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Cytoplasmic delivery of calcein mediated by liposomes modified with a pH-sensitive poly(ethylene glycol) derivative

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Abstract

Previously, as a new type of pH-sensitive liposome, we prepared egg yolk phosphatidylcholine (EYPC) liposomes bearing succinylated poly(glycidol), that is a poly(ethylene glycol) derivative having carboxyl groups, and showed that fusion ability of the liposomes increases under weakly acidic and acidic conditions (Kono, K., Zenitani, K. and Takagishi, T. (1994) Biochim. Biophys. Acta 1193, 1–9). In this study, we examined intracellular delivery of a water-soluble molecule, calcein, mediated by the succinylated poly(glycidol)-modified liposomes. When CV-1 cells, an established line of African green monkey kidney cells, were incubated with bare EYPC liposomes containing calcein at 37°C, only weak and vesicular fluorescence of calcein was observed by using a fluorescence microscope. In contrast, the cells treated with the polymer-modified liposomes containing calcein displayed more intensive and diffuse fluorescence, indicating that calcein was transferred into the cytoplasm. Uptake of the polymer-modified liposomes by the cells was shown to decrease slightly as amount of the polymer fixed on the liposome increases. However, the fluorescence of calcein observed in the liposome-treated cell was, on the contrary, enhanced as amount of the polymer fixed on the liposome increases, indicating that the liposome modified with a higher amount of the polymer transfers its content into cytoplasm more efficiently after internalization into the cell. Fusion assay by resonance energy transfer using *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine and lissamine rhodamine B-sulfonylphosphatidylethanolamine suggested occurrence of fusion between the polymer-modified liposomes and endosomal and/or lysosomal membranes. Moreover, the liposome with a higher polymer content revealed higher percent fusion after internalization into the cell. These results imply that the polymer-modified liposomes transfer the content into the cytoplasm by fusing with the endosomal membrane after internalization into the cells through an endocytic pathway.

Keywords: Poly(ethylene glycol) derivative; pH-sensitive liposome; Cytoplasmic delivery; Membrane fusion; Phosphatidylcholine

Abbreviations: SucPG, succinylated poly(glycidol); EYPC, egg yolk phosphatidylcholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PBS-CM, phosphate-buffered saline containing calcium and magnesium

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1. Introduction

pH-sensitive liposomes have been developed to improve efficiency of the cytoplasmic delivery of various polar molecules and macromolecules and many studies have shown effectiveness of pH-sensitive liposomes as a carrier for these molecules [1,2]. pH-sensitive liposomes are stable under a neutral condition, but are destabilized and become fusogenic under an acidic condition. Therefore, it is thought that after uptake by cells through an endocytic pathway, the liposomes exposed to the acidic pH of the endosome destabilize and/or fuse with the endosomal membrane and then release their contents into the cytoplasm without degradation by the lysosomal enzymes [3].

So far, a number of pH-sensitive liposomes have been prepared using unsaturated phosphatidylethanolamine (PE) and amphipathic stabilizers [4–10]. While their efficiency as a cytoplasmic delivery system has been shown to be superior to pH-insensitive liposomes, stability of the liposomes is relatively poor as compared to phosphatidylcholine (PC)-based liposomes [11,12]. Moreover, it is known that extensive leakage of contents from the PE-based liposomes occurs during fusion [5,7,9]. These problems should be attributable partly to their high tendency to revert to the hexagonal II phase.

As another approach for preparing pH-sensitive liposomes, modification of liposomes with molecules that generate fusogenic activity depending on pH has been attempted [13–15]. It is well known that several viral fusion proteins, such as hemagglutinin of influenza virus, induce membrane fusion efficiently [16]. Also, fusion peptides of viral proteins and their synthetic analogs have been shown to reveal fusogenic activity similar to the viral proteins [17–19]. Furthermore, a number of synthetic, amphipathic peptides [20–23] have been shown to reveal fusogenic activity. Because these molecules are able to induce fusion of vesicles made from PC which form a stable bilayer membrane, it is possible to prepare fusogenic liposomes with high stability by conjugating these molecules to PC-based liposomes. A synthetic, amphipathic 30-residue peptide with a repeat unit of Glu-Ala-Leu-Ala, GALA, was designed by Szoka et al. to mimic the behaviors of the fusion peptides of viral fusion proteins [24] and they showed that GALA induces fusion of PC liposomes in a pH-dependent fashion [20]. Puyal et al. designed a 14-residue GALA-type peptide-attached liposomes and showed that intermixing of lipid between the liposomes occurs in a pH-dependent fashion [14]. Recently, Vogel et al. also prepared liposomes bearing a 30-residue peptide with a sequence similar to that of GALA and demonstrated that the liposomes release the content into cytoplasm after internalization into cells by endocytosis [15].

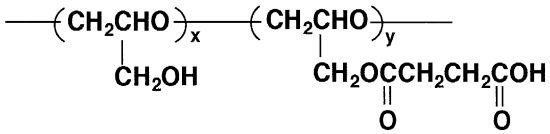

Fig. 1. Structure of succinylated poly(glycidol).

On the other hand, poly(ethylene glycol) is a well-known fusogenic polymer and hence, modification of liposomes with this polymer is expected to give the liposomes with ability to fuse. In a previous study [13], we synthesized succinylated poly(glycidol) (SucPG) (Fig. 1) as a fusogenic polymer with pH-sensitivity. This polymer has a main chain structure similar to that of poly(ethylene glycol). Also this polymer has carboxyl groups on the side chains. It was shown that fusion ability of egg yolk phosphatidylcholine (EYPC) liposomes bearing the polymer is elevated with decreasing pH because ionized carboxylate groups on the polymer chain become protonated and hence the polymer interacts with the liposomal membrane strongly.

Since fusion ability of SucPG-modified EYPC liposomes increases under an acidic condition, it is expected that after internalization into cells through an endocytic pathway, the liposomes transfer their content into the cytoplasm by fusing with the endosomal membranes. Thus, in this study, we examined whether this novel pH-sensitive liposome is able to deliver its content into cytoplasm as the conventional pH-sensitive liposome, namely PE-based one, does.

2. Materials and methods

2.1. Materials

Poly(epichlorohydrin) was purchased from Aldrich (Milwaukee, WIS). Egg yolk phosphatidylcholine

(EYPC) was obtained from Nippon Fat and Oil (Tokyo, Japan). Calcein was supplied by Sigma (St. Louis, MO). Fluorescent lipids, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were from Avanti Polar Lipids (Birmingham, AL). Dulbecco's modified Eagle's medium (DMEM) was from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT). All other reagents were of analytical grade.

2.2. Synthesis of SucPG having long alkyl chains

Succinylated poly(glycidol) was synthesized according to the method previously reported [13]. In brief, poly(glycidol) was synthesized via the method of Cohen [25] by the conversion of poly(epichlorohydrin). The most frequent molecular weight of poly(glycidol) obtained was estimated to be 4600, taking poly(ethylene glycol) as a standard by high-performance liquid chromatography on Asahipak GS-510 column. SucPG was obtained by the reaction of poly(glycidol) with succinic anhydride (1.5 equivalent to hydroxyl groups of the polymer) in *N*,*N*-dimethylformamide at 60°C for 3 h. Unit mol percent of succinylated residue in the resultant polymer was determined to be 94 by ^{1}H-NMR (JEOL JNM-GX-270).

Decylamine was attached to SucPG as anchor to liposomal membranes as follows. SucPG was dissolved in water at pH 5. *n*-Decylamine (0.1 equivalent to carboxyl groups of SucPG) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (0.1 equivalent to carboxyl groups of SucPG) were added to the solution at 4°C and the solution was stirred overnight. The resultant polymer was purified by liquid chromatography on a Sephadex LH-20 column using methanol. Unit mol percent of *n*-decylamine-attached residue in the resultant polymer was determined to be 6 by ^{1}H-NMR.

2.3. Cell culture

CV-1 cell, an African green monkey kidney cell line, was cultured in DMEM supplemented with 10% FBS in a humidifier incubator (5% CO_2) at 37°C. The cells were maintained in monolayer culture.

2.4. Preparation of SucPG-modified liposomes containing calcein

SucPG-modified liposomes were prepared as previously reported [13]. A dry thin membrane of mixture of EYPC and SucPG was made by evaporation of solution of the lipid and the polymer in methanol, and subsequent drying under vacuum. The membrane was dispersed in aqueous 200 mM calcein solution (pH 7.4) and was sonicated for 30 min using a ultrasonic disruptor (Tomy Seiko, UD-200). Free calcein was removed by gel chromatography on a Sephadex G-75 (Pharmacia, Sweden) column using phosphate-buffered saline (PBS) consisting of 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl (pH 7.4) supplemented with 0.36 mM calcium and 0.42 mM magnesium. Bare EYPC liposome containing calcein was prepared according to the above procedure using dry membrane of EYPC without the polymer.

2.5. Fluorescence microscopy of cells treated with liposomes

CV-1 cells grown on cover slips for 24 h were washed three times with PBS containing 0.36 mM calcium and 0.42 mM magnesium (PBS-CM). For experiments in which chloroquine was used, the appropriate volume of 10 mM chloroquine solution was added to the original growth medium 1 h prior to the wash with PBS. Chloroquine was present in all buffers thereafter.

Cells were incubated with liposomes containing 200 mM calcein at 1.5 mmol lipid/l for 3 h. After the incubation, the cells were washed 5 times with PBS-CM and viewed with an Olympus microscope (IMT-2) equipped with a phase contrast and epifluorescence with an excitation filter set that produces excitation in the range 470–500 nm and allows observation of fluorescence emission in the range 515–540 nm with a long wave pass dichroic mirror and barrier filter.

2.6. Evaluation of liposome uptake by cells

Liposomes containing NBD-PE were prepared via the above method using EYPC/NBD-PE (95:5,

mol/mol) as the membrane lipids. Cells (5×10^5) were plated in 25 cm^2-flask containing 5 ml of DMEM-10% FBS 48 h prior to the experiments. The cells were washed three times with PBS-CM and then incubated with the liposomes (1.0 mM) dispersed in PBS-CM at 37°C or 4°C for desired periods.

After incubation, cells were washed five times with PBS-CM and once with PBS and were dislodged by treatment with PBS containing 1 mM EDTA for 5 min. Fluorescence intensity of NBD-PE associated with the cell at 525 nm was measured at excitation wavelength of 450 nm by using a spectrofluorometer (Shimadzu RF-5000). For calibration, the fluorescence intensities for the liposome suspensions alone were also measured. Amount of liposomes associated with a cell was estimated from the fluorescence intensity of a cell using the calibration curve. It was confirmed that fluorescence intensity of NBD-PE is proportional to concentration of NBD in the lipid membrane under the experimental condition, indicating that self-quenching of NBD-PE is negligible. The cell number was determined by estimating cell protein concentration by the Lowry assay [26,27].

2.7. Fluorometric detection of fusion between liposomes and cellular membranes

Fusion between liposomes and cellular membranes was detected by measuring resonance energy transfer between NBD-PE and Rh-PE [28]. Liposomes containing NBD-PE and Rh-PE were prepared via the above method using EYPC/NBD-PE/Rh-PE (94:5:1, mol/mol/mol) as the membrane lipids. Cells (5×10^5) were plated in 25 cm^2-flask containing 5 ml of DMEM-10% FBS 48 h prior to the experiments. The cells were washed three times with PBS-CM and were incubated with the liposomes (1.0 mM) dispersed in PBS-CM at 37°C or 4°C for 3 h.

After the incubation, cells were washed five times with PBS-CM and once with PBS and were dislodged by exposing the cells to PBS containing 1 mM EDTA for 5 min. Fluorescence emission spectra associated with the cell suspension irradiated at 450 nm were measured. Fraction of liposomes fused and/or degraded was determined from the fluorescence spectra as described in the results and discussion section. We assumed that ratio of fluorescence intensity of NBD-PE to that of Rh-PE for the liposomes taken up by the cells is expressed as Eq. (1).

$$R = \left[I_T^{NBD} \times F + I_0^{NBD} \times (1 - F) \right] / \left[I_T^{Rh} \times F + I_0^{Rh} \times (1 - F) \right] \quad (1)$$

where R represents ratio of NBD-PE fluorescence intensity at 522 nm to Rh-PE fluorescence intensity at 586 nm for the liposome-treated cell suspension. I_0^{NBD}, I_0^{Rh}, I_T^{NBD}, and I_T^{Rh} are fluorescence intensities of NBD-PE and Rh-PE associated with the liposome suspension used for liposome-cell incubation before and after addition of Triton X-100 (final concentration 0.24%), respectively. F expresses fraction of liposomes fused and/or degraded in the liposome-treated cells. F value was determined using Eq. (2) which is derived from Eq. (1).

$$F = \left(R \times I_0^{Rh} - I_0^{NBD} \right) / \left[R \times \left(I_0^{Rh} - I_T^{Rh} \right) - \left(I_0^{NBD} - I_T^{NBD} \right) \right] \quad (2)$$

Autofluorescence and influence of addition of the detergent were corrected from these values.

For observation using a fluorescence microscope, incubation of cells was performed as mentioned above except that cells were grown on cover slips. After incubation with liposomes containing NBD-PE and Rh-PE (1.5 mM) for 3 h, the cells were washed 5 times with PBS-CM and viewed with the microscope with an excitation filter set that produces excitation in the range of 470–500 nm and allows observation of fluorescence emission above 515 nm.

2.8. Other methods

The phospholipid concentration was determined by an assay using Phospholipids B-Test Wako reagent supplied by Wako (Osaka, Japan). The size of the vesicles was measured by dynamic light scattering (Otsuka Electronics, DLS-700).

3. Results and discussion

3.1. Delivery of calcein into CV-1 mediated by SucPG-modified liposomes

Previously, we demonstrated that fusion ability of EYPC liposomes modified with SucPG increases be-

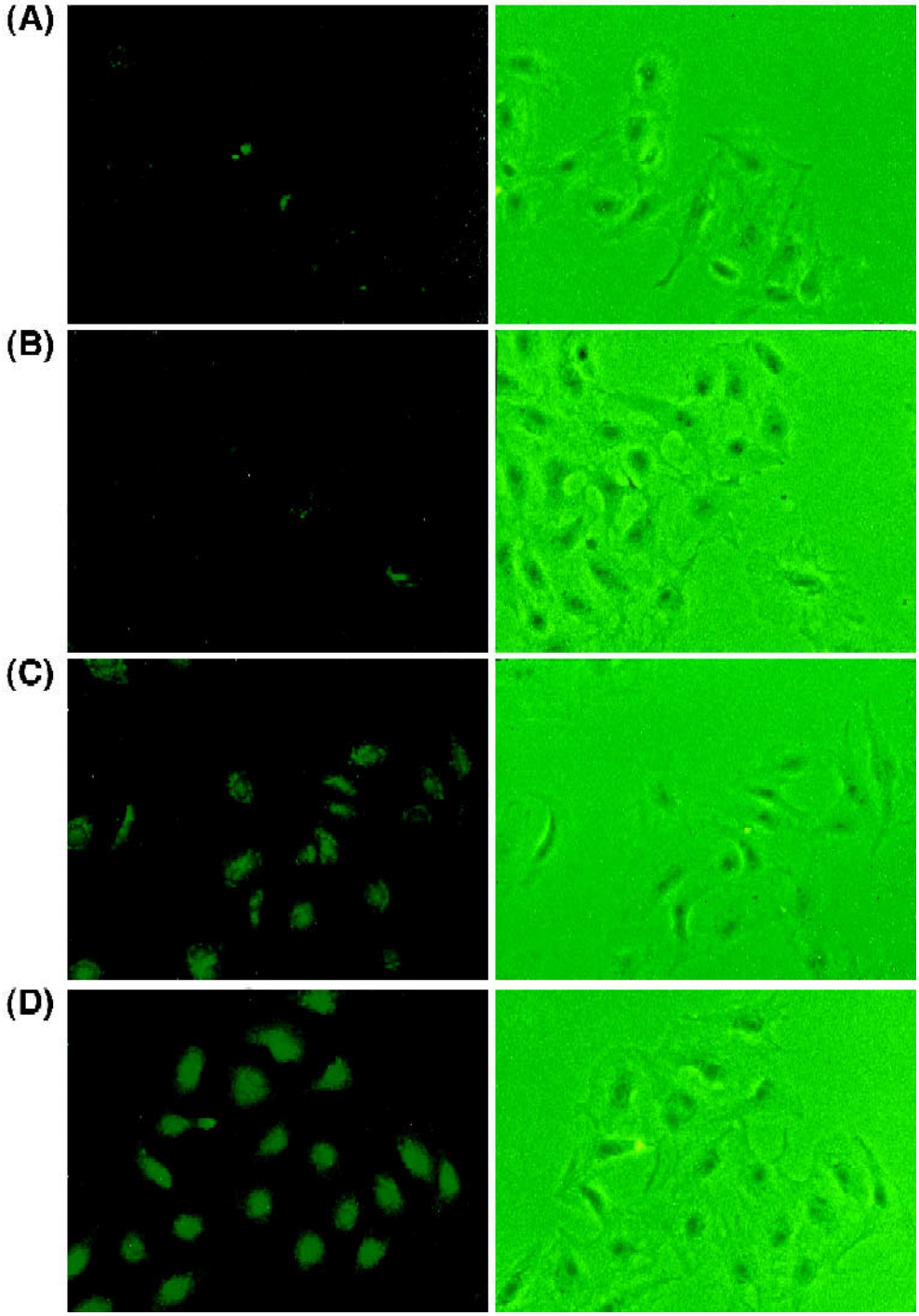

Fig. 2. Fluorescence micrographs (left) and phase contrast micrographs (right) of CV-1 cells treated with various EYPC/SucPG liposomes containing calcein. Ratios of EYPC to SucPG (w/w) for the liposomes are 10:0 (A), 9:1 (B), 8:2 (C), and 7:3 (D). The cell-liposome incubation was performed in phosphate-buffered saline containing calcium and magnesium for 3 h at 37°C. The concentration of liposomal lipid in the incubation was 1.5 mM. Time of exposure to photographic film for these fluorescence micrographs was 28 s.

low pH 6 [13]. Therefore, it was expected that after uptake by cells through an endocytic pathway, the polymer-modified liposomes transfer their contents into the cytoplasm by fusing with the endosomal membrane. Thus, the polymer-modified liposome-mediated delivery of a membrane-impermeable molecule, calcein, into cells was examined. Here, we chose CV-1 because interaction of the cell with liposomes has been well characterized [29–31].

Fusion ability of SucPG-modified liposomes under an acidic condition has been shown to increase with increasing amount of the polymer [13]. Therefore, if the polymer-modified liposomes transfer the content by fusing with endosome, efficiency of cytoplasmic delivery by the liposomes is expected to depend on the amount of the polymer fixed on the liposomes. In the previous study [13] it was shown that liposomes prepared from a mixture of SucPG and EYPC with a ratio of up to 3:7 (w/w) carries the polymer supplied quantitatively and tightly. Thus, four kinds of liposomes consisting of EYPC and SucPG in the ratio of 10:0, 9:1, 8:2, 7:3 (w/w) were prepared. Mean diameters of these liposomes were estimated to be 40.1, 40.3, 44.9, and 47.9 nm, respectively, by dynamic light scattering. The diameter increased slightly as the polymer content increased. Since we used poly(glycidol) with peak molecular weight of 4600,

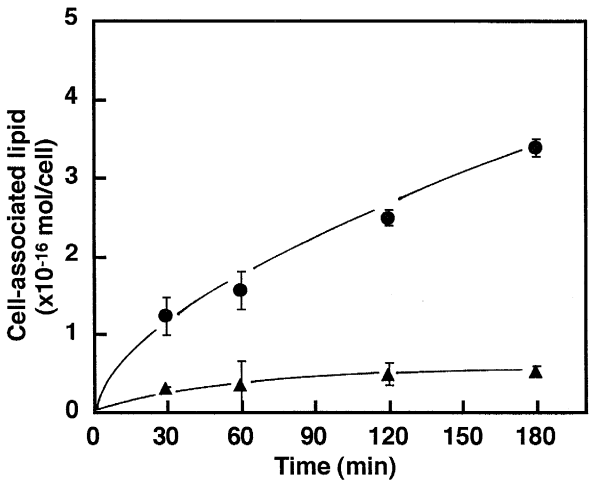

Fig. 3. Uptake kinetics for SucPG/EYPC (7:3, w/w) liposome by CV-1 cell in phosphate-buffered saline containing calcium and magnesium at 37°C (●) and at 0°C (▲). The concentration of liposomal lipid in the cell-liposome incubation was 1.0 mM. The experiments at 37°C and at 0°C were done in triplicate and in duplicate, respectively.

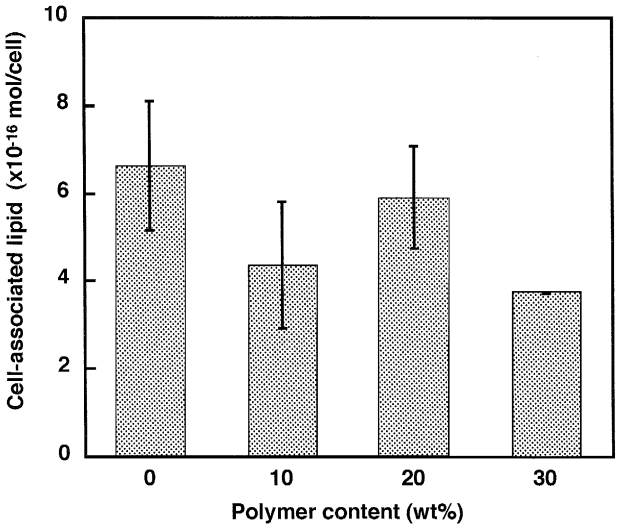

Fig. 4. Influence of SucPG content in the liposomal membrane on uptake by CV-1 cell. The cell-liposome incubation was performed in phosphate-buffered saline containing calcium and magnesium for 3 h at 37°C. The concentration of liposomal lipid in the incubation was 1.0 mM. The experiments were done in triplicate.

molecular weight of SucPG can be calculated to be ca. 10000. It is likely that fixation of the polymer onto the liposome increases apparent size of the liposome.

Fig. 2 shows fluorescence and phase contrast micrographs of CV-1 cells treated with these liposomes containing calcein. When the cells were incubated with the bare EYPC liposome, weak and vesicular fluorescence of calcein was observed. Similarly, vesicular fluorescence was seen in the cells after incubation with an aqueous calcein solution (data not shown). In contrast, when the cells were treated with the SucPG-modified liposomes, fluorescence seen in the cells was quite different. The cells treated with EYPC/SucPG (7:3) liposomes displayed strong and diffuse fluorescence of calcein. This result suggests that calcein molecules were transferred into cytoplasm for the cells treated with the SucPG-modified liposome, whereas calcein molecules existed in endosome and/or lysosome for the cells treated with the bare liposome or with calcein solution. Moreover, the cells exhibited more intensive fluorescence when the cells were treated with the liposome with a higher content of the polymer, suggesting that the liposome with a higher amount of the polymer delivers the content into cytoplasm more efficiently.

Fig. 3 shows the uptake kinetics for the SucPG-modified liposome by the cells. The amount of the liposome associated with the cells is constant and independent of time when the incubation is performed at 0°C. Since endocytosis is inhibited at 0°C, the liposome is likely to bind onto the cell membrane under this condition. In contrast, at 37°C the amount of liposome associated with the cell increases with time, indicating that the liposome is taken up by the cell through an endocytic pathway at 37°C, as shown in previous studies [29–31].

As mentioned above, delivery of calcein is affected by SucPG content of the liposome. Therefore, influence of the polymer content on uptake by the cell was examined. The result is shown in Fig. 4. The amount of the liposome taken up by the cells appears to decrease slightly as the polymer content in the liposomal membrane increases. Since surface of the liposome bearing a higher amount of the polymer should be covered more effectively by the highly hydrophilic polymer chains, the polymer chains attached on the liposome surface might reduce interaction between the liposome and the cell and suppress uptake by the cell. Also, difference in diameter of the liposome may affect uptake by the cells, although the difference is not significant.

As shown in Fig. 4, the bare liposome-treated cells contained the highest amount of the liposome among these liposome-treated cells. Nevertheless, the bare liposome-treated cells displayed much weaker fluorescence of calcein than the cells treated with the polymer-modified liposomes as shown in Fig. 2. Already, we showed that the modification of the polymer hardly affect the captured volume of the liposomes [13]. Therefore, it is considered that most calcein molecules were still trapped and self-quenched in the bare liposome in the cell. On the contrary, SucPG/EYPC (3:7) liposome-treated cells contained the lowest amount of the liposome, but displayed the most intensive fluorescence, suggesting that the liposome can transfer their content into the cytoplasm most efficiently.

3.2. Fusion between SucPG-modified liposomes and endosomal / lysosomal membranes

We expected that the liposomes, after uptake by cells through an endocytic pathway, deliver the content into cytoplasm by fusing with endosomal and/or lysosomal membranes because the inside of these compartments is acidic. In fact, CV-1 cells treated with the SucPG-modified liposomes containing calcein exhibited diffuse fluorescence, indicating calcein molecules were transferred into cytoplasm. If the polymer-modified liposomes transfer their content into the cytoplasm by fusing with the endosomal and/or lysosomal membranes, transfer of calcein from the endosome into the cytoplasm will be interfered in the presence of chloroquine because it effectively elevates the endosomal and lysosomal pH [32,33] and hence will prevent the liposomes from becoming fusogenic.

Fig. 5 depicts fluorescence and phase contrast micrographs of CV-1 cells treated with EYPC/SucPG (7:3) liposome containing calcein in the presence of chloroquine. Apparently, fluorescence intensity of calcein observed in the cells is significantly reduced in the presence of chloroquine, compared with that in the absence of chloroquine shown in Fig. 2, although the fluorescence does not disappear completely. Because fusion ability of the SucPG-modified liposomes increases with decreasing pH from neutral pH [13], under even slightly acidic condition the liposomes become fusogenic to some extent and hence, some fraction of the liposomes might be destabilized and fuse with the endosomal and/or lysosomal membranes. Alternatively, there may be another pathway to deliver calcein into cells, such as direct fusion between the plasma membrane and the liposomes. However, this result indicates that at least predominant pathway for generation of calcein fluorescence requires an acidic environment.

Connor and Huang reported that when L929 cells were treated with calcein-loaded liposomes composed of dioleoylphosphatidylethanolamine and palmitoylhomocysteine, namely pH-sensitive liposome of conventional type, vesicular fluorescence of calcein was observed in the cells in the presence of chloroquine, instead of diffuse fluorescence which was seen in the absence of chloroquine, because acid-induced fusion of the liposome with endosomal membrane does not occur in the former case [34]. However, in this study we observed significant reduction of fluorescence intensity of calcein instead of generation of vesicular fluorescence they observed. This is possibly due to difference in stability of these liposomes. In fact, a

large part of EYPC liposomes were still not degraded in the cell after 3 h-incubation even in the absence of chloroquine as shown below. Therefore, it is likely that in the presence of chloroquine the polymer-modified liposomes retain the content in their inside. Similar effect was seen when the cells were treated

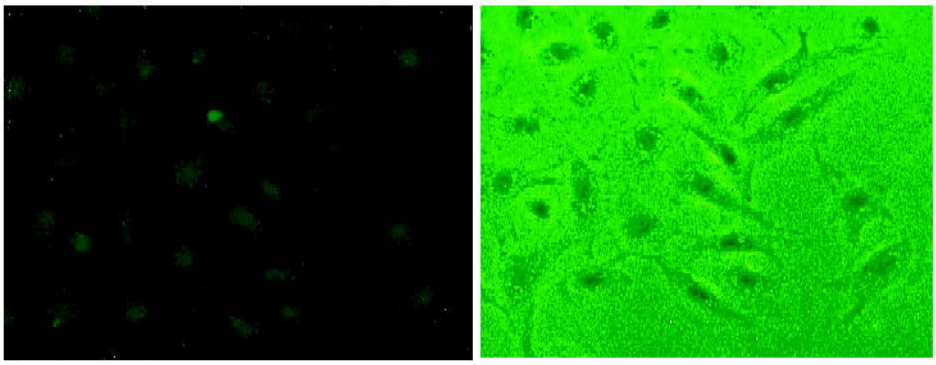

Fig. 5

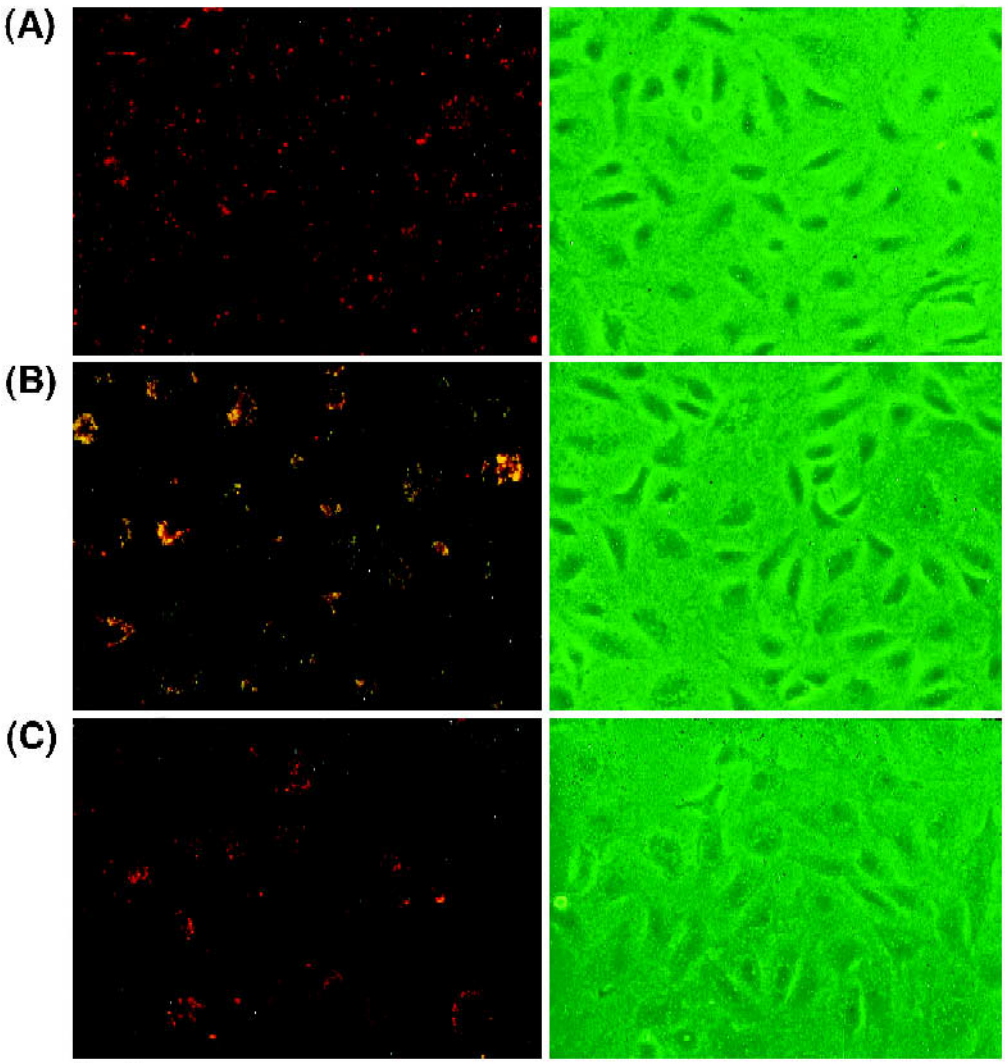

Fig. 6

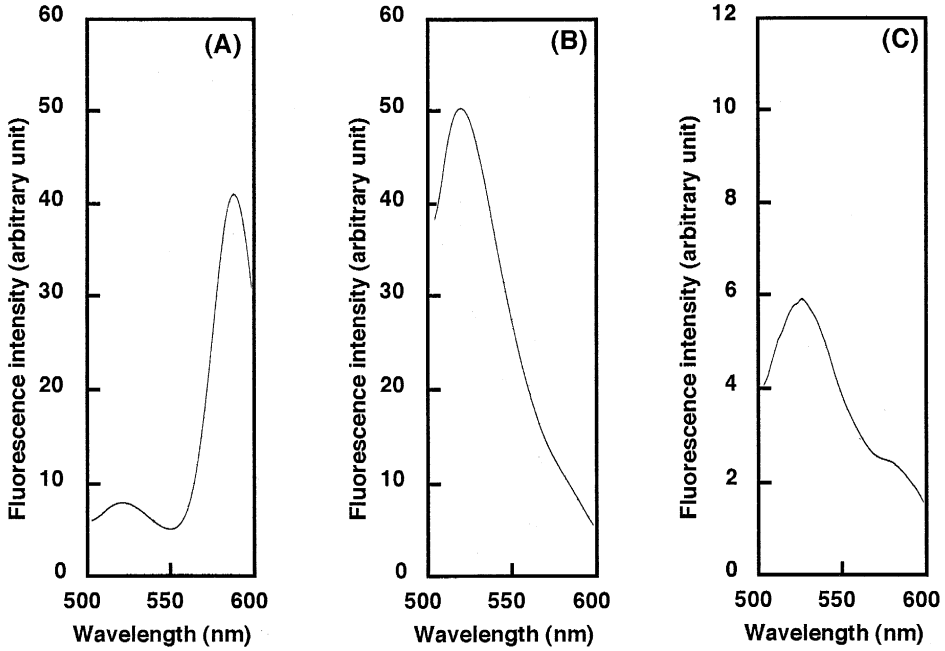

Fig. 7. Fluorescence emission spectra of EYPC/SucPG (7:3, w/w) liposomes containing NBD-PE and Rh-PE: (A) emission spectra of the starting liposome; (B) the liposome solubilized by adding Triton X-100 (final concentration: 0.24%); (C) the liposome-treated cells. λex: 450 nm. The cell-liposome incubation was performed in phosphate-buffered saline containing calcium and magnesium for 3 h at 37°C. The concentration of liposomal lipid in the incubation was 1.0 mM.

with the liposomes in the presence of ammonium chloride, which is also known to increase the endosomal pH [35] (result not shown).

In order to confirm the occurrence of fusion between SucPG-modified liposomes and the endosomal and/or lysosomal membranes, fusion assay by resonance energy transfer was performed [28]. The liposomes containing NBD-PE and Rh-PE were prepared and CV-1 cells were incubated with the liposomes. Fig. 6 represents fluorescence and phase contrast micrographs of the cells treated with the liposomes containing the fluorescent lipids when NBD-PE was excited (λex: 470–500 nm). These fluorescence micrographs reveal striking difference. In the cells treated with bare EYPC liposome displays vesicular, red fluorescence, namely fluorescence of Rh-PE, indicating that in a large fraction of liposomes energy transfer from NBD-PE to Rh-PE occurs efficiently. This suggests that most liposomes are still intact even after 3 h incubation. In contrast, vesicular, yellow

Fig. 5. Fluorescence micrograph (left) and phase contrast micrograph (right) of CV-1 cells treated with EYPC/SucPG (7:3, w/w) liposome containing calcein. The cell-liposome incubation was performed in phosphate-buffered saline containing calcium and magnesium for 3 h at 37°C in the presence of 0.2 mM chloroquine. The concentration of liposomal lipid in the incubation was 1.5 mM. Time of exposure to photographic film for the fluorescence micrograph was 31 s.

Fig. 6. Fluorescence micrographs (left) and phase contrast micrographs (right) of CV-1 cells treated with various liposomes containing NBD-PE and Rh-PE: (A) bare EYPC liposome; (B) and (C) EYPC/SucPG (7:3, w/w) liposome. The cell-liposome incubation was performed in phosphate-buffered saline containing calcium and magnesium in the absence (A and B) or presence (C) of chloroquine (0.2 mM) for 3 h at 37°C. The concentration of liposomal lipid in the incubation was 1.5 mM. Time of exposure to photographic film for these fluorescence micrographs was 11 s.

fluorescence, namely fluorescence of NBD-PE, can be seen in the cells treated with SucPG-modified liposome. In this case most liposomes are considered to fuse with cellular membranes and/or degraded in the cells. When incubation was performed in the presence of chloroquine, apparently fluorescence of Rh-PE is enhanced in the cells, compared to the cell treated in the absence of chloroquine, indicating that fusion and degradation of the polymer-modified liposomes are partly inhibited in the presence of chloroquine. This is consistent with the result of the liposome-mediated calcein delivery.

As is seen in Fig. 2, when the cells were treated with the calcein-loaded polymer-modified liposomes, the cells displayed diffuse fluorescence. However, when the liposomal membrane was labeled with the fluorescent lipids, vesicular fluorescence was observed in the liposome-treated cells. This result indicates that sites where the liposome content exists and where the liposomal lipid exists are different. It is considered that the liposomal lipids exist mainly in endosome and/or lysosome.

For quantitative estimation of fusion, emission spectra of the liposome-treated cells were investigated. Fig. 7 shows fluorescence emission spectra of the starting liposomes (A), the liposomes solubilized by adding Triton X-100 (B), and the liposome-treated cells (C). Apparently, increase of NBD-PE fluorescence near 520 nm and decrease of Rh-PE fluorescence near 585 nm can bee seen in the spectrum of the liposome-treated cells, compared to that of the starting liposomes. This change of the spectrum indicates reduction of surface density of NBD-PE and Rh-PE in the membranes, compared to that of the starting liposomes.

We assumed that there are two distinct fractions of the liposomes existing in the liposome-treated cells, namely the liposomes fused and those not fused with cellular membranes. When the liposome fuses with a cellular membrane, the liposome-derived lipids can freely intermix with those present in the cellular membrane and hence, efficiency of resonance energy transfer between NBD-PE and Rh-PE will decrease significantly. Therefore, these fluorescent lipids derived from the fused liposomes will reveal the fluorescence spectrum shown in Fig. 7B. Some portion of the liposomes taken up by the cells may be degraded in lysosome. The fluorescent probes derived from the degraded liposomes also might give the same spectrum. In contrast, the fluorescence lipids existing in the intact liposomes show the spectrum depicted in Fig. 7A.

Table 1
Fraction of liposome fused and/or degraded in cell

Liposome	Fraction
EYPC	0.31 ± 0.01
EYPC/SucPG (9:1, w/w)	0.68 ± 0.01
EYPC/SucPG (8:2, w/w)	0.74 ± 0.01
EYPC/SucPG (7:3, w/w)	0.78 ± 0.02

After 3 h incubation in PBS containing Ca^{2+} and Mg^{2+}. The experiments were done in duplicate.

Because the spectrum shown in Fig. 7C is considered to consist of the spectra shown in Fig. 7A and B, it is possible to estimate fraction of the liposomes fused or degraded in the cells. Fraction of fused or degraded liposome in the liposome-treated cells evaluated via the above procedure is listed in Table 1. Although bare EYPC liposome is not fusogenic, fused or degraded fraction was estimated to be 0.31 possibly because of degradation of liposomal lipids. However, the polymer-modified liposomes show considerably higher values, compared to bare liposome. Moreover, the liposome with a higher polymer content reveals a higher value. These values are thought to contain fraction of liposomes not fused but degraded. However, degradation of lipid of these liposomes might equally occur in the cells. Thus, this result suggests the liposome with a higher amount of the polymer fuses more easily with cellular membranes.

While so far pH-sensitive liposomes have been considered to deliver their contents into cytoplasm by liposome-endosome fusion [3], it is implied that SucPG-modified liposomes also transfer the content via the same mechanism by considering the following facts: (1) delivery of calcein by the liposomes was interfered by addition of chloroquine, (2) significant intermixing of lipids occurred between the liposomes and endosome and/or lysosome, (3) efficiency of delivery is correlated with fusion ability of the liposomes, (4) calcein was transferred into cytoplasm, whereas liposomal lipids existed in endosome and/or lysosome.

Recently, Vogel et al. reported that EYPC liposomes containing an amphipathic peptide, EALA, deliver a fluorescent dye into cytoplasm by interacting with endosomal membranes after internalization into KB cells [15]. They observed that fluorescence intensity of the dye seen in the liposome-treated cells increased gradually with time. The fluorescence in the cytoplasm was weak within 6 h, but increased continuously with time for more than 48 h. In this study, we observed that overnight incubation of the liposome-treated cells hardly affected intensity of the fluorescence in the cells. Moreover, as shown in Table 1, a large fraction of the SucPG-modified liposomes was already fused after 3 h-incubation. These facts imply that SucPG-modified liposomes may be able to achieve faster cytoplasmic delivery.

In conclusion, it was found that the SucPG-modified EYPC liposomes can transfer their content into cytoplasm by fusing with endosomal and/or lysosomal membranes. Efficiency of delivery mediated by the polymer-modified liposomes was shown to be elevated as the polymer content of the liposome increases. In this polymer-liposome system various kinds of lipids can be used as membrane components. In this study we used EYPC, which forms a stable bilayer membrane, as a liposomal lipid. However, if unsaturated PE is added as an additional membrane component, fusion ability of the liposomes is expected to increase. Also, conjugation of ligands or antibodies, which bind their receptors or antigens existing on the cell surface, to the polymer-modified liposomes is possible by using carboxyl groups on the polymer. Such conjugation will give the liposomes with target specificity. When an appropriate ligand which is internalized into endosome after binding to its receptor is combined to the liposomes, more efficient delivery is expected to be achieved. Therefore, this polymer-liposome system might have potential usefulness as a cytoplasmic delivery system with high stability and high efficiency.

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