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Low pH Induced Membrane Fusion of Lipid Vesicles Containing Proton-Sensitive Polymer†

Naoto Oku,*,‡ Sayumi Shibamoto,‡ Fumiaki Ito,‡ Hisanori Gondo,§ and Mamoru Nango§
Department of Biochemistry, Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, Osaka, Japan, and Department of Applied Chemistry, College of Engineering, University of Osaka Prefecture, Sakai, Osaka, Japan
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ABSTRACT: For the purpose of cytoplasmic delivery of aqueous content in liposomes through endosomes, we synthesized a pH-sensitive polymer, cetylacetyl(imidazol-4-ylmethyl)polyethylenimine (CAIPEI), which generates polycations at acidic pH. CAIPEI in its aqueous phase caused aggregation of sonicated vesicles composed of phosphatidylserine (PS) and phosphatidylcholine (PC) (molar ratio 1:4) when the pH of the solution was lowered. The polymer also induced membrane intermixing as measured by resonance energy transfer between vesicles containing *N*-(7-nitro-2,1,3-benz[*d*]oxadiazol-4-yl)phosphatidylethanolamine and those containing *N*-Rhodamine phosphatidylethanolamine at pH 4–5, while the addition of CAIPEI caused neither aggregation of PC vesicles nor the intermixing of liposomal membranes between PC and PC/PS vesicles at any pH. The CAIPEI-induced membrane intermixing was dependent on the polymer/vesicle ratio rather than on the polymer concentration. Then the polymer was incorporated into the bilayers of PC vesicles. These CAIPEI vesicles also caused membrane intermixing with liposomes containing PS under acidic conditions. The reconstituted CAIPEI did not reduce the trapping efficiency of vesicles or increase their permeability to glucose even at low pH. The vesicles caused the low pH induced aggregation and membrane intermixing with other negatively charged liposomes containing phosphatidic acid or phosphatidylglycerol. These results suggest that the protonation of the polymer at acidic pH endows the CAIPEI vesicles with the activity to fuse with negatively charged liposomes.

Many applications of liposomes as microcapsules for drug delivery and as tools for microinjection have been investigated. For the purpose of cytoplasmic delivery via the endosome of molecules encapsulated in liposomes, several pH-sensitive liposomes have been developed (Connor et al., 1984; Diacovo et al., 1986; Collins & Huang, 1986). This delivery system mimics the process that is believed to be used for delivery of nucleocapsids into the cell cytosol by certain enveloped viruses (White et al., 1983). These viruses bind to cell surface receptors and are then internalized by endocytosis. Following these events, viral envelopes fuse with endosomal membranes due to the acidic condition of endosomes, and the nucleocapsid is released into the cytosol. [In the case of nonenveloped viruses, nucleocapsid release might occur through the pH-sensitive damage to the endosomal membrane induced by these viruses (Seth et al., 1984; Blumenthal, 1986).] Furthermore, vesicular stomatitis virus (Yamada & Ohnishi, 1986; Eidelman et al., 1984), whose receptor was suggested to be the lipid bilayer itself (Yamada & Ohnishi, 1986), and influenza virus (Stegmann et al., 1985) were reported to fuse with negatively charged liposomes under acidic conditions. Other proteins such as the HN protein of Sendai virus (Chejanovsky et al., 1986) or clathrin (Blumenthal et al., 1983) are also reported to induce pH-dependent membrane fusion.

Here we report the effects of a synthetic amphiphathic polymer, cetylacetyl(imidazol-4-ylmethyl)polyethylenimine, on acidic phospholipid vesicles. This polymer is readily incorporated into liposomal membranes and becomes cationic by protonation at pH 4–6. The resulting polycation is expected to induce membrane fusion, since many polycations such as mellitin (Eytan & Almary, 1983), polymyxin B (Gad & Eytan, 1983), polylysine (Gad et al., 1982; Lampe & Nelsestuen, 1983), polyhistidine (Wang & Huang, 1984; Uster & Deamer, 1985), and other synthetic polycations (Oku et al., 1986) are known to fuse negatively charged liposomes. Evidence is presented to show that the polymer endows liposomes with fusing activity only at low pH.

Experimental Procedures

Materials. Egg yolk phosphatidylcholine (PC),[1] phosphatidic acid (PA) from egg yolk PC, phosphatidylglycerol (PG) from egg yolk PC, and phosphatidylethanolamine (PE) from hydrated egg yolk PC were gifts of the Research Institute of

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* Address correspondence to this author.
‡ Setsunan University.
§ University of Osaka Prefecture.

[1] Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; N-NBD-PE, *N*-(7-nitro-2,1,3-benz[*d*]oxadiazol-4-yl)phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine; PEI, polyethylenimine; CAIPEI, cetylacetyl(imidazol-4-ylmethyl)polyethylenimine.

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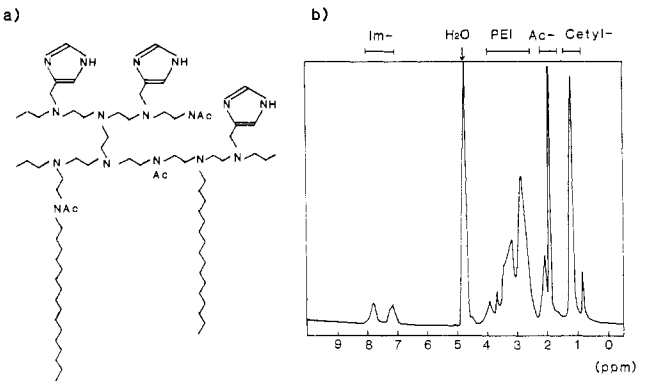

FIGURE 1: (a) Structure of CAIPEI. (b) ^{1}H NMR pattern of CAIPEI.

Nippon Fine Chemical Co., Ltd. (Hyogo, Japan). Bovine brain phosphatidylserine (PS), *N*-(7-nitro-2,1,3-benz[*d*]oxadiazol-4-yl)-PE (N-NBD-PE), and *N*-(lissamine Rhodamine B sulfonyl)dioleoyl-PE (N-Rh-PE) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). All lipids gave a single spot on silica gel thin-layer chromatography.

Polyethylenimine (PEI) with an average molecular weight of about 1800 was a gift from Dr. I. M. Klotz of the Department of Chemistry, Northwestern University (originally purchased from Dow Chemical Co.). This material was purified with an Amicon Diaflo ultrafiltration apparatus, using an Amicon YM-2 membrane. Imidazol-4-ylmethyl chloride monohydrochloride was prepared by a published method (Turner et al., 1949).

For the glucose assay, ATP and NADP were purchased from Sigma Chemical Co. (St. Louis, MO). Glucose-6-phosphate dehydrogenase and hexokinase were from Oriental Yeast Co., Ltd. (Tokyo, Japan). Reduced Triton X-100 came from Aldrich Chemical Co. (Milwaukee, WI).

Synthesis of Cetylacetyl(imidazol-4-ylmethyl)-PEI. Cetylpolyethylenimine (I). Cetyl bromide (0.17 g) was added to polyethylenimine (0.50 g) in absolute ethanol (15 mL). The resulting solution was stirred at 50 °C for 12 h. The polymer-containing solution was dialyzed against 40% ethanol in water and then against water at room temperature, after which the aqueous solution was lyophilized. Integration of the proton magnetic resonance (^{1}H NMR) spectrum of the product in D_2O indicated 0.04 mol of cetyl groups ($C_{16}H_{33}$) per residue mol of polymer (C_2H_4N). Thus, the modified polymer may be represented by the stoichiometric formula $(C_2H_4N)_m(C_{16}H_{33})_{0.04m}$ (I), m = 43. I was obtained almost quantitatively.

Cetyl(imidazol-4-ylmethyl)polyethylenimine (II). This derivative was prepared by alkylation of I (0.28 g) with imidazol-4-ylmethyl chloride (0.21 g) in absolute ethanol (5 mL) as described previously (Klotz et al., 1971; Nango & Klotz, 1978). The modified polymer was purified by the same method as described for I. Integration of the ^{1}H NMR spectrum of the product in D_2O indicated 0.24 mol of imidazol-4-ylmethyl groups ($C_4H_5N_2$) per residue mol of polymer. The stoichiometric formula corresponds to $(C_2H_4N)_m(C_{16}H_{33})_{0.04m}(C_4H_5N_2)_{0.24m}$ (II), m = 43. II was obtained almost quantitatively.

Cetylacetyl(imidazol-4-ylmethyl)polyethylenimine (III). The structure of this derivative is shown in Figure 1a. The derivative was prepared by acetylation of II with acetic anhydride as described previously (Johnson & Klotz, 1974; Kimura et al., 1984). The modified polymer was purified by the same ultrafiltration procedure as described for PEI. The extent of acetylation (C_2H_3O) was determined from the peak area due to the acetyl group in the ^{1}H NMR spectrum (shown in Figure 1b). Stoichiometric composition for the acetylated derivative is $(C_2H_4N)_m(C_{16}H_{33})_{0.04m}(C_4H_5N_2)_{0.24m}(C_2H_3O)_{0.40m}$ (III), m = 43.

Nuclear Magnetic Resonance Spectra. ^{1}H NMR spectra were taken with a JEOL JNM-GX-270 MHz instrument. Samples were prepared at a concentration of approximately 5% by weight in D_2O (99.96%, Merck Co.).

Preparation of Liposomes. Phospholipids dissolved in $CHCl_3$ were dried under reduced pressure and stored in vacuo for at least 1 h. For incorporation of CAIPEI into lipid bilayers, a lipid film was prepared with PC (1 μmol) and CAIPEI (0.05 μmol) and hydrated in saline (2 mL). The resulting liposomal suspension was sonicated for 10 min with a Branson Model 200 Sonifier at 50% duty cycle. For measurement of the barrier function of liposomes, the dried lipid film was hydrated in 0.3 M glucose solution and sonication was omitted. The concentration of liposomes is expressed in terms of lipid content. To examine how much CAIPEI could be incorporated into lipid bilayers, CAIPEI vesicles were applied to a Bio-Gel A 1.5-m column. Then the elution of CAIPEI in each fraction was measured for imidazole content by using Pauly's reagent after addition of 1% reduced Triton X-100. About 65% of imidazole was recovered from liposomal fraction. However, CAIPEI associated to the liposomes was stable since no free CAIPEI was detected by rechromatography of the vesicles (data not shown).

Measurements of Lipid Intermixing. Intermixing of membrane lipids was measured with the resonance energy transfer assay based on the description by Hoekstra (1982). For determination of "the fusion from without", i.e., fusion of vesicles induced by CAIPEI in the aqueous phase, resonance energy transfer between vesicles composed of PC:PS:N-NBD-PE (molar ratio 75:20:5) and those of PC:PS:N-Rh-PE (75:20:5) was determined. In brief, 5 nmol of both donor and acceptor vesicles were mixed in 1 mL of phosphate- (pH 8.0 and 7.0) or acetate- (pH 6.0, 5.0, and 4.0) buffered saline in the presence of various concentrations of CAIPEI. The fluorescence intensity of N-NBD-PE was monitored with a Hitachi fluorescence spectrophotometer Model F-4,000. The excitation and emission monochromators were set at 470 and 530 nm, respectively. All experiments were done at room temperature.

For determination of "the fusion from within", i.e., fusion between CAIPEI vesicles and vesicles containing PS, the former vesicles containing N-Rh-PE (PC:N-Rh-PE:CAIPEI = 98:2:5) in their bilayer were applied to a Bio-Gel A 1.5-m column for removal of unincorporated CAIPEI before being mixed with vesicles containing N-NBD-PE. Donor vesicles (10 nmol) and acceptor vesicles (40 nmol) were mixed, and the fluorescence intensity of N-Rh-PE was monitored with the excitation and emission monochromators set at 470 and 594 nm, respectively.

Determination of Light-Scattering Change of Liposomal Dispersions. CAIPEI-induced light-scattering change of PC/PS vesicles (0.5 mM as lipids) was monitored at 450 nm with Hitachi fluorescence spectrophotometer, Model F-4,000.

Measurement of Hemolytic and Hemagglutinating Activities. Heparinized blood was freshly drawn from a healthy donor and washed 3 times with saline. Then 0.5% erythrocytes in 2 mL of saline was incubated with various amounts of CAIPEI in aqueous phase or in vesicle bilayers at various pHs for 30 min at 37 °C. After centrifugation, released hemoglobin was determined at 541 nm. One hundred percent hemolysis was achieved with hypotonic treatment.

Hemagglutinating activity was measured as follows: CAIPEI in aqueous phase or in vesicle bilayers was diluted

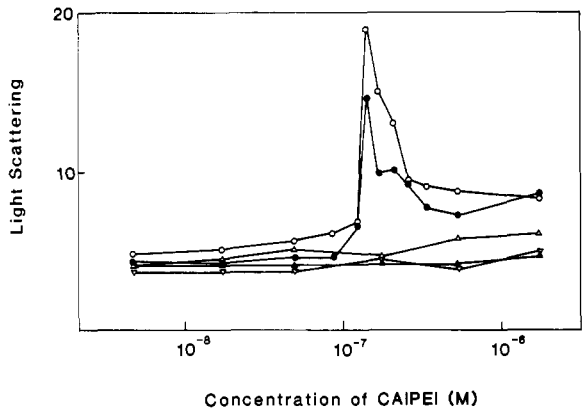

FIGURE 2: CAIPEI-induced light-scattering changes of liposomal solutions. Sonicated liposomes composed of egg yolk PC and bovine brain PS (molar ratio 4:1, final concentration of liposomes was 10 nmol in 1 mL of saline of various pHs) were incubated with various concentrations of CAIPEI for 5 min. Light-scattering changes were monitored at 450 nm after incubation at pH 4.0 (○), 5.0 (●), 6.0 (△), 7.0 (▲), or 8.0 (▽).

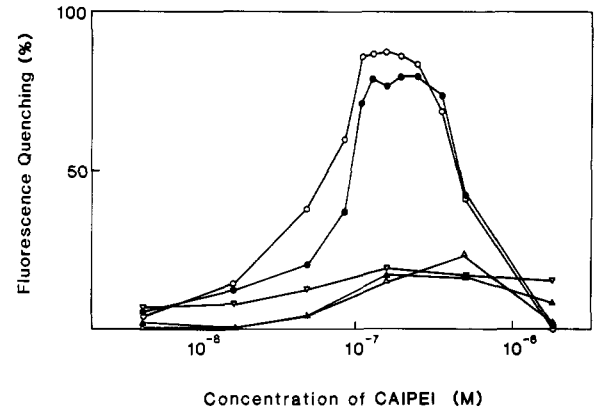

FIGURE 3: Effect of CAIPEI on the fluorescence resonance energy transfer between liposomes containing N-NBD-PE and those containing N-Rh-PE. Sonicated liposomes composed of PC, PS, and N-NBD-PE (molar ratio 75:20:5) were mixed with the same concentration of sonicated liposomes composed of PC, PS, and N-Rh-PE (75:20:5). Then various concentrations of CAIPEI were added to the liposomal solutions (final concentration of liposomes was 10 nmol in 1 mL of solution). Resonance energy transfer was monitored as described under Experimental Procedures. The fluorescence intensity of the liposomal solution without CAIPEI was essentially unchanged during the experiment. The fluorescence intensity of 100% resonance energy transfer was taken as zero, since the fluorescence intensity of sonicated liposomes prepared from PC, PS, N-NBD-PE, and N-Rh-PE (75:20:2.5:2.5) was negligible. Resonance energy transfer was measured after a 5-min incubation at pH 4.0 (○), 5.0 (●), 6.0 (△), 7.0 (▲), or 8.0 (▽).

serially and incubated with 0.5% human erythrocytes for 2 h at 4 °C. The minimum amount required for hemagglutination was then determined.

Other Methods. Phospholipid phosphate was determined by a modification of the Bartlett procedure (Bartlett, 1959). Glucose assay was done by a modification of the method of Kinsky et al. (1969). The amounts of NADPH generated were determined fluorophotometrically.

RESULTS

Fusion from Without: CAIPEI-Induced Aggregation and Membrane Intermixing of Negatively Charged Liposomes. The effect of CAIPEI on liposomal aggregation and membrane intermixing was examined. Figure 2 shows the light-scattering change of PC/PS vesicles induced by CAIPEI at various pHs. No light-scattering change was observed when PC/PS vesicles were incubated at neutral or slightly alkaline pH (shown in Figure 2) or when PC vesicles were used (data not shown). On the other hand, PC/PS vesicles were aggregated by the addition of CAIPEI at low pH, suggesting that negative charges on the liposomes and an acidic condition are essential for the liposomal aggregation. Figure 2 also shows that the light-scattering change reached a maximum at a concentration of about 1.5×10^{-7} M CAIPEI and then declined at higher concentrations of polymers. An increase in the light scattering of liposomal suspensions occurred within a minute after the addition of CAIPEI (data not shown).

Next, we examined CAIPEI-induced membrane fusion. Figure 3 shows the resonance energy transfer between donor (PC/PS/N-NBD-PE) and acceptor (PC/PS/N-Rh-PE) vesicles. No resonance energy transfer was observed in the absence of CAIPEI during the period of measurement (5 min). As is apparent in Figure 3, CAIPEI caused membrane intermixing only under the acidic conditions, indicating the similarity between liposomal aggregation and membrane intermixing with respect to pH dependence. To rule out the possibility that fluorescence quenching is a consequence merely of close contact between two lipid bilayers, we investigated the effect of poly(aspartic acid), which was added after preincubation of liposomes with CAIPEI at low pH. We also investigated the fluorescence quenching when the pH was brought back to neutral after a similar preincubation. Less than 10% of the fluorescence quenching was cancelled by these treatments, suggesting that the fluorescence changes are not due to aggregation of liposomes (data not shown). As shown in Figure 3, the maximum energy transfer obtained was more than 50%, corresponding to more than one round of vesicle fusion. The amount of CAIPEI that would be required to induce the maximum energy transfer observed is about 10^{-10} mol for 2×10^{-9} mol of PS. Thus, if imidazole moieties are completely protonated, the charge ratio of the polycation to PS in the outer layer of liposomes is about one. Membrane intermixing was also monitored between PC/N-Rh-PE and PC/PS/N-NBD-PE, between PC/N-NBD-PE and PC/PS/N-Rh-PE, or between PC/N-NBD-PE and PC/N-Rh-PE vesicles. CAIPEI did not induce membrane intermixing between these sets of vesicles at low pH (data not shown). In our previous experiments (Oku et al., 1986), we observed that polycation-induced membrane fusion was dependent on the polycation:vesicle charge ratio rather than on the concentration of polycation. To elucidate which factor is critical for the CAIPEI-induced fusion, we examined the membrane intermixing by varying the concentration of PC/PS vesicles. The relationship between CAIPEI:vesicle ratio and concentrations of CAIPEI at which 50% resonance energy transfer occurred is shown in Figure 4. Membrane intermixing induced by CAIPEI at low pH was observed to be related to the CAIPEI:vesicle ratio rather than to the CAIPEI concentration, suggesting that the mechanism of CAIPEI-induced fusion is similar to that of polycation-induced fusion, though the latter occurred at neutral pH.

Fusion from Within: Membrane Intermixing of Negatively Charged Liposomes with CAIPEI-Incorporated Vesicles. Since CAIPEI in aqueous phase was shown to have fusogenic effects on negatively charged vesicles at acidic pH, we incorporated CAIPEI into the lipid bilayer and examined the effect of the reconstituted vesicles on PC/PS vesicles. Before that, we examined the effect of the CAIPEI incorporation on liposomal properties. Liposomes were prepared with PC and various amounts of CAIPEI in 0.3 M glucose solution. After removal of untrapped glucose, the amount of glucose encapsulated and the leakage of glucose under various pH conditions were determined. Figure 5 shows that CAIPEI increased the

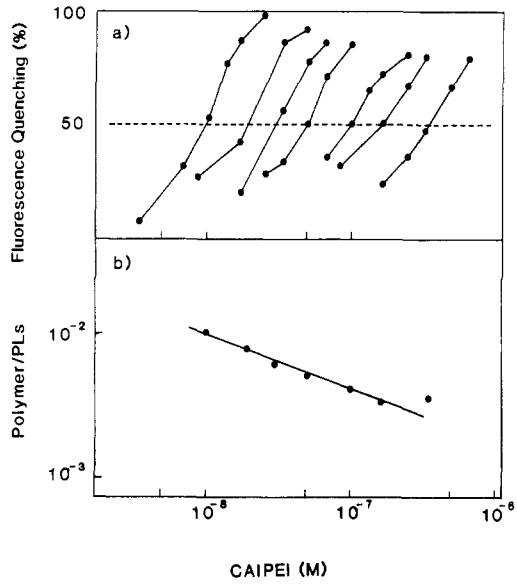

FIGURE 4: Correlation of CAIPEI-induced membrane fusion with liposomal concentrations. (a) Membrane intermixing of PC/PS vesicles was monitored in the presence of various concentrations of CAIPEI as described in the legend of Figure 3 with each concentration of liposomes, i.e., 1, 2.5, 5, 10, 20, 50, and 100 μM as phospholipids (respective curves from left to right). (b) The concentration of CAIPEI that caused 50% resonance energy transfer was read from (a), obtained by the assays with individual liposome concentrations, and the CAIPEI:liposome ratio was plotted against the amount of CAIPEI.

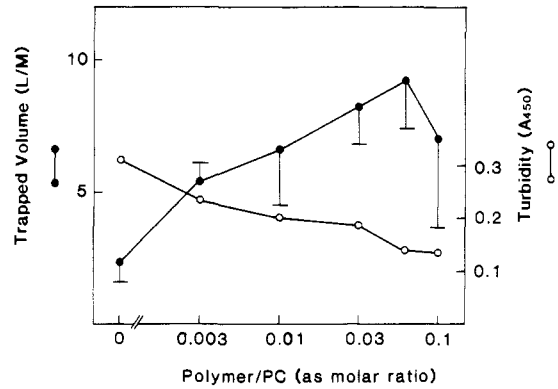

FIGURE 5: Trapped volume and barrier function of CAIPEI vesicles. Liposomes composed of PC and various amounts of CAIPEI were formed in 0.3 M glucose solution (10 mM as PC). Untrapped glucose was removed by three cycles of centrifugation (12000g for 5 min) in the presence of saline. The resulting liposomes (200 nmol as PC) were diluted with 1% reduced Triton X-100 (final volume was 200 μL) and centrifuged at 12000g for 5 min. The amount of glucose in an aliquot of the supernatant was measured enzymatically. Then the trapped volume (●) was calculated. Liposomes were diluted (0.1 mM as PC), and their turbidity (○) was measured at 450 nm.

trapped volume due to the increased hydration of the multilamellar lipid bilayers. Furthermore, since the percent release of glucose from the CAIPEI vesicles was essentially the same as that from the control vesicles under low pH conditions, the incorporation of CAIPEI did not affect the barrier function of the liposomal membranes (data not shown).

Next, we examined the effect of CAIPEI vesicles on PC/PS vesicles. Figure 6 shows the resonance energy transfer induced by the CAIPEI vesicles. The intermixing was observed to be dependent on the pH of the reaction mixture. We also observed the pH-dependent agglutination of PC/PS vesicles induced by CAIPEI vesicles (data not shown).

Since we observed that the presence of CAIPEI in PC vesicles endowed them with fusing activity, we then examined the effect of lipid composition of acceptor liposomes on the

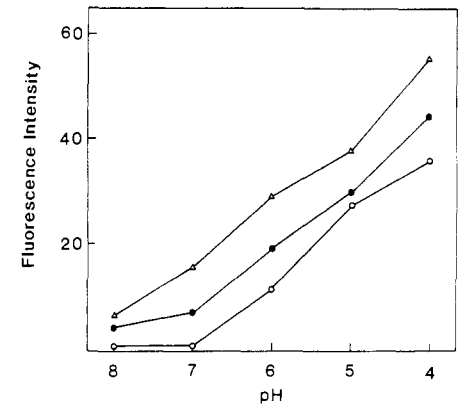

FIGURE 6: Effect of CAIPEI vesicles on PC/PS liposomes. Sonicated vesicles composed of PC, N-Rh-PE, and CAIPEI (98:2:5) were applied to a Bio-Gel A 1.5-m column for removal of free CAIPEI. Then 10 nmol of the vesicles were mixed with 40 nmol of PC/PS vesicles (PC:PS:N-NBD-PE = 75:20:5, ○; PC:PS:N-NBD-PE = 25:70:5, ●) or PS vesicles (PS:N-NBD-PE = 95:5, △) at the indicated pHs in the figure. After a 5-min incubation, resonance energy transfer was determined as described under Experimental Procedures.

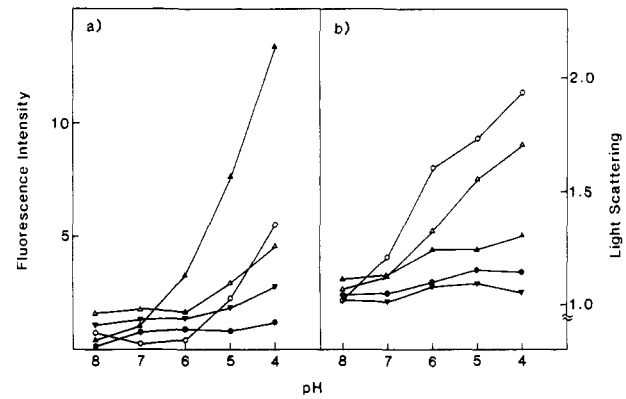

FIGURE 7: Light-scattering changes and membrane intermixing between CAIPEI vesicles and liposomes composed of various lipids. (a) CAIPEI vesicles (PC:N-Rh-PE:CAIPEI = 95:5:5, 10 nmol as phospholipids) were mixed with 50 nmol of PC vesicles (PC:N-NBD-PE = 95:5, ●), PC/PS vesicles (PC:PS:N-NBD-PE = 75:20:5, ○), PC/PA vesicles (PC:PA:N-NBD-PE = 75:20:5, ▲), PC/PG vesicles (PC:PG:N-NBD-PE = 75:20:5, △), or the same amounts of PC/PE vesicles (PC:PE:N-NBD-PE = 75:20:5, ▼). The resonance energy transfer was monitored as an increase in fluorescence intensity with the excitation and emission set at 470 and 594 nm, respectively. (b) Light scattering was monitored after incubation of CAIPEI vesicles (PC:CAIPEI = 100:5, 10 nmol as PC) with 50 nmol of PC vesicles (●), PC/PS (80:20) vesicles (○), PC/PA (80:20) vesicles (▲), PC/PG (80:20) vesicles (△), or PC/PE (80:20) vesicles (▼). The light scattering of lipid vesicles was determined in the absence of CAIPEI vesicles, and the relative increase in light scattering was calculated.

fusion event. As is apparent in Figure 7, reconstituted vesicles fused with negatively charged liposomes containing acidic lipids.

Hemolytic and Hemagglutinating Activities of CAIPEI in Aqueous Phase and in Vesicle Bilayers. The eventual goal of this experiment is the use of liposomes for therapeutic applications. Thus, if the CAIPEI vesicle is hemolytic, it is not favorable for such use, since the vesicle may interact with erythrocytes in the blood stream. So we investigated the effects of CAIPEI in aqueous phase and in vesicle bilayers on human erythrocytes. As is shown in Figure 8, CAIPEI in neither form caused hemolysis at neutral pH. However, hemolysis did occur when the pH of the reaction mixture was lowered. Table I shows the hemagglutinating activities of CAIPEI in its two states. CAIPEI alone (i.e., in aqueous phase) did not induce hemagglutination at neutral pH but did under low pH con-

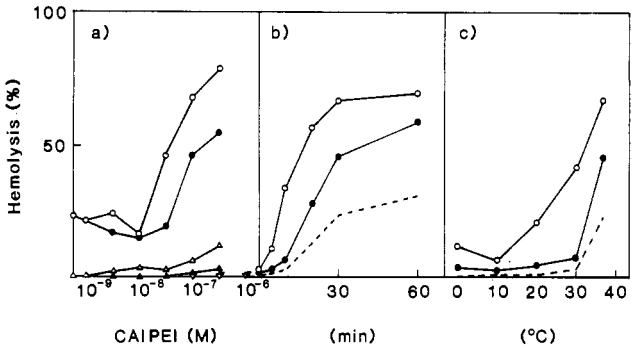

FIGURE 8: Hemolytic activity of CAIPEI in aqueous phase and in vesicle bilayers. (a) Hemolytic activities of CAIPEI in aqueous phase and in vesicle bilayers were determined as described under Experimental Procedures. Human erythrocytes were incubated with various concentrations of CAIPEI in aqueous solution (○, △, ▽) or CAIPEI in liposomal bilayer (●, ▲, ▼) at 37 °C for 30 min at pH 5.0 (○, ●), 6.0 (△, ▲), or 7.0 (▽, ▼). (b) Human erythrocytes were incubated in the presence of 200 μmol of CAIPEI (○) or CAIPEI vesicles (●) at pH 5.0. Dashed line shows the percent hemolysis when erythrocytes alone were incubated at pH 5.0. (c) Same as (b) except that the experiment was done at various temperatures from 0 to 37 °C (30-min incubation).

Table I: Hemagglutinating Activities of CAIPEI in Aqueous Phase and in Vesicle Bilayers[a]

pH	CAIPEI (pmol)	CAIPEI vesicles (pmol)
8.0		>60 000
7.0	>100 000	350
6.0	420	80
5.0	34	40

[a] Hemagglutinating activity of CAIPEI in aqueous vs bilayer state was determined as described under Experimental Procedures. The minimum amounts of CAIPEI and CAIPEI vesicles required for hemagglutination are shown in the table.

ditions. This result is consistent with the data that demonstrated pH dependence of the liposomal agglutination. However, CAIPEI vesicles caused hemagglutination at neutral pH. It might be explained by the cooperative action of partially protonated polymers. This problem should be overcome before use is made of the CAIPEI vesicles as microcapsules of drugs.

Discussion

In this study, we synthesized a pH-sensitive polymer, CAIPEI, which is readily incorporated into the liposomal bilayer, and investigated the effect of this polymer in aqueous phase and in vesicle bilayers on negatively charged liposomes with respect to the induction of liposomal aggregation and fusion.

We designed the polymer on the basis of several facts. First of all, the infection process of some enveloped viruses, such as Semliki forest virus, influenza virus, and vesicular stomatitis virus, has been elucidated to some extent: These viruses initially bind to cell surface receptors and are internalized into endosomes, where the release of nucleocapsids into the cytosol occurs via fusion of viral envelopes with endosomal membranes (White et al., 1983). Spike proteins such as E protein of Semliki forest virus, G of vesicular stomatitis virus, and HA of influenza virus, which are known to be responsible for the initial binding process, are also thought to be responsible for the fusion process at the acidic pH inside endosomes. During the fusion process, spike proteins are believed to interact directly with endosomal lipid bilayers. Indeed, these viruses are reported to fuse with negatively charged liposomes under acidic conditions (Yamada & Ohnishi, 1986; Eidelman et al., 1984; Stegman et al., 1985). Thus, the synthesis of a polymer that causes membrane fusion at acidic pH would enable the cytoplasmic delivery of aqueous contents of liposomes.

Second, negatively charged liposomes are known to fuse in the presence of positively charged metal ions or polycations including polylysine (Gad et al., 1982; Lampe & Nelsestuen, 1983) and synthetic polymers such as polyethylenimine and poly(allylamine) (Oku et al., 1986) at the neutral pH.

These findings prompted us to synthesize a polymer that becomes a polycation at low pH. Indeed, polyhistidine caused liposomal fusion only at low pH, at which it becomes a strong polycation (Wang & Huang, 1984; Uster & Deamer, 1985). In this paper, we described the synthesis of CAIPEI, which has 2 alkyl chains for its embedment into lipid bilayers and 10 imidazole moieties for induction of fusion by protonation at low pH. Furthermore, acetylation was carried out to block any residual primary amines and some secondaries on the polymer. The polymer indeed induced the membrane fusion of acidic liposomes at low pH (shown in Figure 3).

The mechanism of membrane fusion observed in this experiment is possibly the same as that underlying polycation-induced fusion of negatively charged liposomes. CAIPEI was observed to have an optimum concentration for inducing aggregation or fusion of liposomes (shown in Figures 2 and 3), which is frequently observed in polycation-induced liposomal changes, since an excess of polycations may reduce the vesicular aggregation and fusion due to the electrostatic repulsion between liposomal surfaces coated with polycations. In this paper, we used polymer-linked imidazole instead of directly embedding imidazole derivatives into the lipid bilayer, since polycations bind to liposomes stronger than monovalent cations due to cooperative binding of the cationic residues to negatively charged head groups of phospholipids. This strong binding is thought to be important for fusion. CAIPEI-induced membrane intermixing was observed to be dependent on the polymer:vesicle ratio rather than on polymer concentration (see Figure 4). Such dependence might result from the cooperative multivalent binding. Indeed, a similar dependence was observed in polycation-induced liposomal fusion (Oku et al., 1986). Furthermore, the optimal CAIPEI-induced membrane fusion was observed at a ratio of about one imidazole per available PS, which is close to that observed in polycation-induced liposomal fusion (Oku et al., 1986).

Next, we incorporated CAIPEI into bilayers of PC vesicles and examined the effect of these liposomes on PS/PC vesicles. CAIPEI vesicles fused with negatively charged vesicles at low pH (shown in Figure 6). The membrane intermixing was not due to the CAIPEI in the aqueous phase which had not been incorporated in lipid bilayer but due to that incorporated in lipid bilayers, since CAIPEI in the aqueous phase could not induce membrane fusion between PC and PC/PS vesicles. Vesicles containing PS were reported to fuse at a pH below 4.5 when agglutination of liposomes was mediated by polylysine (Walter et al., 1986) or lectin (Bondeson et al., 1984). In these cases, the trigger for the fusion was suggested to be protonation of PS or PA and, in fact, liposomes composed of other acidic lipids such as phosphatidylinositol did not fuse under such a condition. In this study, CAIPEI vesicles fused with liposomes containing PG as well as PS or PA (shown in Figure 7), suggesting that protonation of CAIPEI molecules rather than of head groups of liposomes affects the fusion event.

Taken together, our results indicate that the fusion of CAIPEI vesicles at low pH is triggered by the binding of polycations generated by protonation of imidazole moieties to the acidic phospholipids. The hydrophobic interaction of the

PEI moiety with the lipid bilayer may also be involved in the fusogenic action. However, further experiments must be done to elucidate the mechanism of fusion. In this paper, we only show the membrane intermixing. We also tried to determine the intermixing of aqueous contents according to the methods described by Ellens et al. (1985). However, we could not determine the intermixing of aqueous contents since ANTS, an aqueous marker, bound to CAIPEI due to the negative charges of this fluorophore.

A number of pH-sensitive liposomes have been developed, including liposomes that become leaky at low pH (Nayer & Schroit, 1985). For the purpose of fusion with the endosomal membrane, palmitoylhomocysteine (Connor et al., 1984), *N*-acyl-2-aminopalmitic acid and its amide conjugates with serine or histidine (Diacovo et al., 1986), and oleic acid (Collins & Huang, 1986) have been used. However, these liposomes were composed mainly of PE, a typical nonbilayer lipid. We used liposomes containing PC as the main component, since our eventual goal is the therapeutic application of liposomes, and PC liposomes are rather stable against changes in a variety of parameters such as pH and salt concentration. Indeed, PC vesicles with CAIPEI in their bilayers were stable, and little increase in permeability to glucose was observed even at low pH. Furthermore, no hemolysis was observed in the presence of these CAIPEI vesicles at neutral pH. In light of these results, the CAIPEI vesicle seems to be a good candidate for liposomal application as a cytoplasmic delivery system.

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