FEBS Letters 456 (1999) 306–310 FEBS 22424

Temperature-dependent interaction of thermo-sensitive polymer-modified liposomes with CV1 cells

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Received 3 May 1999; received in revised form 6 July 1999

Abstract Egg yolk phosphatidylcholine liposomes modified with a copolymer of *N*-acryloylpyrrolidine and *N*-isopropylacrylamide having a lower critical solution temperature at ca. 40°C were prepared and an effect of temperature on their interaction with CV1 cells was investigated. The unmodified liposomes were taken up by the cells approximately to the same extent after 3 h incubation at 37 and 42°C. In contrast, uptake of the polymer-modified liposomes by CV1 cells decreased slightly at 37°C but increased greatly at 42°C, compared to the unmodified liposomes. Proliferation of the cells was partly prohibited by the incubation with the unmodified liposomes encapsulating methotrexate at 37 and 42°C. The treatment with the polymer-modified liposomes containing methotrexate at 37°C hardly effected the cell growth. However, the treatment at 42°C inhibited the cell growth completely. It is considered that the highly hydrated polymer chains attached to the liposome surface suppressed the liposome-cell interaction below the lower critical solution temperature of the polymer but the dehydrated polymer chains enhanced the interaction above this temperature. Because interaction of the polymer-modified liposomes with cells can be controlled by the ambient temperature, these liposomes may have potential usefulness as efficient site-specific drug delivery systems.

Key words: Temperature-sensitive liposome; Lower critical solution temperature; Poly(*N*-acryloylpyrrolidine); Poly(*N*-isopropylacrylamide); Liposome-cell interaction; Drug delivery system

1. Introduction

With respect to site-specific delivery of drugs and bioactive molecules, a variety of stimuli-sensitive liposomes have been designed [1]. Among them are temperature-sensitive liposomes [2,3], pH-sensitive liposomes [4–6] and light-sensitive liposomes [7–9]. The release of the contents from these liposomes can be triggered by these chemical and physical stimuli.

We [3,10–13] and other groups [14,15] have attempted temperature-sensitization of liposomes by the modification with thermo-sensitive polymers. For example, poly(*N*-isopropylacrylamide) (poly(NIPAM)), which is a well-known thermo-sensitive polymer, exhibits a lower critical solution temperature (LCST) at 32–35°C in aqueous solutions [16,17]. The polymer is highly hydrophilic and soluble in water below its LCST. However, it becomes hydrophobic and water-insoluble above this temperature [18]. Therefore, if this polymer is conjugated to a liposome surface, the liposome is stabilized by the hydrated polymer chains below its LCST, whereas destabilization of the liposome is induced by the interaction between the liposome membrane and the hydrophobic polymer chains above this temperature [3,10–13].

In addition, the modification with poly(NIPAM) can provide liposomes with a temperature-sensitive surface property: the liposome surface is covered with a layer of hydrated polymer chains below its LCST, whereas the dehydrated polymer chains are absorbed on the liposome surface above this temperature [12]. In fact, we observed that intensive aggregation of phosphatidylcholine liposomes modified with a NIPAM copolymer took place above the LCST of the copolymer and below the gel to liquid crystalline phase transition temperature of the lipid membranes, suggesting that the hydrophobic polymer chains bound on the liposome membrane in a gel phase increase hydrophobicity of the liposome surface [19].

Liposome-cell interaction is known to depend on surface properties of liposomes. For example, liposome binding to cells and liposome endocytosis by cells are influenced by the structure of head groups of lipids and surface charge density of liposomes [20–22]. Also, attachment of hydrophilic polymers, such as polyethylene glycol, to the liposome surface reduces its uptake by cells [22,23].

Since the surface property of thermo-sensitive polymer-modified liposomes changes, depending on the temperature, interaction of these liposomes with cells is expected to be controlled by the ambient temperature. In this study, we prepared egg yolk phosphatidylcholine (EYPC) liposomes modified with a copolymer of *N*-acryloylpyrrolidine (APr) and NIPAM having two dodecyl groups at the terminal (poly(APr-co-NIPAM)-2C_{12}) (Fig. 1), which exhibits a LCST near the physiological temperature [11]. The influence of temperature on uptake of the polymer-modified liposomes by CV1 cells, an African green monkey kidney cell line, was investigated. The effect of methotrexate (MTX) encapsulated in the polymer-modified liposomes on the cell growth was also examined. Suppression and enhancement of interaction between the polymer-modified liposomes and the cells, depending on the temperature, have been reported.

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Abbreviations: NIPAM, *N*-isopropylacrylamide; APr, *N*-acryloylpyrrolidine; LCST, lower critical solution temperature; Poly(APr-co-NIPAM)-2C_{12}, copolymer of APr and NIPAM having two dodecyl groups at the terminal; EYPC, egg yolk phosphatidylcholine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; MTX, methotrexate; DMEM, Dulbecco's modified Eagle's medium; Tris, tris(hydroxymethyl)aminomethane; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PBS-CM, PBS containing 0.36 mM $CaCl_2$ and 0.42 mM $MgCl_2$

PII: S0014-5793(99)00975-8

2. Materials and methods

2.1. Chemicals

EYPC and MTX were purchased from Sigma. *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) was obtained from Avanti Polar Lipids. Dulbecco's modified Eagle's medium (DMEM) was from Nissui Pharmaceutical. Fetal bovine serum (FBS) was from Hyclone Laboratories. Tris(hydroxymethyl)aminomethane (Tris) was from Kishida Chemical. Synthesis and characterization of poly(APr-co-NIPAM)-2C_{12} were described elsewhere [11].

2.2. Liposome preparation

Liposomes were prepared via the method previously reported [11]. A thin membrane of a mixture of lipid (10 mg) and polymer (10 or 15 mg) was obtained by evaporating their solution in chloroform. The membrane was further dried under vacuum overnight and dispersed in 1.5 ml of 10 mM Tris-HCl and 140 mM NaCl solution of pH 7.4 using a bath-type sonicator. For the preparation of liposomes encapsulating MTX, 1.5 ml of 10 mM Tris-HCl and 140 mM NaCl solution containing 1.8 mM MTX (pH 7.4) was added to the lipid-polymer membrane. The liposome dispersion was extruded through a polycarbonate membrane with a pore size of 100 nm at 0°C. Free polymer and free MTX were removed by gel permeation chromatography on a Sepharose 4B column at 4°C using a 10 mM Tris-HCl-buffered solution containing 140 mM NaCl at pH 7.4. The amount of MTX encapsulated in liposomes was determined by the method reported by Jones and Hudson [24].

2.3. Liposome uptake by cells

Liposomes containing NBD-PE were prepared via the above method using EYPC/NBD-PE (97:3, mol/mol) as the membrane lipid. The cells (1.5×10^5/well) were plated into six well trays in 1 ml of DMEM supplemented with 10% FBS, 24 h prior to the experiment. The cells were washed three times with phosphate-buffered saline (PBS) containing 0.36 mM $CaCl_2$ and 0.42 mM $MgCl_2$ (PBS-CM). Then, DMEM (1 ml) with or without 10% FBS and the liposome dispersion (150 μl) were added to the cells and incubated for 3 h at a given temperature. The cells were washed seven times with ice-cooled PBS-CM and twice with ice-cooled PBS and lysed by adding 0.3 ml of Luc-PGC-50 detergent (Toyo, Japan). The fluorescence intensity of NBD-PE in the cell lysate at 525 nm was measured at an excitation wavelength of 450 nm by using a spectrofluorometer (Shimadzu RF-5000). The cell number was determined from the protein concentration of the lysate using BCA protein assay reagent (Pierce, IL, USA).

2.4. Interaction of MTX-loaded liposomes with cells

1 Day before the experiment, the cells (3×10^4/well) were plated into six well trays in 1 ml DMEM with 10% FBS. Aliquots (150 μl) of the MTX-loaded liposome suspensions were added to the cells growing in fresh DMEM with 10% FBS and incubated for 10, 30 and 60 min at 37 or 42°C. After each incubation, the cells were washed seven times with ice-cooled PBS-CM and then incubated in 1 ml DMEM with 10% FBS for 24 h at 37°C and 5% CO_2. The cells were washed seven times with PBS-CM and twice with ice-cooled PBS and lysed by adding 0.3 ml of Luc-PGC-50 detergent. The cell number was determined from the protein concentration of the lysate using BCA protein assay reagent.

2.5. Other methods

The size of liposomes was evaluated by dynamic light scattering using a 380 ZLS instrument (Nicomp). The amount of polymer bound to liposomes was estimated as previously reported [11,19]. The phospholipid concentration was measured by an assay using Phospholipids B-Test Wako (Wako Pure Chemical Industries).

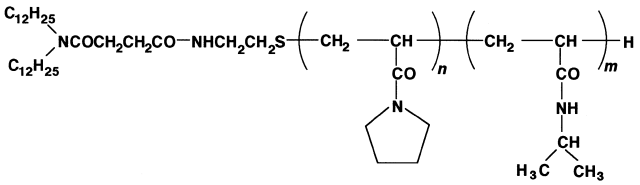

Fig. 1. Structure of poly(APr-co-NIPAM)-2C_{12}.

3. Results and discussion

Poly(APr-co-NIPAM)-2C_{12} used in this study has been shown to have the APr/NIPAM molar ratio of 81.6/18.4 in the previous study [11]. The weight and the number average molecular weights of its polymer chain moiety were 9200 and 4100, respectively. The LCST of its polymer chain moiety was 39.6 ± 0.1°C. We have shown that dioleoylphosphatidylethanolamine liposomes modified with this polymer exhibit a limited extent of calcein release below 35°C, but a drastic contents release takes place around 40°C due to a bilayer to hexagonal transition in the liposome membrane [11]. This fact indicates that the polymer chain undergoes a hydrated coil to dehydrated globule transition at this temperature on the liposome membrane.

Two kinds of the polymer-modified EYPC liposomes as well as the unmodified EYPC liposome were prepared in this study. As shown in Table 1, about half of the amount of polymer supplied was bound to liposomes. The amounts of the polymer of 0.46 and 0.71 mg/mg lipid are calculated to correspond to 7.2 and 10.6 mol%, respectively, on the basis of the average molecular weight of the polymer. These liposomes were shown to have similar sizes with diameters of 140–160 nm at 37°C by dynamic light scattering. Because the polymer chain attached to the liposome changes its nature between hydrophilic and hydrophobic around 40°C, this change may affect stability of the liposomes. Thus, we measured the diameter of the polymer-modified EYPC liposomes at 42°C. The liposomes revealed essentially the same diameter at 37 and 42°C and their diameter did not change after 120 min incubation at 42°C. This result indicates that the polymer-modified EYPC liposomes are stable even after the polymer chain becomes hydrophobic above its transition temperature. We have already reported that aggregation of thermo-sensitive polymer-modified liposomes takes place above the polymer's LCST only when the liposome membranes are in a gel phase [19].

The influence of the incubation temperature on liposome uptake by CV1 cells was examined. The cells were incubated with either the polymer-modified or the unmodified liposomes at 37, 41 or 42°C for 3 h. The amount of liposomal lipid associated with the cell after the incubation is shown in Fig. 2. For the unmodified liposome, approximately the same amount of liposome was taken up by the cell between 37

Table 1
Preparation of liposomes

Liposome	Polymer in feed (mg/mg lipid)	Polymer fixed (mg/mg lipid)	Polymer content (mol%)	Diameter (nM)
Unmodified	0	0	0	156
Polymer-modified				
Liposome-1	1.0	0.46	7.2	161
Liposome-2	1.5	0.71	10.6	140

and 42°C, indicating that temperature does not affect the liposome uptake in this temperature region. In the case of the polymer-modified liposome, at 37°C, the amount of liposome taken up by the cell was lower than that of the unmodified liposome at the same temperature. However, the amount of cell-associated liposome increased with raising the temperature. This suggests that the polymer chains attached to the liposome surface affect the liposome-cell interaction. In comparison with the unmodified liposome, the polymer-modified liposome was taken up by the cell less readily at 37°C and more readily at 42°C. Because the LCST of the polymer chain was ca. 40°C, the polymer chain is highly hydrated and takes on a random coil or extended conformation at 37°C. It has been reported that polyethylene glycol chains grafted to liposomes weaken liposome-cell interactions and reduce the liposome uptake by cells, probably because of steric repulsion by the highly hydrated polymer chains [22,23,25]. Similarly, hydrated chains of the poly(APr-co-NIPAM) might cover the liposome surface and suppress the liposome-cell interaction below the polymer's LCST. In contrast, at 42°C, the cells treated with the polymer-modified liposomes exhibited a higher amount of the cell-associated liposome than those treated with the unmodified liposomes. Above the LCST, the grafted polymer chains become hydrophobic and might be adsorbed on the liposome surface. We have shown that poly(NIPAM) chains attached to EYPC liposomes exist around the surface of the membrane above the polymer's LCST [12]. The adsorbed polymer chains should increase hydrophobicity of the liposome surface. It is likely that the hydrophobic polymer chains bound on the liposome surface facilitate binding to the cell and recognition of the liposome by the cell.

Fig. 3 represents the effect of the amount of the grafted polymer on liposome uptake by CV1 cells. The modification with the polymer content of 7.2 mol% decreased the uptake to some extent. However, a further increase in the polymer content was not effective for suppression of the liposome-cell interaction. Du et al. have reported that only 0.7 mol% of polyethylene glycol 5000-grafted lipid is needed to fully cover the liposome surface, when the polymer chain takes on a mushroom conformation [25]. Therefore, the surface of the liposome with the polymer content of 7.2 mol% might be fully

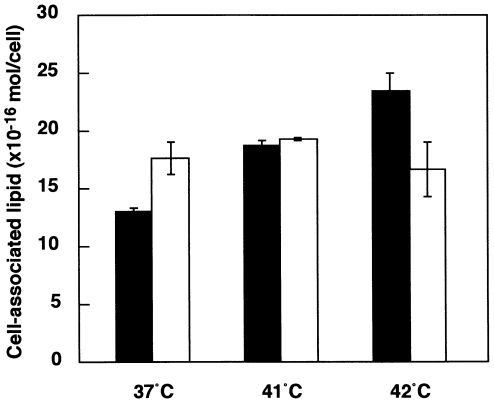

Fig. 2. The effect of temperature on uptake of polymer-modified (■) and unmodified (□) EYPC liposomes by CV1 cells after 3 h incubation in DMEM at varying temperatures. The bars represent the S.D. ($n=3$). Liposomes with a polymer content of 7.2 mol% were used. The concentration of liposomal lipid in the incubation was 0.6 mM.

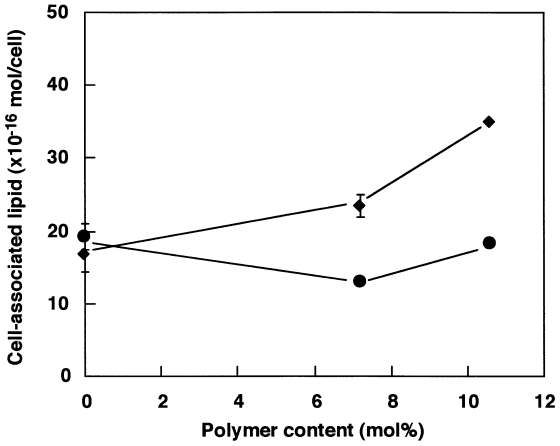

Fig. 3. Influence of the amount of the grafted polymer on uptake by CV1 cells at 37 (●) and 42°C (◆) in DMEM. The bars represent the S.D. ($n=3$). The concentration of liposomal lipid in the incubation was 0.6 mM.

covered by the hydrated polymer chains. The liposome with the polymer content of 10.6 mol% revealed a slightly higher uptake than the liposome with the polymer content of 7.2 mol%. As the surface density of the polymer chain on the liposome increases, its conformation might change from the mushroom structure to a stretched brush structure [25,26]. Such a conformational change of the grafted polymer might affect the liposome-cell interaction. By contrast, at 42°C, the amount of cell-associated liposome increased with an increasing polymer content. Above the LCST, the polymer chain takes on a dehydrated globule, which is much smaller than the hydrated coil structure [18]. Probably, for the liposome with the polymer content of 7.2 mol%, the liposome surface is not fully covered with the polymer chains when they are in the globule state. Therefore, the liposome with the higher amount of the grafted polymer should have a more hydrophobic surface, resulting in further enhancement of the uptake.

Since serum proteins bound on liposome surface are known to affect liposome-cell interactions [27], uptake of the liposomes by CV1 cells was examined in the presence of serum (Fig. 4). In comparison with the liposome uptake in the absence of serum, the uptake was significantly reduced in the presence of serum, probably due to adsorption of serum proteins on the liposome surfaces. However, promotion of the liposome uptake was observed for the polymer-modified liposomes at 42°C, compared with the uptake at 37°C and the case of unmodified liposomes as well.

We have reported that an efficient contents release occurs from thermo-sensitive polymer-modified liposomes of dioleoylphosphatidylethanolamine above the polymer's LCST [10–12]. However, when phosphatidylcholine is used as the liposomal lipid, only a limited portion of the contents is released from thermo-sensitive polymer-modified liposomes under the same condition [3,12,15]. In this study, we examined retention of calcein encapsulated in the polymer-modified EYPC liposomes and found that 81 and 91% of the loaded calcein molecules were retained after 3 h incubation in DMEM with and without 10% FBS at 42°C, respectively. Also, we did not observe a remarkable difference in their release behaviors at 37 and 42°C, indicating that the temper-

ature-dependent hydrophobicity change of the polymer hardly influences the contents release from the liposomes.

Since it was shown that the polymer-modified EYPC liposomes can retain hydrophilic molecules at 37 and 42°C, we investigated temperature-induced control of delivery of MTX to CV1 cells mediated by the polymer-modified EYPC liposomes. MTX, which has been used for the treatment of various malignant diseases [28], prohibits proliferation of cells by binding tightly to cytoplasmic dihydrofolate reductase. Because MTX can be readily entrapped in liposomes, many studies have been performed about their use as potential carrier systems for MTX [24,29,30].

The effect of the polymer-modified and the unmodified EYPC liposomes encapsulating MTX on growth of CV1 cells was explored. These liposomes were added to a prescribed number (ca. 5×10^4) of the cells and incubated for 10, 30 or 60 min at 37 or 42°C. After washing with PBS-CM, the cells were incubated for another 24 h at 37°C and then, the cell number was measured (Fig. 5). For the cells treated with the unmodified liposomes, the cell growth was partly prohibited. The prohibition became more significant as the period of the liposome treatment increased. While it seems that the liposome treatment at 42°C was slightly more efficient for prohibition of the cell growth than the treatment at 37°C, this difference was not remarkable. For the polymer-modified liposomes, however, a significant difference in the cell growth was seen between the cells treated at 37°C and those treated at 42°C. When the cells were incubated with the liposomes at 37°C, prohibition of the cell proliferation hardly occurred even for the cells having the 60 min incubation. In contrast, when the treatment was done at 42°C, the cell growth was completely prohibited even by the 10 min treatment. It was confirmed that the cell growth was not influenced by the treatment with the liposomes which did not contain MTX at 37 and 42°C. Also, free MTX at this concentration did not inhibit cell proliferation at 37 and 42°C. Thus, it is unlikely that MTX leaked out of the liposomes at 42°C effected the cell growth. Therefore, it can be considered that the grafted polymer chains with a hydrophobic nature promoted the liposome-cell interaction and a larger quantity of MTX was delivered into the cells at 42°C than at 37°C. In fact, a 2.3 times larger amount of liposomal lipid was found in the cells treated with the polymer-modified liposomes at 42°C, compared to the cells treated at 37°C (Fig. 4).

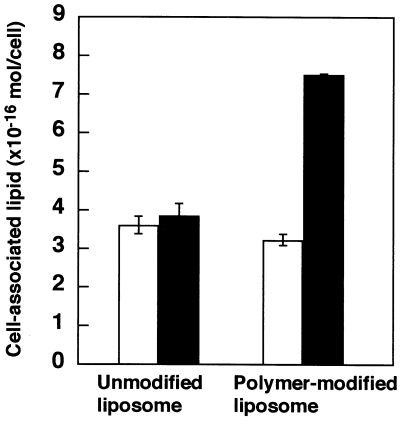

Fig. 4. Uptake of polymer-modified and unmodified EYPC liposomes by CV1 cells after 3 h incubation in DMEM with 10% FBS at 37 (□) and 42°C (■). The bars represent the S.D. ($n=3$). Liposomes with a polymer content of 10.6 mol% were used. The concentration of liposomal lipid in the incubation was 0.6 mM.

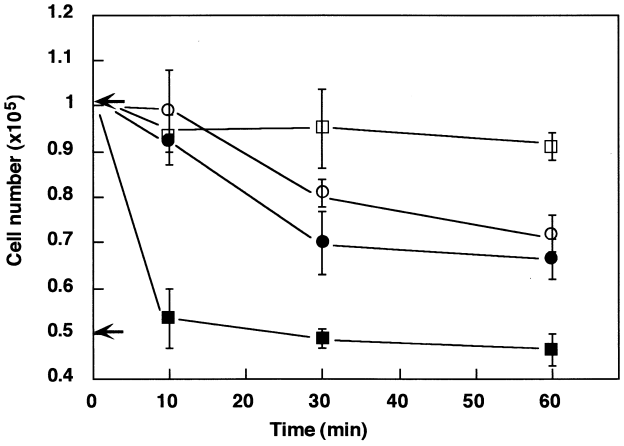

Fig. 5. The effect of MTX-loaded liposomes on growth of CV1 cells. The cells were incubated with polymer-modified (□, ■) or unmodified (○, ●) EYPC liposomes encapsulating MTX in DMEM with 10% FBS for various periods at 37 (open symbols) or 42°C (closed symbols). The cells were washed with PBS-CM and incubated for 24 h at 37°C. The cell number after the 24 h incubation was shown as a function of the initial incubation time. The lower and upper arrows represent the initial number of cells and the number of cells after the 24 h incubation at 37°C without the liposome treatment, respectively. Liposomes with a polymer content of 10.6 mol% were used. The concentrations of liposomal lipid and MTX in the incubation were 0.54 mM and 6.2×10^{-7} M, respectively.

As is seen in Fig. 4, uptake of the polymer-modified liposomes by CV1 cells was suppressed at 37°C, compared with the uptake at 42°C. However, the cells took up the polymer-modified liposomes and the unmodified liposomes approximately to the same extent at 37°C. Nevertheless, MTX encapsulated in the polymer-modified liposomes was much less effective than that entrapped in the unmodified liposomes. Since MTX must be taken up in cytoplasm to be active, it might be necessary for the anionic MTX to be delivered into low-pH compartments, such as the lysosome, where MTX should be protonated and thus diffuse into cytoplasm [29]. O'Brien and collaborators showed that attachment of polyethylene glycol chains to liposome surface reduces uptake of liposomes in low-pH compartments of HeLa cells [22]. Thus, even though the same amount of the liposome was taken up by CV1 cells during the incubation, a smaller fraction of the liposomes might be taken up in low-pH compartments for the polymer-modified liposomes than for the unmodified liposomes. At present, the reason why the activity of these MTX-loaded liposomes is different is unclear. However, it seems that the location of the liposomes in the cell affects the appearance of the MTX activity.

The present study demonstrated that interaction of the poly(APr-co-NIPAM)-$2C_{12}$-modified EYPC liposomes with CV1 cells varied, depending on the temperature. The interaction of the polymer-modified liposomes with cells can be enhanced by increasing the temperature slightly above the physiological temperature. Therefore, these liposomes may have potential usefulness for site-specific drug delivery.

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