cloud points under acidic conditions. The cloud point tends to increase with increasing pH; it disappears at neutral pH, demonstrating that the temperature sensitivity of the copolymer changes depending on the environmental pH. Carboxylate ions on the copolymer chain increase their hydration at neutral pH, but their protonation might decrease their hydration at acidic pH, thereby generating their temperature sensitivity. This result indicates that the temperature sensitivities of the polymer–copolymer chains were provided by MD units and that their temperature sensitivity was controlled by pH-sensitive MAA units. In comparison of the cloud point between MD-MAA<sub>20</sub> and MD-MAA<sub>20</sub>-LT, the attachment of anchor moieties decreased the cloud point between pH 6.5 and pH 5.5 and abolished water-solubility at pH 5.0. In addition, a water-solubility change of the



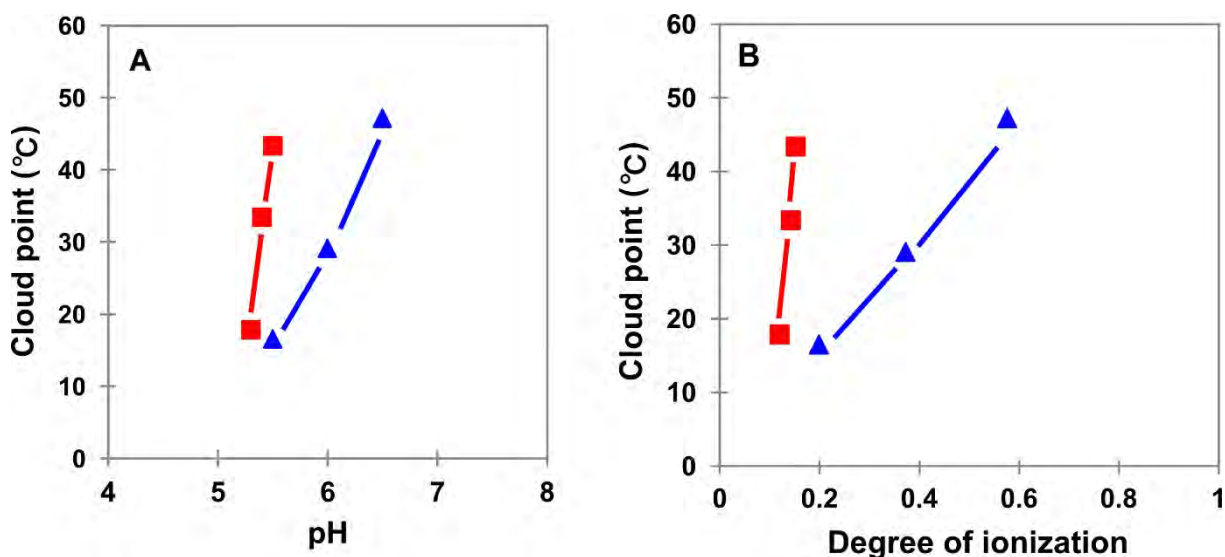
copolymer chains took place in a broader temperature region after incorporation of LT units. It is likely that lauryl groups on the LT unit-containing copolymer chain mutually associate through hydrophobic interaction, which might decrease the copolymer chain mobility and restrict freedom of the chain conformation. Probably, such constraints on the mobility and on the conformational freedom for the copolymer chains suppress efficient interaction among the temperature-sensitive methoxy diethylene glycol chains on the copolymer chains, resulting in the water-solubility change of the copolymer in the broad temperature region. However, it is noteworthy that the copolymer chain retained its temperature sensitivity after the attachment of anchors. In addition, compared to MD-MAA<sub>20</sub>-LT copolymer, MD-MAA<sub>50</sub>-LT copolymer exhibited the water-solubility change in a higher pH region. This fact might be important because the anchor-bearing copolymers which exhibit a temperature-induced water-solubility change at a desired pH are obtainable by controlling the MD and MAA contents in the copolymer chains.

pH-Dependence of the charged states for MAA unit-containing copolymers, MD-MAA<sub>20</sub>-LT and MD-MAA<sub>50</sub>-LT, were examined using acid–base titration (Figure S2). Although these copolymers show decreased carboxylate anions with decreasing pH from pH 10 to pH 4, MD-MAA<sub>50</sub>-LT with apparent  $pK_a$  of 7.0 showed a somewhat

lower degree of ionization than MD-MAA<sub>20</sub>-LT with apparent pK<sub>a</sub> of 6.5 at the same pH of neutral and mildly acidic pH region (pH > 5.5). This fact reflects that MD-MAA<sub>50</sub>-LT tends to lose negative charges more readily than MD-MAA<sub>20</sub>-LT in neutral and weakly acidic pH regions. It is likely that the higher content of MAA units in the copolymer chain of MD-MAA<sub>50</sub>-LT enhances protonation of carboxylate ions to avoid their electric repulsion. As a result, this copolymer exhibited a slightly higher pK<sub>a</sub> value than MD-MAA<sub>20</sub>-LT did.

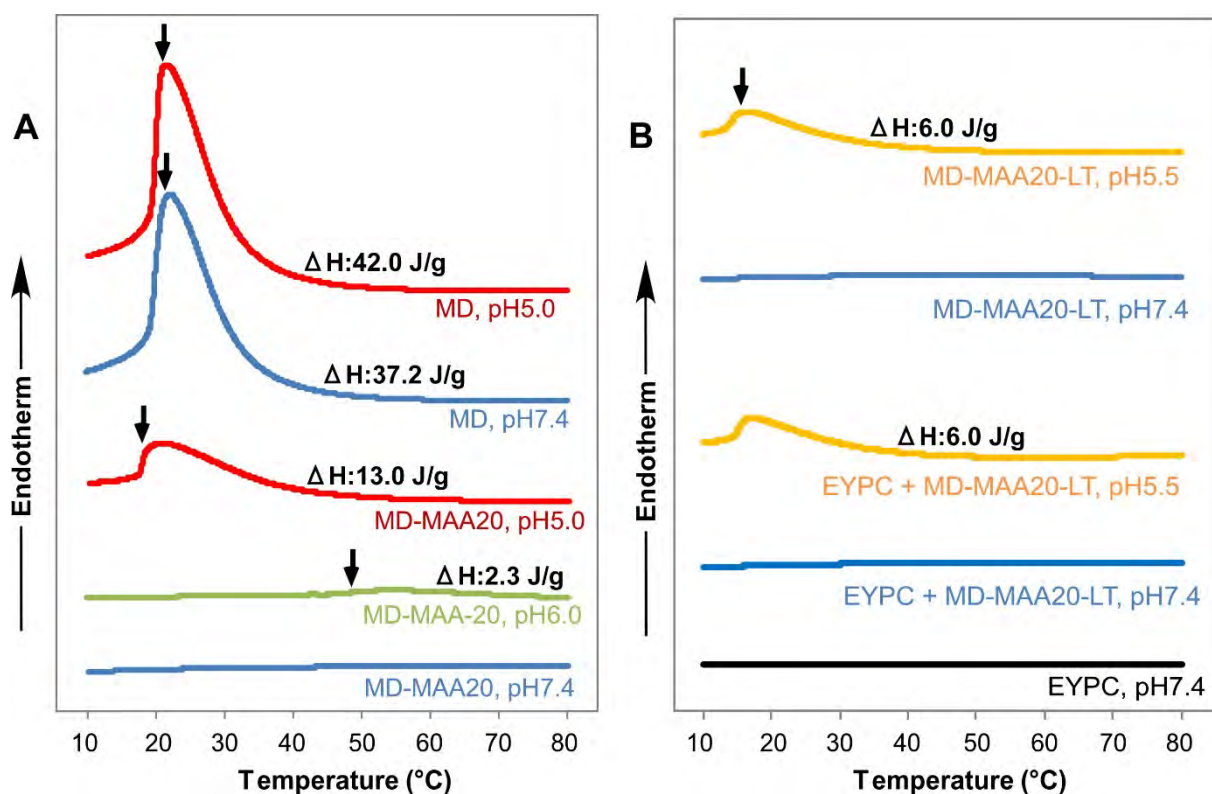
Based on both temperature-dependent turbidity changes for the copolymer solutions at various pHs (Figure S1) and pH-dependent ionization for the copolymers (Figure S2), effects of pH and degree of ionization on the cloud points are portrayed in Figure 2. For MD-MAA<sub>20</sub>-LT, the cloud point, which is indicative of the copolymer's temperature sensitivity, decreased gradually with decreasing pH in the mildly acidic pH region around pH 6.5–5.5, where the copolymer showed a large decrease of ionization degree from 0.6 to 0.2. In contrast, MD-MAA<sub>50</sub>-LT showed a steep decrease of the cloud point with pH decrease in acidic pH region around pH 5.5–5.3, where the copolymer showed only a tiny degree of ionization change from 0.15 to 0.13. MD-MAA<sub>50</sub>-LT chain includes a small number of MD units and numerous MAA units.

Therefore, protonation of such a large fraction (ca. 85%) of MAA units might be necessary for generation of temperature-sensitivity induced by MD units.



**Figure 2.**

The coil–globule transition of temperature-sensitive polymers taking place at cloud point is known to be detectable using DSC [44,45]. Therefore, the transition of the polymer and copolymers prepared in this study was also examined using DSC (Figure 3). Their DSC charts showed an endotherm, which is indicative of the occurrence of the coil–globule transition.



**Figure 3.**

The transition enthalpy and the cloud point for the polymer and copolymers are also presented in Figure 3. Their endothermic peak temperatures mostly agreed with cloud points of the turbidity measurements because the endotherms are derived from dehydration of the copolymer chains upon their conformational change [46,47]. However, their transition enthalpies tend to decrease when comonomer units such as MAA units are included in the copolymer chains. For example, when compared between MD homopolymer and MD-MAA<sub>20</sub> copolymer, the latter exhibits much lower transition enthalpy irrespective of pH. Inclusion of these units in the copolymer chains

is expected to affect their conformation by electrostatic repulsion of MAA units as described above. Therefore, such difference in the copolymer chain conformation might suppress interaction among diethylene glycol chains and disturb the efficient transition of the copolymer chains. In addition, the copolymer having LT units, MD-MAA<sub>20</sub>-LT, exhibited a transition roughly under the same pH and temperature conditions in the presence of EYPC, suggesting that the copolymer undergoes the conformation transition on lipid membranes, just as they do in aqueous solutions.

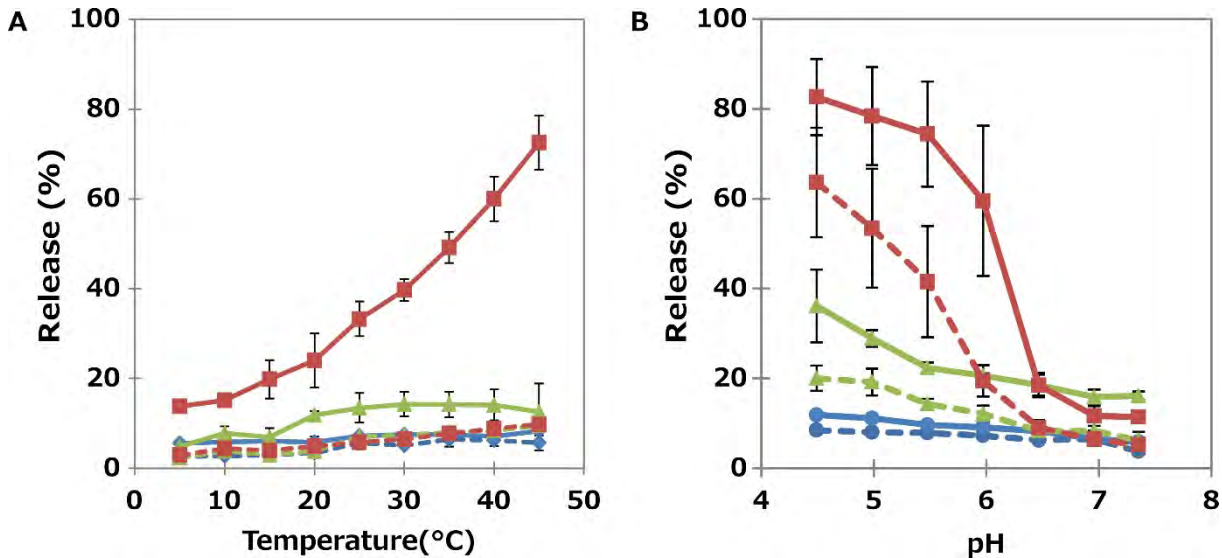
### 3.2. Dual signal-responsive properties of liposomes modified with copolymers

To support progress in the construction of dual-signal responsive liposomes, we examined the effects of copolymer modification on content release behaviors of EYPC liposomes. We prepared liposomes of three kinds using extrusion with a 100 nm pore filter (see the *Experimental section*): MD-MAA<sub>20</sub>-LT-modified liposomes, MD-MAA<sub>50</sub>-LT-modified liposomes, and unmodified liposomes. Their sizes, polydispersity index and zeta potentials are listed in Table S1. The MD-MAA<sub>50</sub>-LT-modified liposomes exhibited a somewhat smaller diameter than those of other liposomes, probably because the negatively charged and highly hydrated copolymer chains can increase the surface stability of the liposomes. In fact,

MD-MAA<sub>50</sub>-LT chains cover the lipid membrane surface more efficiently than MD-MAA<sub>20</sub>-LT chains do, as described later.

The copolymer-modified liposomes encapsulating pyranine and its quencher DPX were prepared. Their content release behaviors were estimated by following pyranine fluorescence (Figure 4). Pyranine release from the unmodified EYPC liposomes was extremely low and affected neither by temperature nor by pH. The MD-MAA<sub>20</sub>-LT-modified liposomes also only slightly released the content at neutral pH but that release was enhanced with decreasing pH and/or increasing temperature to some extent. In contrast, MD-MAA<sub>50</sub>-LT-modified liposomes exhibited superior pH-responsive and temperature-responsive content release behavior. As presented in Figure 4A, pyranine was released only slightly from the liposomes at neutral pH, irrespective of the solution temperature, although the release was markedly enhanced at temperatures higher than 20 °C at pH 6.0. Regarding the pH-dependence of the same liposomes (Figure 4B), the pyranine release was enhanced below pH 6.0, but the enhancement was much greater at 45 °C than at 20 °C. This result indicates that the content release of the MD-MMA<sub>50</sub>-LT-modified liposomes was induced in response to both weakly acidic pH and mildly elevated temperature. Probably, a cooperative effect of protonation of carboxyl groups and dehydration of diethylene glycol chains on the

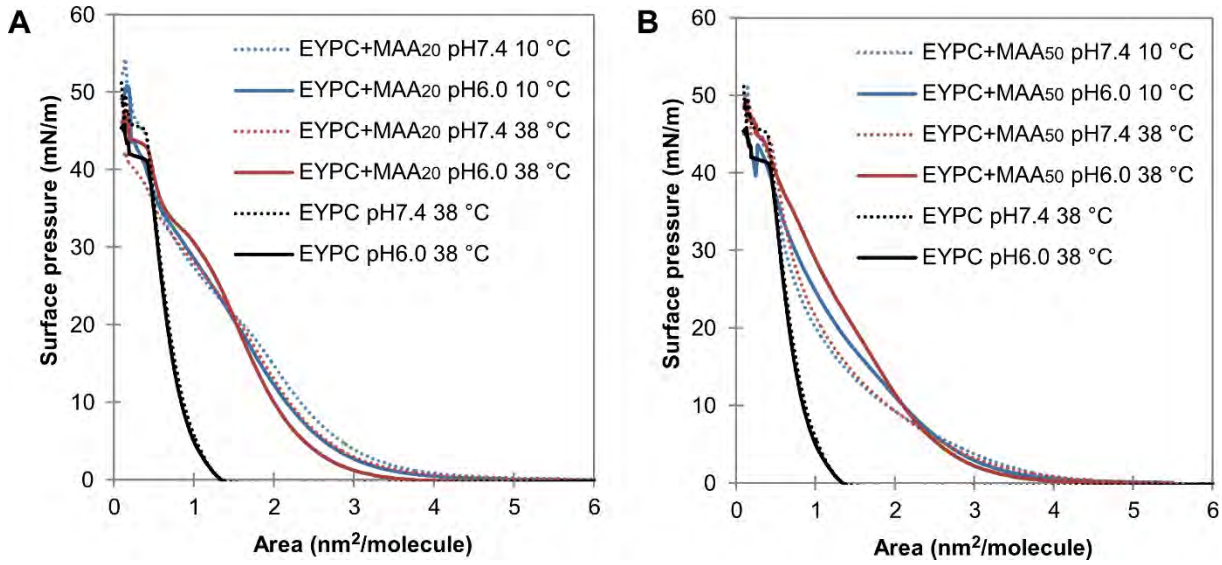
copolymer chains can generate the pH and temperature-dependent content release from the liposomes.



**Figure 4.**

We also examined surface pressure-area ( $\pi$ -A) isotherms of monolayer of EYPC with or without copolymers to investigate copolymer–liposome membrane interactions further (Figure 5). In general, the isotherm of the lipid monolayer is regarded as containing liquid expanded and condensed phases [48]. However, monolayers of EYPC, with a phase transition temperature of around -5.8 °C [49], are regarded as taking on a liquid-expanded phase where the hydrocarbon chains are in a highly fluid and mobile state at the temperatures used for these experiments [50,51]. The  $\pi$ -A isotherms of the

EYPC monolayer obtained in this study (Figure 5) closely resemble those reported by other researchers [51], suggesting the reliability of the measurements.



**Figure 5.**

We prepared monolayers using EYPC or mixtures of EYPC and the copolymers (1/1, wt/wt) as we did for preparation of the copolymer-modified liposomes. Their  $\pi$ -A isotherms were measured under neutral or weakly acidic pH and at 10 °C or 38 °C. As presented in Figure 5A, the EYPC monolayer without copolymer showed fundamentally identical isotherms at pH 6.0 and at pH 7.4 because the charged state of phosphorylcholine moiety of EYPC is not altered between pH 3 and pH 13 [52,53]. For the EYPC/MD-MAA<sub>20</sub>-LT mixed monolayers, a much larger apparent molecular area of



EYPC was observed, compared to those in the absence of the copolymer at the same surface pressure, irrespective of temperature and pH (Figure 5A). This result indicates that the copolymer chains were distributed in the monolayer and that they expanded the monolayers, thereby causing a marked increase of distance between the EYPC molecules.

Comparison of the isotherms of EYPC/MD-MAA<sub>20</sub>-LT monolayers reveals that the monolayers exhibited larger apparent EYPC molecular area below 20 mN/m, but a smaller apparent EYPC molecular area above 20 mN/m at lower temperatures and higher pH (Figure 5A). A similar tendency was apparent for the EYPC/MD-MAA<sub>50</sub>-LT monolayers in Figure 5B, although their molecular area change became greater for areas above 10 mN/m, compared to the EYPC/MD-MAA<sub>20</sub>-LT monolayers. At low surface pressures of below 10 mN/m or 20 mN/m, monolayers containing either copolymer exhibited larger values of the apparent EYPC molecular area under conditions, such as neutral pH and low temperature conditions, in which hydration of the copolymer chains is enhanced. In contrast, at surface pressures higher than 10 mN/m or 20 mN/m, these monolayers tend to show smaller values of the apparent EYPC molecular area under those conditions. Probably, under low surface pressure conditions, highly hydrated copolymer chains taking on an expanded conformation are produced on the monolayers

through hydrophobic interaction between the copolymer anchor groups and the monolayers, and spread through the monolayers, which is expected to induce monolayer expansion and increase the apparent EYPC molecular area. However, when the surface pressure becomes high, the monolayer contraction might induce elimination of the highly hydrated copolymer chains, which are expected to have high affinity to the aqueous phase rather than to the hydrophobic region of the lipid monolayers, resulting in elimination of the copolymer chains from the monolayers. In contrast, the copolymer chains that are less hydrated under low pH and high temperature conditions might have stronger affinity to the monolayers and would therefore induce expansion of the monolayer more effectively.

The area per molecule in the EYPC bilayer membranes in excess water has been estimated as 0.67–0.71 nm<sup>2</sup>, which corresponds to the EYPC molecular area in the EYPC monolayers compressed to 20–25 mN/m [54]. Therefore, we examined the temperature-dependence of apparent molecular area of EYPC for the EYPC/copolymer mixed monolayers further at the surface pressure of 20 mN/m, where the EYPC molecular area was about 0.7 nm<sup>2</sup> (Figure 5A).

As presented in Figure S3, EYPC monolayers exhibited similar molecular area values irrespective of temperature and pH, indicating that EYPC monolayers change the

characters depending neither on temperature nor on pH under the experimental conditions. The EYPC/MD-MAA<sub>20</sub>-LT monolayers also fundamentally did not exhibit temperature-dependence of the apparent molecular area of EYPC at both of pH 7.4 and pH 6.0. However, the monolayer exhibited two-fold larger apparent molecular area values of around 1.5 nm<sup>2</sup>, compared to those of the EYPC monolayers without the copolymer (about 0.7 nm<sup>2</sup>). This fact implies that the MD-MAA<sub>20</sub>-LT copolymer chains penetrated into the monolayers efficiently and expanded the monolayers irrespective of temperature and pH. Probably, the high content of amphiphilic character of diethylene glycol chains and the low content of negatively charged carboxylate ions for the MD-MAA<sub>20</sub>-LT copolymer chains enable their efficient distribution in the monolayer under the experimental conditions.

In contrast, influences of temperature and pH were observed on the apparent molecular area for the EYPC/MD-MAA<sub>50</sub>-LT monolayers. The monolayers exhibited apparent molecular area values of about 1.1 nm<sup>2</sup> at pH 7.4, irrespective of temperature. However, the monolayers exhibited larger values of apparent molecular area at pH 6.0 than at pH 7.4. The values increased significantly with increasing temperature, indicating that penetration of the copolymer chains into the monolayers was enhanced at weakly acidic pH and increasing temperature. Indeed, the copolymer chains have

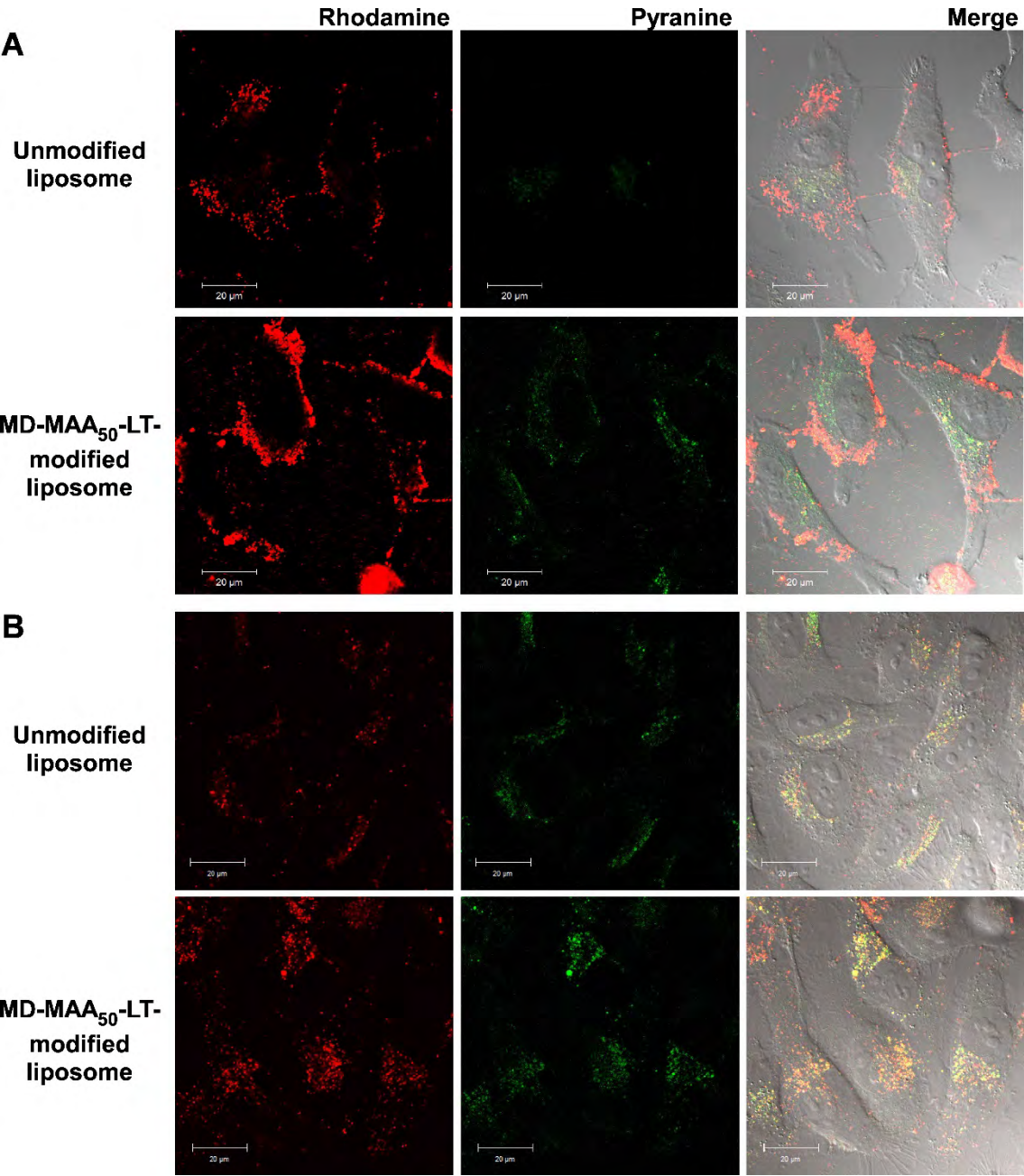
negatively charged carboxylate anions at neutral pH, which is expected to suppress their distribution in the monolayers, irrespective of temperature. However, at weakly acidic pH, both protonation of the carboxylate ions on the copolymer chains and temperature-induced dehydration of the temperature-sensitive diethylene glycol side chains might promote their penetration into the monolayers, resulting in the temperature dependence and larger values of the apparent molecular area. This fact suggests that penetration of MD-MAA<sub>50</sub>-LT chains into the EYPC membranes is enhanced with decreasing pH and increasing temperature, which is consistent with the result of content release behavior of the same copolymer-modified liposomes.

### *3.3. Liposome-cell interaction*

As described above, MD-MAA<sub>50</sub>-LT-modified liposomes exhibited dual-signal-responsive properties in which the liposomes enhanced pyranine release under mildly acidic pH and elevated temperature (Figure 4). Therefore, we investigated the interaction of MD-MAA<sub>50</sub>-LT-modified liposomes with HeLa cells using confocal laser scanning microscopy (CLSM). We compared the results with those of the unmodified liposomes, which were used as control liposomes. HeLa cells were treated for 2 h with MD-MAA<sub>50</sub>-LT-modified or unmodified liposomes encapsulating pyranine

442 and its quencher DPX and being labeled with rhodamine-lipid. After washing with PBS,  
443 the cells were observed with CLSM (Figures 6A). Cells treated with the unmodified  
444 liposomes showed the punctate fluorescence of rhodamine, suggesting that the  
445 unmodified liposomes were taken up by the cells and were trapped in endosome and/or  
446 lysosome. In addition, punctate fluorescence of pyranine was observed in the same cells,  
447 but the fluorescence intensity was very low. This result indicates that, after  
448 internalization into the cells, the unmodified liposomes were trapped in endosomes  
449 and/or lysosomes, but a large fraction of the liposomes retained their contents in their  
450 interior after 2 h-incubation. In contrast, the cells treated with MD-MAA<sub>50</sub>-LT-modified  
451 liposomes showed much stronger fluorescence in the cell periphery, suggesting that  
452 MD-MAA<sub>50</sub>-LT-modified liposomes have a strong affinity to cellular membranes.  
453 Rhodamine fluorescence in the cellular periphery was much stronger than pyranine  
454 fluorescence. Therefore, it is likely that the liposomes were adsorbed onto the cellular  
455 membrane without releasing their contents. However, punctate green fluorescence of  
456 pyranine was also observed in the cells. Their merge image displayed yellow dots in the  
457 intracellular spaces, suggesting that a fraction of the liposomes was taken up by the cells  
458 and their contents were released in endosomes and lysosomes during the short 2  
459 h-incubation. It is likely that the copolymer chains bound onto the liposomes induced

destabilization of liposomes quickly upon exposure to the weakly acidic environment of endosomes and released their contents there.



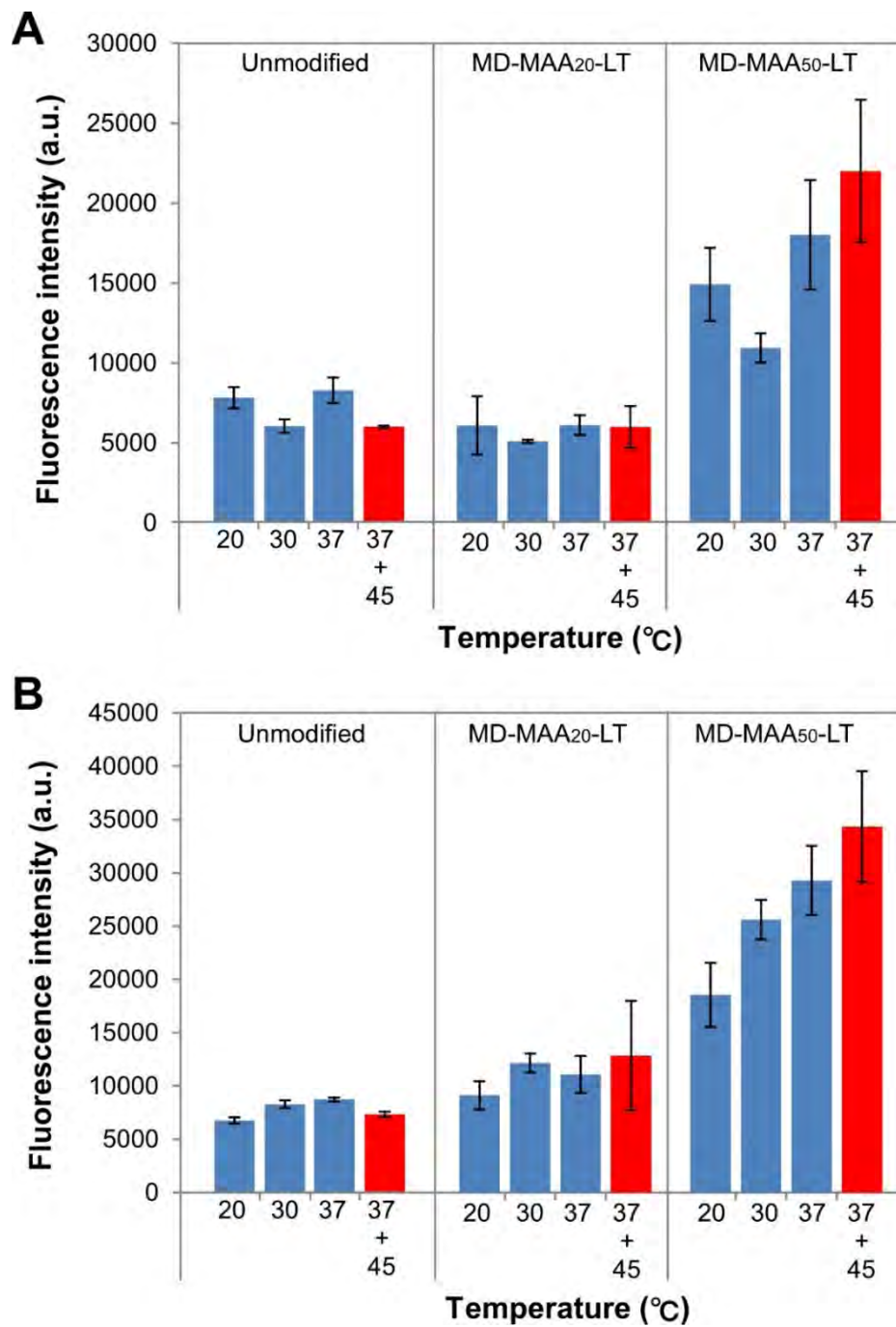
**Figure 6.**

These liposome-treated cells were also observed after further incubation in the medium for 8 h with CLSM (Figures 6B). Cells treated with unmodified liposomes exhibited stronger pyranine fluorescence than the same cells before the additional 8 h culture, indicating that the liposomes were decomposed by lysosomal enzymes and that their contents were leaked there in 8 h. For cells treated with MD-MAA<sub>50</sub>-LT-modified liposomes, strong rhodamine fluorescence in the cellular periphery disappeared. Instead, punctate fluorescence of rhodamine in the intracellular space became much stronger than the same cells before 8 h culture (Figure 6B). In addition, the cells increased pyranine fluorescence as well after 8 h culture. This fact indicates that MD-MAA<sub>50</sub>-LT-modified liposomes were adsorbed efficiently and quickly onto the cell surface and that they were gradually taken up by the cell through the endocytosis pathway. Furthermore, these liposomes release the contents in endosomes, which have a mildly acidic interior.

We further estimated the liposomes with cells quantitatively using a flow cytometer (Figure 7). HeLa cells were treated with the unmodified or copolymer-modified liposomes encapsulating pyranine/DPX and were labeled with rhodamine-lipid for 2 h. Then the cellular fluorescence intensity was measured. As Figure 7A shows, cells treated with unmodified or MD-MAA<sub>20</sub>-LT-modified liposomes

484 exhibited similar and low level of rhodamine fluorescence, irrespective of incubation  
485 temperature. These cells displayed a low level of pyranine fluorescence as well (Figure  
486 7B), which indicates that these liposomes have low affinity to HeLa cells and only  
487 slightly released the contents in cells during 2 h-incubation at 20–37 °C.





**Figure 7.**

In contrast, cells treated with MD-MAA<sub>50</sub>-LT-modified liposomes exhibited roughly three times stronger rhodamine fluorescence at 20–37 °C than those treated

with liposomes of other kinds, demonstrating high affinity of MD-MAA<sub>50</sub>-LT-modified liposomes to HeLa cells in this temperature region. Furthermore, the cellular fluorescence of pyranine for the cells treated with MD-MAA<sub>50</sub>-LT-modified liposomes increased as the incubation temperature rose. Considering that the liposome-treated cells exhibited roughly similar levels of rhodamine fluorescence but a marked increase of pyranine fluorescence with rising temperature, it is likely that the liposomes adsorbed the cells at similar efficiency in the experimental temperature region but the release of contents from the liposomes trapped in endosomes might be enhanced with increasing temperature. In fact, among these liposomes, only MD-MAA<sub>50</sub>-LT-modified liposomes were shown to cause temperature-dependent enhancement of content release in the mildly acidic pH region (Figure 4).

Recent reports have described that various types of cells including HeLa cells take up negatively charged nanoparticles, such as oligonucleotide-functionalized nanoparticles, through interaction with scavenger receptors that are known to mediate endocytosis of polyanionic ligands [55-58]. Therefore, it is possible that negatively charged carboxyl anions of MAA units of the copolymer-modified liposomes are recognized by HeLa cells via scavenger receptors. In addition, considering the hydrophobic character of methoxy diethylene glycol chains of MD units, the copolymer

chains might have affinity to the cell membranes through hydrophobic interaction. Although both of MD-MAA<sub>50</sub>-LT and MD-MAA<sub>20</sub>-LT contain MD and MAA units, MD-MAA<sub>50</sub>-LT chains are regarded as exposed more efficiently from the liposome surface, compared to MD-MAA<sub>20</sub>-LT which was revealed to be buried more readily in the lipid membrane (Figure 5). Therefore, it is likely that MD-MAA<sub>50</sub>-LT-modified liposomes exhibit high affinity to HeLa cells through the synergic effect of the MD unit-induced hydrophobic interaction and the MAA unit-derived efficient recognition by scavenger receptor.

#### 4. Conclusions

We developed functional liposomes with destabilization that can be dually controlled by temperature and pH by surface modification of stable EYPC liposomes with a new type of methacrylate-based copolymer having both thermosensitivity and pH-sensitivity. The copolymer-modified liposomes showed content release under weakly acidic pH and elevated temperature conditions. In addition, the copolymer-modified liposomes exhibited strong affinity onto HeLa cells and induced delivery of their contents into the cells. Indeed, the liposomes of the present type release their contents in response to the combination of elevated temperature and weakly acidic

pH. Such properties of the liposomes are expected to be important for improving the accuracy of drug delivery because drug release from the liposomes can be limited precisely in the endosome or lysosome interiors of the target cells of the target tissues to which local heating is applied. In this study, we observed no efficient transfer of the liposome content into cytosol of the cells. Indeed, the cytoplasmic delivery function might be an important function of liposomes. However, instilling this function to the liposomes might be achieved by incorporating fusogenic lipids, such as phosphatidylethanolamines with unsaturated acyl chains, as membrane components. Therefore, results of this study are expected to lead to effective strategies for improving the accuracy of liposome-based drug delivery.

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**Figure captions**

**Figure 1.** Preparation of dual-stimulus-responsive liposomes using copolymers having both temperature-sensitivity and pH-sensitivity.

**Figure 2.** Cloud point for MD-MAA<sub>20</sub>-LT (triangles) and MD-MAA<sub>50</sub>-LT (squares) as a function of pH (A) and the degree of ionization (B).

**Figure 3.** DSC charts for MD and MD-MAA<sub>20</sub> (A) and for MD-MAA<sub>20</sub>-LT and a mixture of MD-MAA<sub>20</sub>-LT and EYPC (1/1, wt/wt) (B) dissolved in 10 mM phosphate buffer containing 140 mM NaCl of various pH. For reference, DSC chart for EYPC dispersion at pH 7.4 is also shown in (B). Arrows indicate cloud points estimated in Figure S1. The polymer (copolymer) concentration was 10 mg/mL. The heating rate was 1 °C/min.

**Figure 4.** Pyranine release after 10 min incubation from unmodified liposomes (circles) and liposomes modified with MD-MAA<sub>20</sub>-LT (triangles) or MD-MAA<sub>50</sub>-LT (squares). (A) Pyranine release as a function of temperature at pH 7.4 (dotted lines) or pH 6.0 (solid lines). (B) Pyranine release as a function of pH at 45 °C (solid lines) or 20 °C (dotted lines). Copolymer/EYPC was 8/2 (wt/wt). The lipid concentration was 0.02 mM.

**Figure 5.** Surface pressure-molecular area curves for EYPC membrane in the presence

of MD-MAA<sub>20</sub>-LT (A) or MD-MAA<sub>50</sub>-LT (B) at various temperatures and pH.

**Figure 6.** Confocal laser scanning microscopy (CLSM) images of HeLa cells treated with pyranine/DPX-loaded, rhodamine-lipid-labeled unmodified and MD-MAA<sub>50</sub>-LT modified liposomes. (A) Cells were incubated in the liposome-containing DMEM medium with FBS (10%) for 2 h and were observed using CLSM. (B) After treatment with liposomes, the cells were cultured for 8 h in the DMEM medium with FBS (10%) and were observed with CLSM. Rhodamine fluorescence, pyranine fluorescence, and merge images are presented from left to right.

**Figure 7.** Rhodamine (A) and pyranine (B) fluorescence intensities of HeLa cells treated with pyranine-loaded, rhodamine-lipid-labeled unmodified liposomes or liposomes modified with MD-MAA<sub>20</sub>-LT or MD-MAA<sub>50</sub>-LT at 20, 30, or 37 °C for 2 h. For liposome-treated cells at 37 °C, additional heating at 45 °C for 10 min was also performed. Cellular fluorescence intensity was evaluated using flow cytometry. The average fluorescence intensity is shown.