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Novel pH-sensitive liposomes: liposomes bearing a poly(ethylene glycol) derivative with carboxyl groups

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Abstract

Three kinds of succinylated poly(glycidol)s were synthesized as poly(ethylene glycol) derivatives having carboxyl groups by the reaction of poly(glycidol) with varying amounts of succinic anhydride in dimethylformamide. These polymers promoted fusion of egg-yolk phosphatidylcholine liposome more intensively with decreasing content of carboxyl groups at pH 7.4, although the extent of fusion was limited. However, the polymer with 56% of succinylated residues induced fusion of the liposome much more strongly at pH 4.0. Egg-yolk phosphatidylcholine liposomes bearing the succinylated poly(glycidol) which was combined with long alkyl chains as anchors to the liposomal membrane were prepared. The leakage of calcein entrapped in the inner aqueous phase of the liposomes was slight at pH 7.4. However, the leakage increased with decreasing pH. The turbidity measurement and the fusion assay indicate that the liposomes bearing the polymer fuse more intensively with decreasing pH and with increasing amount of the polymer bound to the liposomes.

Key words: Membrane fusion; pH-sensitive liposome; Liposome; Phosphatidylcholine; Poly(ethylene glycol) derivative; Drug delivery system

1. Introduction

pH-sensitive liposomes have been developed to improve the efficiency of the cytoplasmic delivery of various polar materials and macromolecules such as antitumor drugs, proteins, and DNA, and the effectiveness of the liposomes as a delivery system for those compounds into cells has been shown by many studies [1–7]. These liposomes are stable at physiological pH, but after being internalized by cells through an endocytic pathway, the liposomes exposed to the acidic pH of the endosome are destabilized and possibly fuse with the endosome membrane, resulting in release of their contents into the cytoplasm without degradation by the lysosomal enzymes [8].

Most pH-sensitive liposomes have been prepared using unsaturated phosphatidylethanolamine (PE) as the phospholipid component [9–16], with some exceptions [17]. Unsaturated PE has a strong tendency to form a nonbilayer structure at neutral pH [18] and a great ability to support fusion [19–21]. Unsaturated PE does not form liposomes by itself, but liposomes can be prepared by adding another component to PE. As such stabilizers, titratable amphiphiles having a carboxyl group have been used for preparation of pH-sensitive liposomes [9–16]. Because the stabilizing ability of these amphiphiles decreases under acidic conditions, destabilization and fusion of the liposomes occur.

However, this type of pH-sensitive liposome has several disadvantages. The stability of the liposomes is relatively poor as compared to phosphatidylcholine-based liposomes. It has been reported that pH-sensitive liposomes composed of unsaturated PE and oleic acid rapidly aggregate and become leaky in the presence of plasma [22]. Destabilization of PE-based pH-sensitive liposomes is also caused by the interaction with human erythrocyte ghosts [23]. Furthermore, if the disintegration of the liposomes occurs in competition with fusion of the liposomes with the endosomal mem-

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Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; EYPC, egg-yolk phosphatidylcholine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; Rh-PE, lissamine rhodamine B-sulfonyl phosphatidylethanolamine.

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brane upon exposure to an acidic environment in the endosome, effective transport of drugs into the cytoplasm cannot be expected for the liposomes. In fact, extensive leakage of contents in the inner phase of the PE-based liposomes was observed when fusion of the liposomes occurs [10,13,15,24]. These problems should be attributable to their tendency to revert to the H_{II} phase. Several attempts to increase the stability of PE-based pH-sensitive liposomes were made by selecting titratable stabilizers [13,16], by adding cholesterol as a liposome component [25] or by treatment with human plasma [26].

Another approach for preparing stable pH-sensitive liposomes is to use a lipid with a higher bilayer-forming tendency such as phosphatidylcholine (PC). Several molecules, for example amphiphilic peptides [27–31] and viral fusion proteins [32], are known to induce fusion of PC liposomes in a pH-dependent fashion. Most of them acquired pH-sensitivity for fusion activity by altering the charge of the carboxyl groups of the acidic amino acid residues. Incorporation of fatty acids as membrane components has been shown to give a pH-dependent fusion property to PC liposomes, although these liposomes have not been applied as pH-sensitive liposomes [33,34]. Poly(ethylene glycol) is a well-known fusogenic polymer [35] and has been used to increase the fusion ability of egg-yolk phosphatidylcholine (EYPC) liposomes by incorporation into the liposome membrane [36]. Incorporation of carboxyl groups into the polymer might render its fusion activity pH-sensitive. By combining PC liposomes with such molecules, pH-sensitive liposomes can be made.

In the present work, we have synthesized poly(glycidol) with various amounts of succinylated residues (Fig. 1) as poly(ethylene glycol) derivatives having carboxyl groups and we did prepare EYPC liposomes bearing the derivatives. The fusogenic activities of the poly(ethylene glycol) derivatives and the fusion behavior of the liposomes bearing the derivatives have been investigated.

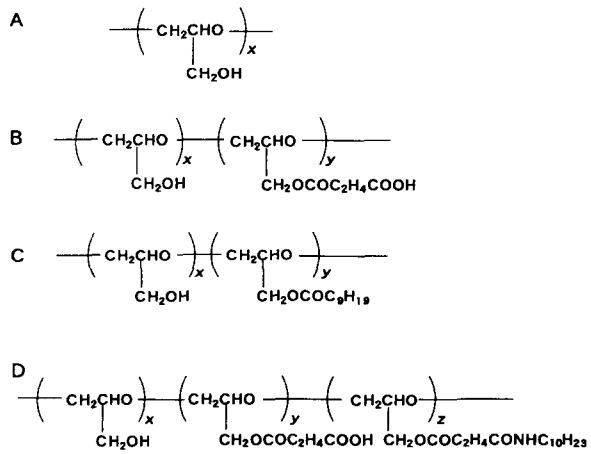

Fig. 1. Structures of poly(glycidol) (A), succinylated poly(glycidol) (B), poly(glycidol) having alkyl chains (C), and succinylated poly(glycidol) having alkyl chains (D).

2. Materials and methods

2.1. Materials

Epichlorohydrin, *N,N*-dimethylformamide, *N,N'*-carbonyldiimidazole, n-decylamine, n-capric acid, and Triton X-100 were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was purchased from Nacalai Tesque (Kyoto, Japan). Egg-yolk phosphatidylcholine (EYPC) and calcein were supplied by Sigma (St. Louis, MO, USA). Fluorescent probes, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dioleoylphosphatidylethanolamine (NBD-PE) and lissamine rhodamine B-sulfonyl phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). These fluorescent probes exhibited a single spot on thin-layer chromatography [37,38]. Epichlorohydrin and *N,N*-dimethylformamide were distilled before use.

2.2. Synthesis of succinylated poly(glycidol)

Poly(glycidol) was synthesized according to the method of Cohen [39] by the conversion of poly(epichlorohydrin). The most frequent molecular weight of poly(glycidol) obtained was estimated to be 7600 taking poly(ethylene glycol) as standard by high-performance liquid chromatography analysis on Asahipak GS-510 column (Asahi, Japan). Poly(glycidol) with varying amounts of carboxyl groups were prepared by the reaction of poly(glycidol) with various amounts of succinic anhydride in *N,N*-dimethylformamide at 60°C for 3 h. Amount of succinylated residues introduced were determined by the potentiometric titrations using a Horiba pH meter (M-8).

2.3. Synthesis of succinylated poly(glycidol) having long alkyl chains

The succinylated poly(glycidol) having long alkyl chains was synthesized by combining n-decylamine to a part of the carboxyl groups of the polymer. The succinylated poly(glycidol) was dissolved in water at pH 5. n-Decylamine (0.1 equivalent to carboxyl groups of the derivative) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (0.1 equivalent to carboxyl groups of the derivative) were added to the solution at 4°C and the solution was stirred overnight. The resultant derivative was purified by liquid chromatography on a Sephadex LH-20 (Pharmacia, Sweden) column using methanol.

2.4. Synthesis of poly(glycidol) having long alkyl chains

Poly(glycidol) having long alkyl chains was synthesized as follows. Poly(glycidol) was dissolved in *N,N*-dimethylformamide. n-Capric acid (0.08 equivalent to glycidol residues) and *N,N'*-carbonyldiimidazole (0.08 equivalent to glycidol residues) were mixed in *N,N*-dimethylformamide at 0°C. The solution was stirred for 1 h and then the poly(glycidol) solution was added to the solution at 0°C. The resultant polymer was purified by liquid chromatography on a Sephadex LH-20 column using methanol.

2.5. Calcein leakage release from liposomes

Calcein-containing EYPC liposomes were prepared as reported previously [31]. Dry thin membranes composed of EYPC and poly(ethylene glycol) derivatives were made by evaporation of a mixed methanol solution of EYPC and the derivatives and subsequent drying under vacuum. The membranes were dispersed in aqueous 100 mM calcein solution (pH 7.4) and were sonicated for 30 min using a ultrasonic disruptor (UD-200, Tomy Seiko). Free calcein was removed by gel chromatography on a Sephadex G-75 (Pharmacia, Sweden) column using 10 mM acetate buffer containing 100 mM NaCl. Calcein-loaded liposomes were dispersed in 10 mM acetate and 100 mM NaCl buffer of preadjusted pH and the fluorescence change was monitored. Excitation and monitoring wavelengths were 495 and 515 nm, respectively. The percent leakage of liposome was defined as

$$\% \text{ leakage} \frac{F^t - F^0}{F^f - F^0} \times 100$$

where F^0 and F^t mean the initial and intermediate fluorescence intensities of liposome, respectively. F^f is the fluorescence intensity after the addition of Triton X-100 (final concentration 0.15%). It was confirmed that the leakage percentage determined at a given pH agreed with the value evaluated after adjusting the pH of the sample to 7.4. The errors between these values were within 5%.

2.6. Fusion of liposomes

The fusion assay was performed as described elsewhere [31]. Fusion of liposomes was determined by measuring the fluorescence energy transfer between NBD-PE and Rh-PE [38]. Sonicated liposomes were obtained according to the procedure mentioned above. The liposome containing 1 mol% of NBD-PE and Rh-PE was mixed in a 1:1 mole ratio with the probe-free liposome. The suspension was excited at 450 nm and fluorescence emission spectra were measured at various time intervals. The R value [9,29,31], the ratio of fluorescence intensity at 530 nm to that at 590 nm, was calculated. This ratio was converted into the apparent concentration of these fluorophores in the membrane using a standard curve of the concentration of the fluorophores vs. R value. The percent fusion was defined as

$$\% \text{ fusion} = \frac{C^t - C^0}{C^f - C^0} \times 100$$

where C^0 and C^t mean the initial and intermediate concentrations of the fluorophores, respectively. C^f, which equals 0.5 mol% of each fluorophore, represents the concentration at which complete fusion of the liposomes occurs.

2.7. Aggregation of liposomes

For detection of aggregation of liposomes the absorbance of the liposome suspension was measured by a spectrophotometer (Ubest-30, Japan Spectroscopic) using a 280 nm light source.

2.8. Estimation of amount of succinylated poly(glycidol) bound to liposome

The amount of succinylated poly(glycidol) bound to the liposome was calculated from the amount of free polymer found when gel permeation chromatography on a Sepharose 4B column was performed for a liposome suspension prepared by sonicating a mixture of EYPC and the polymer in 10 mM acetate buffer containing 100 mM NaCl at pH 7.4. The polymer was detected using a UV detector at 210 nm.

2.9. Other methods

The phospholipid concentration was determined by an assay using Phospholipids B-Test Wako reagent supplied by Wako (Osaka, Japan). Nuclear magnetic resonance spectra were taken with a JEOL JNM-GX-270 MHz instrument.

3. Results

3.1. Interaction of poly(ethylene glycol) derivatives with the EYPC liposome

In order to obtain succinylated poly(glycidol)s with different amounts of carboxyl groups, succinic anhydride was reacted with poly(glycidol) in a residual mole ratio of 0.5, 1.0, and 1.5. Unit percent of succinylated residues of the resultant polymers were 12.2, 56.9, and 85.5, which were designated P-12, P-56, and P-85,

respectively. The acid-base titration for the polymers was performed. The titration curves of these polymers were essentially identical. The result showed that the charge density of the polymers changed between pH 3.4 and 9.0 and the apparent pK_a of these polymers was 5.4 (results not shown).

Interaction of the succinylated poly(glycidol)s and also poly(glycidol), P-0, with EYPC liposome was investigated. Figs. 2A and 2B show fusion of EYPC liposome in the presence of various concentrations of these polymers at pH 7.4 and 4.0, respectively. At pH 7.4 these polymers promoted the fusion of liposome very slightly. Enhancement of fusion by these polymers decreases upon increasing the amount of carboxyl group. On the other hand, marked promotion of fusion was observed in the presence of P-56 at pH 4.0. A higher concentration of P-85 also enhanced the fusion, while P-0 and P-12 hardly enhanced the fusion of the liposome at pH 4.0. Fusion of the EYPC liposome was also observed in the absence of the polymers, although the extent of the fusion was very slight. Because the extent of fusion decreased when a liposome containing 0.5 mol% of each fluorescent probe was used as the labeled liposome, the fluorescent probes in the membrane may enhance fusion of the liposomes to a slight extent.

3.2. EYPC liposomes bearing poly(ethylene glycol) derivatives

Many studies show that hydrophilic polymers can be fixed on lipid membranes by conjugation of hydrophobic molecules to the polymers [40–42]. The hydrophobic moieties conjugated to the polymer act as anchors to lipid membranes. In order to obtain succinylated poly(glycidol) with anchors to liposome membrane, n-decylamine was attached to a part of the carboxyl groups of the succinylated poly(glycidol).

As is apparent from the result of Fig. 2, P-56 promotes fusion of the EYPC liposome at pH 7.4 as well as at pH 4.0. This means that P-56 fixed on the liposome surface may interact with and destabilize the liposome membrane at neutral pH. Moreover, when a part of the carboxyl groups of the polymer was combined with alkyl chains, the resultant polymer should interact more strongly with the liposome membrane due to the loss of carboxyl groups. On the other hand, P-85 does not destabilize the liposome at pH 7.4. Although the polymer, at least in an aqueous solution, has a lower ability to promote fusion than P-56 under acidic conditions, possibly due to formation of intra- and/or intermolecular hydrogen bonding, it was expected that the fusogenic activity of P-85 increases in the lipid membrane as mentioned in Discussion. Moreover, the polymer possesses many carboxyl groups after combination with anchors and hence is expected to reveal pH-sensitivity. Therefore, P-84, which was the second preparation of P-85, was chosen as a liposome modifier and P-84 with anchors (P-84M) was prepared. The percentage of succinylated residues of P-84 was determined to be 84.0 by acid-base titration. Poly(glycidol) having long alkyl chains (P-0M) was also synthesized as a control for P-84M. The percentages of alkyl chain-attached residue of P-84M and P-0M were determined by analyzing the ^{1}H-NMR spectra of these polymers to be 7.4 and 4.7, respectively.

Several EYPC liposomes bearing succinylated poly(glycidol) and EYPC liposomes bearing poly(glycidol) were prepared by sonication of a mixture of EYPC and P-84M or P-0M in varying ratios in aqueous solutions. In order to determine the amount of the

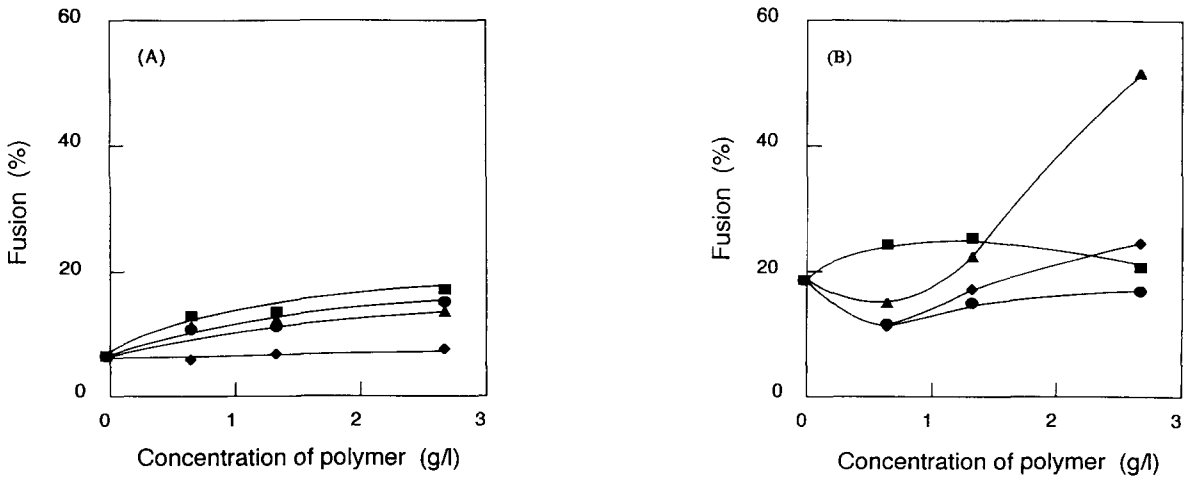

Fig. 2. Fusion of EYPC liposomes in the presence of various concentrations of poly(ethylene glycol) derivatives at pH 7.4 (A) and 4.0 (B) in 10 mM acetate and 0.1 M NaCl buffer solution at 35°C. P-0 (■), P-12 (●), P-56 (▲), P-85 (◆). Incubation time 30 min. The concentration of EYPC was $5.1 \cdot 10^{-4}$ M.

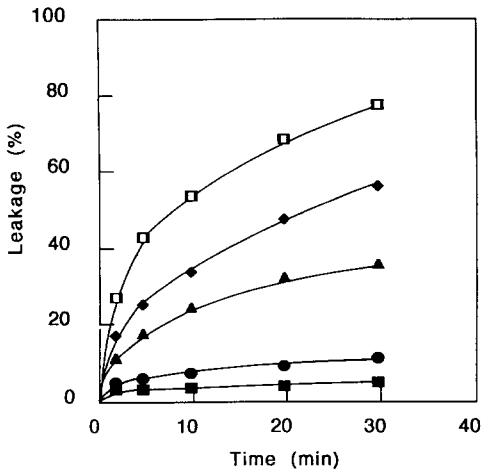

Fig. 3. Time-course of leakage of calcein from P-84M/EYPC (1:4, w/w) liposome in 10 mM acetate and 0.1 M NaCl buffer solution containing 0.1 mM ethylenediaminetetraacetic acid at 35°C at pH: 7.4 (■), 6.0 (●), 5.5 (▲), 5.0 (♦), and 4.5 (□). The concentration of EYPC was $5.1 \cdot 10^{-4}$ M.

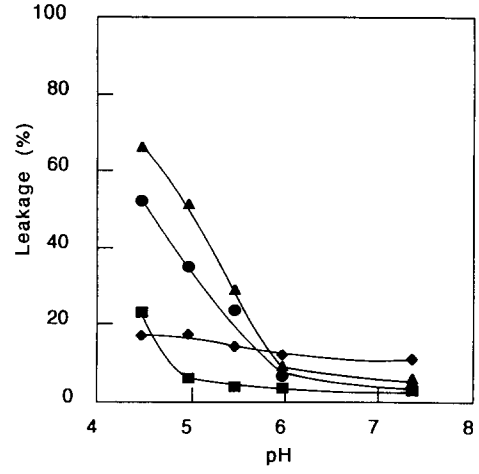

Fig. 4. pH-dependence of calcein-leakage from EYPC liposomes bearing various amounts of poly(ethylene glycol) derivatives in 10 mM acetate and 0.1 M NaCl buffer solution containing 0.1 mM ethylenediaminetetraacetic acid at 35°C. P-84M/EYPC (w/w): 3:7 (▲), 1:4 (●), 1:9 (■); P-0M/EYPC (w/w): 3:7 (♦). Incubation time 10 min. The concentration of EYPC was $5.1 \cdot 10^{-4}$ M.

poly(ethylene glycol) derivative bound on the liposome, gel permeation chromatography was performed for the liposome suspension prepared by sonicating a mixture of EYPC and the derivative in a buffer solution. This assay showed that the P-84M/EYPC (3:7, w/w) liposome, which contained the highest amount of the derivative used in the study, carried 97% of the derivative supplied in the preparation. Therefore, it can be concluded that the derivative supplied was quantitatively and tightly bound on the liposome.

The pH effect on leakage, aggregation, and fusion of the liposomes was investigated. Fig. 3 depicts the typical profiles of calcein leakage from the P-84M/EYPC (1:4, w/w) liposome at various pH values. Leakage was negligible at pH 7.4, but increased with decreasing pH. The pH-dependence of leakage of calcein from various EYPC liposomes bearing poly(ethylene glycol) derivatives is shown in Fig. 4. It is apparent that leakage from the P-84M/EYPC liposomes increases below pH 5.5, which corresponds to the apparent pK_a of the succinylated poly(glycidol). Extent of leakage from the liposomes also increased with increasing content of succinylated poly(glycidol) in the liposomes. On the other hand, leakage from the P-0M/EYPC liposome is approximately constant in the experimental pH region. It was observed that when the amounts of lipid molecules composing the liposomes were equal, almost the same amounts of calcein molecules were released from the polymer-modified liposomes and unmodified liposome after the addition of Triton X-100 at pH 4.5 as well as at pH 7.4, indicating that the polymer did not act as a carrier for calcein and all calcein molecules loaded in the liposomes were released by the addition of the detergent.

The aggregation of the P-84M/EYPC liposome was examined by the turbidity measurement. Fig. 5 shows the time-course of apparent absorbance of the liposome suspension when the liposome was put in the buffer solution of various pH values. Increase in absorbance was observed under acidic conditions, indicating that aggregation and/or fusion of the liposome occurred. A more significant increase in absorbance was observed when the bare EYPC liposome was present in the polymer-modified liposome suspension. Since absorbance of the bare liposome suspension did not change at experimental pH values, the result suggests that the polymer-modified liposome interacted with the bare liposome as well as the modified liposome (data not shown).

The fusion of EYPC liposomes bearing the poly(ethylene glycol) derivatives was studied by measuring lipid

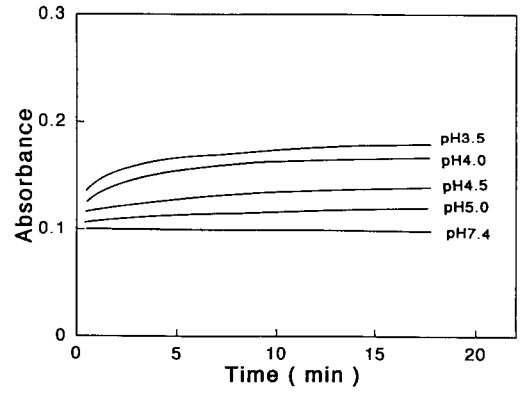

Fig. 5. Time-course of absorbance of P-84M/EYPC (3:7, w/w) liposome suspension at 280 nm in 10 mM acetate and 0.1 M NaCl buffer solution at 35°C at various pH values. The concentration of EYPC was $5.1 \cdot 10^{-4}$ M.

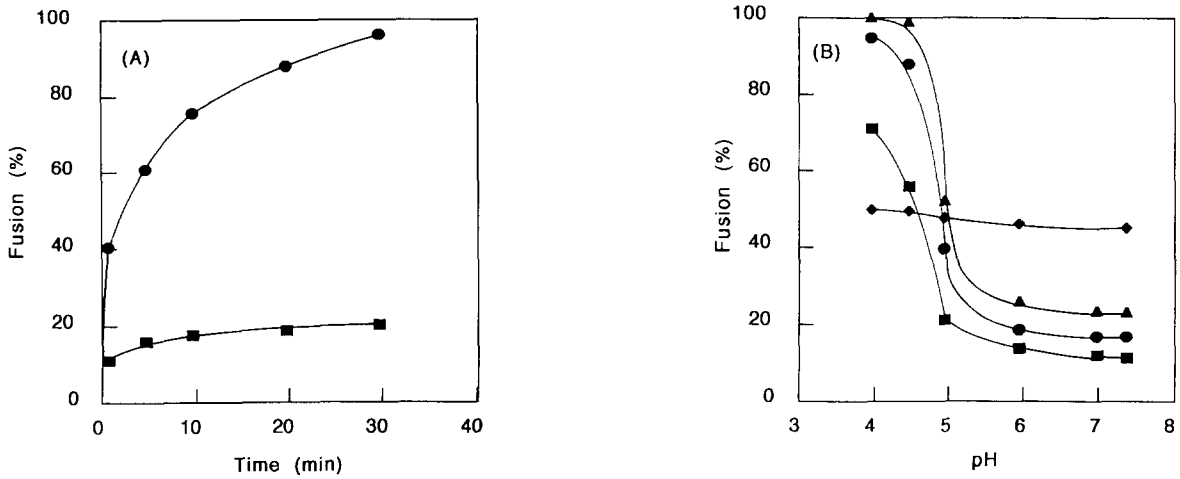

Fig. 6. (A) Time-course of fusion of P-84/EYPC (1:4, w/w) liposomes in 10 mM acetate and 0.1 M NaCl buffer solution at 35°C at pH 7.4 (■) and pH 4.0 (●). (B) Percent fusion of EYPC liposomes bearing various amounts of poly(ethylene glycol) derivatives after 30 min as a function of pH in 10 mM acetate and 0.1 M NaCl buffer solution at 35°C. P-84M/EYPC (w/w): 3:7 (▲), 1:4 (●), 1:9 (■); P-0M/EYPC (w/w): 3:7 (◆). The concentration of EYPC was $1.8 \cdot 10^{-4}$ M.

intermixing using NBD-PE and Rh-PE as fluorescent probes. Fig. 6(A) represents the typical profiles of fusion of the P-84M/EYPC (1:4, w/w) liposome. While fusion of the liposome was hardly observed at pH 7.4, remarkable fusion occurred at pH 4.0. pH-dependence of fusion of EYPC liposomes modified with poly(ethylene glycol) derivatives is shown in Fig. 6(B). Fusion of the P-84M/EYPC liposomes was remarkably enhanced below pH 5. Liposomes with the higher polymer content resulted in more extensive fusion in the acidic region and also in the neutral region. Fusion behavior of the P-0M/EYPC (3:7, w/w) liposome is also depicted in Fig. 6(B). Fusion percent of the liposome reveals approximately the same value: around 50%.

It is important to estimate the ability of the liposomes modified with poly(ethylene glycol) derivatives to fuse with bare liposome because the liposomes are required to fuse with the endosomal membrane which is not a destabilized membrane. Therefore, fusion between the P-84M/EYPC liposome and the bare EYPC liposome was also examined. The result is shown in Fig. 7. Compared with fusion between the modified liposomes, fusion of the modified liposome with bare liposome is shown to be less above pH 7.0 and below pH 4.5, but higher between pH 5 and pH 6.

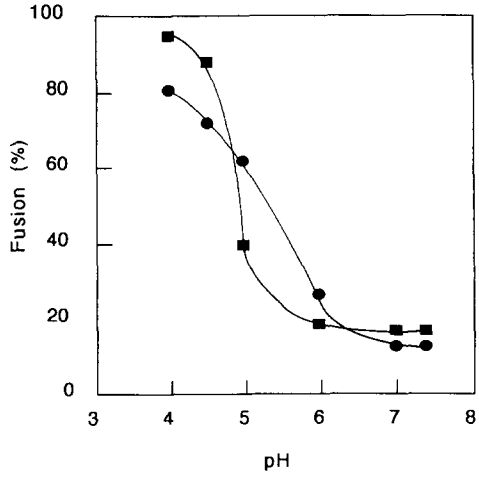

Fig. 7. Percent fusion between P-84M/EYPC (1:4, w/w) liposomes (■) or between P-84M/EYPC (1:4, w/w) liposome and EYPC liposome (●) after 30 min as a function of pH in 10 mM acetate and 0.1 M NaCl buffer solution at 35°C. The concentration of EYPC was $1.8 \cdot 10^{-4}$ M.

4. Discussion

The objective of the present study was to develop a novel pH-sensitive liposome composed of PC. Our strategy for pH-sensitization of PC liposomes was the modification of the liposomes with fusogen which generates fusogenic activity in response to pH. We chose poly(ethylene glycol) as a fusogen and synthesized pH-sensitive derivatives of the polymer.

Poly(glycidol) was used to prepare poly(ethylene glycol) derivatives because the polymer has a similar structure of main chain with poly(ethylene glycol) and side chains which can be modified to carboxyl groups. Three kinds of succinylated poly(glycidol)s were prepared as poly(ethylene glycol) derivatives having carboxyl groups (Fig. 1).

The ability of the poly(ethylene glycol) derivatives to promote fusion of EYPC liposomes in an aqueous solution seems to be somewhat weak, but is clearly pH-dependent (Fig. 2). At pH 7.4 a lower percent

fusion of liposome was observed when the liposome was incubated with the poly(ethylene glycol) derivative having larger amounts of carboxyl groups. Since distribution of the polymer in the liposome membrane depends on hydrophilicity of the polymer, binding of the polymer to the liposome membrane should decrease with increasing negatively charged carboxyl groups on the polymer. Therefore, it is likely that the interaction of the polymer chain with the liposome membrane is depressed more strongly by larger amounts of charged carboxyl groups.

On the other hand, the fusion behavior of the liposome induced by the poly(ethylene glycol) derivatives under acidic conditions is complicated. While poly(glycidol), P-0, revealed a slight effect on fusion of the liposomes in the experimental concentrations of the polymer, the succinylated poly(glycidol)s, P-12, P-56, and P-85, suppressed fusion of the liposomes at a polymer concentration of 0.66 g/l. However, percent fusion increased with increasing concentration of these polymers above 1.32 g/l. As is distinct from the case of pH 7.4, affinity of the polymers for the liposome membrane should be enhanced due to protonation of carboxyl groups at pH 4.0. When a few amounts of the polymer are adsorbed on the liposome, the liposome is not destabilized sufficiently to bring about fusion. Since it was reported that poly(ethylene glycol) chains in the liposome surface lead to an increase of the repulsive force between the liposome membranes [43], the poly(ethylene glycol) derivatives on the liposome may likewise hinder the interaction between the liposomes. However, with increasing amounts of the polymer adsorbed on the liposome, the bilayer membrane is destabilized more intensively, resulting in an increase of fusion of the liposome.

Among the poly(ethylene glycol) derivatives prepared, P-56 showed the most significant effect on the promotion of fusion. This result suggests that an optimum amount of succinylated residues exists for the maximum fusion-promoting ability of the polymer. Combination of succinic acid with the polymer increases affinity of the polymer for the liposome under acidic condition possibly due to an increase in hydrophobic interactions between succinate groups and the lipid membrane and the formation of hydrogen bonding between unionized carboxyl groups of the polymer and the phosphodiester groups of the liposome surface [44]. However, since carboxyl groups are well known to form hydrogen bonding with oxyethylene units [45], introduction of too great an amount of the succinate group causes reduction of the dehydrating ability of the polymer due to formation of intramolecular and/or intermolecular hydrogen bonding. It is generally recognized that the dehydrating ability of poly(ethylene glycol) is important for the ability of the polymer to induce membrane fusion. Therefore, P-85 may show a weak ability to enhance fusion of the liposome.

pH-sensitivity of EYPC liposomes bearing the poly(ethylene glycol) derivatives was studied. As is seen in Figs. 3 and 4, the liposomes containing P-84M are stable above pH 6.0, but become leaky below pH 5.5. Since this pH value corresponds to apparent pK_a of the poly(ethylene glycol) derivative, it is likely that the increase of leakage under mildly acidic conditions is related to protonation of carboxyl groups of the succinylated poly(glycidol). While several PE-based pH-sensitive liposomes have been reported to reveal appreciably fast leakage immediately after exposure to acidic conditions [9,11,15], leakage from the liposomes bearing the succinylated poly(glycidol) is relatively slow under such conditions. This may be because the bilayer membrane of liposomes prepared in this study are not destabilized as extremely as those of PE-based liposomes. On the other hand, the liposome containing P-0M released calcein to a similar extent over experimental pH values. Interestingly, this liposome was somewhat more leaky than the liposome containing the same amount of P-84M at neutral pH. This suggests that polymer P-0M bound on the liposome interacts with the lipid membrane and destabilizes it to a certain extent. This is also shown in the fusion behavior of the P-0M-modified liposome, which had a higher tendency to fuse than the liposome modified with P-84M above pH 5 (Fig. 6(B)).

The pH-sensitive property of the liposomes bearing P-84M is also observed in turbidity (Fig. 5) and fusion measurements (Figs. 6 and 7). As pH was decreased, turbidity of the liposome suspension increased, suggesting vesicle aggregation and/or an increase in vesicle size due to fusion of the liposome. It has been reported that poly(methacrylic acid) and poly(2-ethylacrylic acid) solubilize phosphatidylcholines in aqueous solutions under acidic conditions [46,47]. These polymers are considered to provide hydrophobic sites for hydrocarbon solubilization at low pH [48]. Since the poly(ethylene glycol) derivatives have relatively a hydrophilic polymer backbone and side chains, the derivatives should interact mainly with the polar group of phospholipid and hence do not solubilize the lipid.

Occurrence of the fusion of the liposome was confirmed by detecting dilution of the fluorescent probes. Fusion of the liposome composed of EYPC and P-84M proceeded very slowly under neutral conditions and the fusion rate at neutral pH was comparable with that of bare EYPC liposome. However, with decreasing pH, the fusion rate of the liposome modified with P-84M was enhanced remarkably. In contrast, the liposome modified with P-0M did not exhibit pH-sensitivity, but revealed a much stronger ability to fuse than bare EYPC liposome. The liposome bearing P-0M fused more intensively than the liposome containing the same

amount of P-84M above pH 5. But the liposome modified with P-84M fused more readily than that bearing P-0M below pH 5.0. These results suggest that poly(glycidol) chains of the poly(ethylene glycol) derivative bound on the liposome surface enhance fusion of the liposome and carboxyl groups of the derivative control interaction between the polymer chains and the liposome responding to pH as mentioned above. Although P-85 showed a weak activity to enhance fusion of EYPC liposome under acidic conditions, the liposomes coated with P-84M revealed a marked ability to fuse under these conditions possibly because carboxyl groups of the polymer bound to the liposome surface form hydrogen bonding with phosphodiester groups of the lipid in preference to ether groups of the polymer due to the presence of a large excess of phosphodiester groups [44].

In order to determine the ability of the P-84M/EYPC liposome to fuse with a stable membrane, fusion between the modified EYPC liposome and bare EYPC liposome was investigated (Fig. 7). The result obtained indicates that the EYPC liposome bearing P-84M can fuse with bare EYPC liposome as well as the modified liposome. The extent of fusion is shown to be smaller for fusion between the modified liposome and the bare liposome than that between the modified liposomes below pH 4.5. However, the modified liposome fused more easily with the bare liposome under weak acidic conditions. Fusion between the liposomes bearing the polymer might be suppressed under weak acidic conditions because of the electrostatic repulsion between negatively-charged liposome surfaces due to the ionized carboxyl groups of the polymer bound on the liposome.

In conclusion, EYPC liposomes bearing the succinylated poly(glycidol), which is a poly(ethylene glycol) derivative having carboxyl groups, were shown to change ability to fuse in response to pH. The fusion ability of the liposomes was low at neutral pH, but increased with decreasing pH. In addition, since the hydrophilic polymer chains such as poly(ethylene glycol) attached on the liposome surface reduce interactions of the liposome with plasma components [49–52], stability of the poly(ethylene glycol) derivative-modified liposomes in the blood may be superior to conventional pH-sensitive liposomes. Therefore, the liposomes prepared in this study are expected to be effective carriers of various materials into the cytoplasm. Efficiency of cytoplasmic delivery by the liposomes is being studied now.

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