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pH-Dependent Fusion of Lipid Vesicles Induced by Proton-Sensitive Polymer

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A novel pH-sensitive polymer, acetyl-imidazole-polyethylenimine, was synthesized. The polymer caused aggregation and membrane intermixing of sonicated phospholipid vesicles containing phosphatidylserine following lowering of the pH of the solution. This polymer seems useful for many liposomal applications.

The membrane fusion is an important process in many biological phenomena. However, the mechanism of membrane fusion is still poorly understood. A fusogen whose activity is regulated by differing conditions of the reaction, such as pH, might be useful for elucidating the mechanism of membrane fusion. In this paper, we report a pH-sensitive fusogen which induces liposomal fusion only under acidic conditions. This fusogen might be of interest for studying the infection process of some enveloped viruses, and the cytoplasmic delivery through the endosome of molecules encapsulated in liposomes.

We synthesized a pH-sensitive polymer, acetyl-imidazol-polyethylenimine (**2**), and investigated the fusogenic effects of the polymer on acidic phospholipid vesicles. The polymer becomes a polycation by protonation at pH 4-6. Thus the polymer is expected to induce membrane fusion, since many polycations such as polylysine,[1] and other synthetic polycations[2] are known to fuse negatively charged liposomes. Furthermore, polyhistidine, which also becomes polycationic by protonation under acidic conditions, is reported to induce membrane fusion at low pH.[3] In this report, imidazole was linked to branched polyethylenimine (PEI, M.W.=1 800), since polymer-linked imidazole seems much more active than imidazole monomer due to cooperative binding of the cationic residues to negatively charged head groups of the liposomal phospholipids.[4] This strong binding is thought to be important for fusion. We modified the PEI with ten imidazole moieties for inducing fusion by protonation at low pH.

Polymers **1**, **2**, and **3** were prepared by the following sequence of steps:[5] To obtain **1**, alkylation of PEI with imidazolemethylenechloride was used to introduce methyleneimidazole groups ($C_4H_5N_2$) into the PEI, $(C_2H_4N)_m$ (m=43). Polymer **2** or **3** was produced from either **1** or PEI, respectively, by subsequent treatment with acetic anhydride, by which any residual primary amines and some secondaries on the polymers were blocked with acetyl groups (C_2H_3O). Integration

of the peaks in the ^{1}H-NMR spectra of these polymers dissolved in D_2O indicated the following stoichiometric compositions for **1**, **2**, and **3**:

1: $(C_2H_4N)_m(C_4H_5N_2)_{0.24m}$ m=43

2: $(C_2H_4N)_m(C_4H_5N_2)_{0.24m}(C_2H_3O)_{0.51m}$ m=43

3: $(C_2H_4N)_m(C_2H_3O)_{0.75m}$ m=43

All lipids, i.e., egg yolk phosphatidylcholine (PC), bovine brain phosphatidylserine (PS), N-4-nitrobenzo-2-oxa-1,3-diazole PE (NBD-PE), and N-(lissamine Rhodamine B sulfonyl)dioleoyl-PE (Rh-PE) gave a single spot on silica gel thin-layer chromatography, and were used without further purification. Liposomes were prepared in saline and sonicated for 10 min using a Branson Sonifier at 50% duty cycle. Liposomal concentrations were indicated as phospholipid concentrations.

Since liposomal aggregation is a prerequisite for membrane fusion, we examined the polymer-induced agglutination of liposomes as determined by the change in light scattering of liposomal dispersions in the presence of the polymers. Ten nmol of lipid vesicles composed of PC:PS (4:1) were mixed with various amounts of polymers in 1 ml of saline at various pH's and incubated for 5 min at room temperature. Then the change in light scattering of the vesicles was measured at 450 nm using a fluorescence spectrophotometer. Figure 1 shows the relative increase in light scattering of PC/PS liposomes. The addition of PEI caused aggregation of the vesicles in a concentration-dependent manner. An increase in the light scattering of liposomal suspensions occurred within one minute after the addition of PEI (data not shown). PEI did not induce any change in light scattering when PC liposomes were used (data not shown), suggesting that negative charges on the liposomes are essential for the liposomal aggregation. **1** induced a pattern of liposomal aggregation very similar to that of Fig.1a (data not shown). On the other hand, acetylation of PEI (**3**) completely blocked the formation of liposomal aggregates. Considering these observations, it is expected that **2** does not interact with acidic phospholipids at neutral pH due to the acetylation of free amines, but interacts with these at low pH due to the protonation of the imidazole moiety.[6] Indeed, **2** induced aggregation of PC/PS vesicles only under low pH conditions (shown in Fig.1c).

Alternatively, we examined the fusogenic effects of PEI and its derivatives on liposome as determined by membrane intermixing. The intermixing of membrane lipids was measured with the resonance energy transfer assay based on the method of Hoekstra[7] using NBD-PE as a donor and Rh-PE as an acceptor lipid. Five nmol of vesicles composed of PC:PS:NBD-PE (molar ratio 15:4:1) and the same amount of those of PC:PS:Rh-PE (15:4:1) were mixed in the presence of various concentrations of polymers. Then the fluorescence intensity of NBD-PE was monitored (ex. 470 and em. 530 nm). Figure 2 shows the resonance energy transfer between donor and acceptor vesicles at various pH's. As is apparent in Fig.2, PEI caused membrane intermixing at all pH's, while **2** caused it only under acidic conditions, indicating the similarity between liposomal aggregation and membrane intermixing

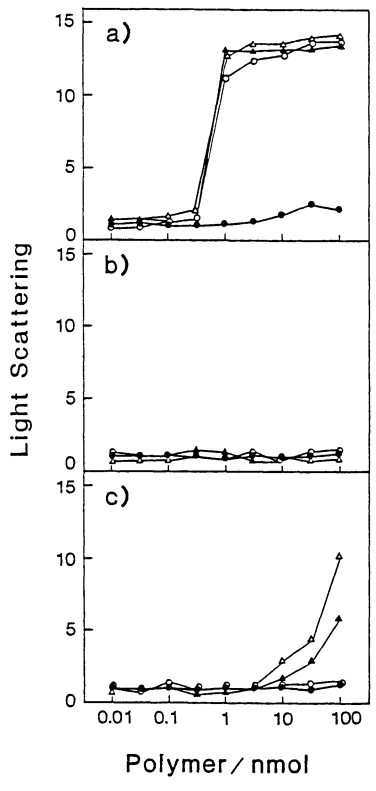

Fig. 1.

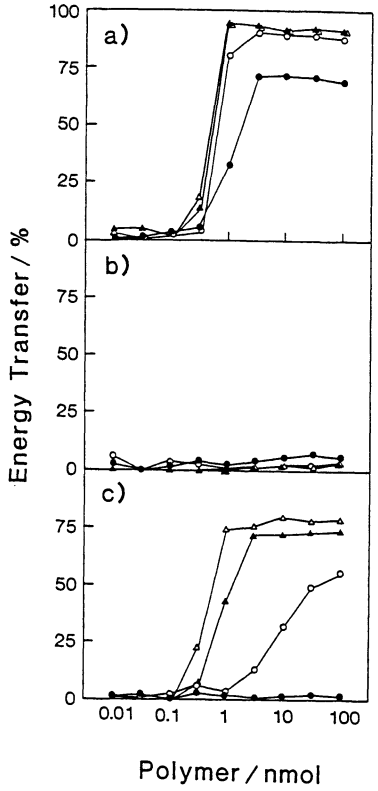

Fig. 2.

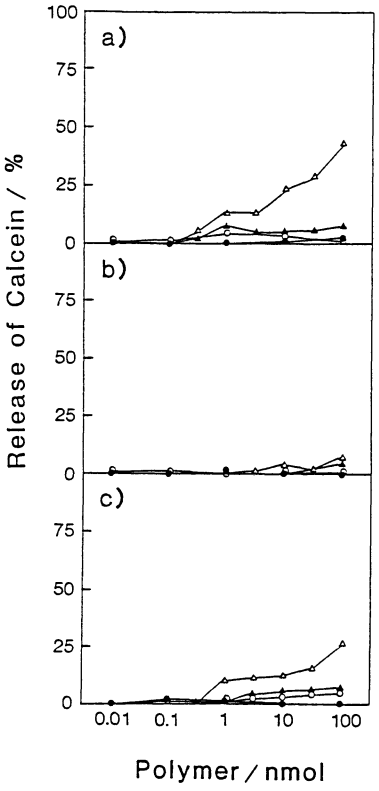

Fig. 3.

Fig.1. Changes in light scattering of liposomal suspensions induced by synthetic polymers. Sonicated liposomes composed of PC and PS (molar ratio 4:1, final concentration of liposomes was 10 nmol in 1 ml saline of various pH's) were incubated with various concentrations of PEI a), $\underset{\sim}{3}$ b), or $\underset{\sim}{2}$ c) for 5 min. Changes in light scattering were monitored at 450 nm after a 5 min incubation at pH 7.0 (●), 6.0 (o), 5.0 (▲), or 4.0 (△).

Fig.2. Effect of synthetic polymers on the fluorescence resonance energy transfer between liposomes containing NBD-PE and those containing Rh-PE. Sonicated liposomes containing NBD-PE were mixed with the same concentration of sonicated vesicles containing Rh-PE. Then various concentrations of PEI a), $\underset{\sim}{3}$ b), or $\underset{\sim}{2}$ c) were added to liposomal solution. NBD fluorescence was monitored as described in the text, after incubation at pH 7.0 (●), 6.0 (o), 5.0 (▲), or 4.0 (△).

Fig.3. Effect of synthetic polymers on the permeability properties of liposomal membrane. PC/PS liposomes were formed in 10 mM Mops-buffer (pH 7.2) containing 75 mM calcein. Vesicles were separated from free calcein by gel filtration on a Sephadex G-50 (column size, 15 mm x 250 mm; elution, with saline containing 0.01 mM EDTA). Liposomes (10 nmol in 1 ml of saline at various pH's) were incubated for 5 min with various concentrations of PEI a), $\underset{\sim}{3}$ b), or $\underset{\sim}{2}$ c). Released calcein efflux at pH 7.0 (●), 6.0 (o), 5.0 (▲), or 4.0 (△) was measured by fluorophotometry as described in the text.

with respect to pH dependence. 3 showed the absence of fusogenic activity which is in agreement with its negligible effect on liposomal agglutination. The effect of 1 on the membrane intermixing was quite similar to the effect of PEI on it (data not shown). The maximum energy transfer obtained in the presence of PEI or 2 was more than 50%, corresponding to more than one round of vesicle fusion.

Next, we examined the effect of the polymers on the permeability properties of liposomes as determined by calcein efflux from liposomes. Ten nmol of PC/PS liposomes containing 75 mM calcein were diluted to 1 ml with saline at various pH's and then mixed with various amounts of PEI or its derivatives. The fluorescence of the calcein was continuously monitored with a fluorescence spectrophotometer (ex. 488 and em. 520 nm). One hundred percent efflux of calcein was determined after addition of reduced Triton X-100. Figure 3 shows the efflux of calcein induced by PEI and its derivatives at various pH's. 3 did not induce any permeability changes even at pH 4.0, while PEI and 2 induced partial release of calcein at low pH's, suggesting that these polymers perturbed lipid bilayers at low pH. The difference between calcein efflux at pH 5.0 and that at pH 4.0 may be partly explained by the changes in the ionic form of calcein, for the carboxyl groups of calcein are less completely ionized at pH 4.0.

2 is a hopeful fusogen for studying membrane fusion since the effect is easily regulated by the pH of solutions. The fusion of vesicles at low pH is most likely to be triggered by a polycation which is generated by protonation of some group, such as the imidazole moiety used in the present experiments. The hydrophobic interaction of the PEI backbone with the lipid bilayer may also be involved in the fusion event. However, the further elucidation of the mechanism of membrane fusion must be examined.

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