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# Liposome Fusion Rates Depend upon the Conformation of Polycation Catalysts

Alexander A. Yaroslavov,*[,†] Andrey V. Sybachin,[†] Ellina Kesselman,[‡] Judith Schmidt,[‡] Yeshayahu Talmon,[‡] Syed A. A. Rizvi,[§] and Fredric M. Menger*[,§]

[†]Department of Chemistry, M. V. Lomonosov Moscow State University, Leninskie Gory, Moscow 119992, Russian Federation

[‡]Department of Chemical Engineering, Technion—Israel Institute of Technology, 32000 Haifa, Israel

[§]Department of Chemistry, Emory University, Atlanta, Georgia 30322, United States

Supporting Information

**ABSTRACT:** Cryo-TEM and NaCl-leakage experiments demonstrated that the cationic polymer polylysine induces fusion of anionic liposomes but that the cationic polymer poly(*N*-ethyl-4-vinylpyridinium bromide) (PEVP) does not, although both polymers bind strongly to the liposomes. The difference was traced to the thickness of the coatings at constant charge coverage. Polylysine is believed to form planar $\beta$-sheets that are sufficiently thin to allow membrane fusion. In contrast, looping and disorganization among adsorbed PEVP molecules physically prevent fusion. A similar effect is likely to be applicable to important polycation-induced fusion of cell membranes.

Polymer-induced cell fusion forms the basis of modern hybridoma technology.[1] Nonionic polymers (e.g., polyoxyethylene) and cationic polymers (e.g., polylysine) can both effectively provoke cell fusion.[2,3] The mechanisms whereby polymers induce cell fusion have been addressed using native cells and model systems. Among the latter, one finds liposomes (spherical bilayer vesicles) composed of lipids or lipid-like surfactants.[4] Liposomes have the advantage that their physicochemical properties and structural attributes (surface charge, phase state, presence of rafts, etc.) are relatively simple to modulate.[5,6] For example, bound water can be cleared from liposome surfaces by polyoxyethylene, thereby allowing direct contact among the membranes, which subsequently fuse.[7]

Cationic polylysine induces cell fusion at a low concentration because of its effective electrostatic binding to anionic cell surfaces.[3] Important details of polylysine-induced fusion of biological membranes have been revealed via the use of anionic liposomes with diameters not exceeding 100 nm. Adsorption of polylysine onto such liposomes is accompanied by neutralization of the anionic membrane charge. In addition, lateral segregation of the anionic lipid within the liposomal membrane (domain formation) can occur.[8] These events, which lead to disordered lipid packing, favor polylysine-induced liposome fusion.[9] On the other hand, polycations with different structures (e.g., diethylaminoethyldextran and spermine) actually prevent liposome fusion.[10,11] In the present communication, we show that poly(*N*-ethyl-4-vinylpyridinium bromide) (PEVP) lacks the fusogenic capabilities of polylysine although both polymers bind efficiently to anionic liposomes. Since this marked difference in the behaviors of the two polycations is nonintuitive, it prompted the study described herein.

Polylysine hydrobromide [degree of polymerization (DP) = 360] was commercially available, while PEVP (DP = 600) was synthesized by exhaustive quaternization of poly(4-vinylpyridine) with ethyl bromide.[12] Unilamellar liposomes (20−100 nm in diameter) were prepared in a pH 9 buffer by sonicating zwitterionic phosphatidylcholine [egg lecithin (EL)] containing 10 mol % doubly anionic diphosphatidylglycerol [cardiolipin ($CL^{2-}$)]. Polylysine was then mixed with the liposomes in sufficient amount that ∼75% of the negative liposome surface charge (i.e., the negative charge on the external leaflet) was neutralized by the positive charge of the bound polycation. This 75% neutralization (which maintained a small negative charge on the liposomes) was computed from (a) the known concentrations of the anionic and cationic components and (b) the assumption that all of the bound amino groups of polylysine were protonated. The latter assumption was verified by an electrophoretic titration at pH 9, in which the liposome mobility in an electric field ceases when the amount of polylysine added corresponds to roughly 50% neutralization of the total $CL^{2-}$. (The remaining 50% of the $CL^{2-}$ resides within the inner leaflets and, being charge-neutralized by counterions, does not contribute to mobility).[13−15] Since PEVP is known to induce $CL^{2-}$ flip-flop from the interior to the exterior leaflet, it was necessary to add twice the monomer equivalent used with polylysine in order to achieve a similar 75% charge neutralization of the liposomes. By this means, PEVP- and polylysine-covered surfaces with identical charges could be compared.

The behavior of the liposome/polylysine complex was monitored by cryo-transmission electron microscopy (cryo-TEM). The image in Figure 1a shows that the polydispersity of the system remained unchanged 5 min after the liposomes were mixed with polylysine. After 2 h, however, huge fused structures with varying morphologies, together with individual liposomes, were observed (Figure 1b). In contrast, PEVP did not initiate fusion after either 5 min (Figure 1c) or 2 h (Figure 1d), despite the fact that the vesicles came into direct contact (at least according to microscopic resolution). In other words, neither the size nor the morphology of the liposomes was modified in the presence of PEVP.

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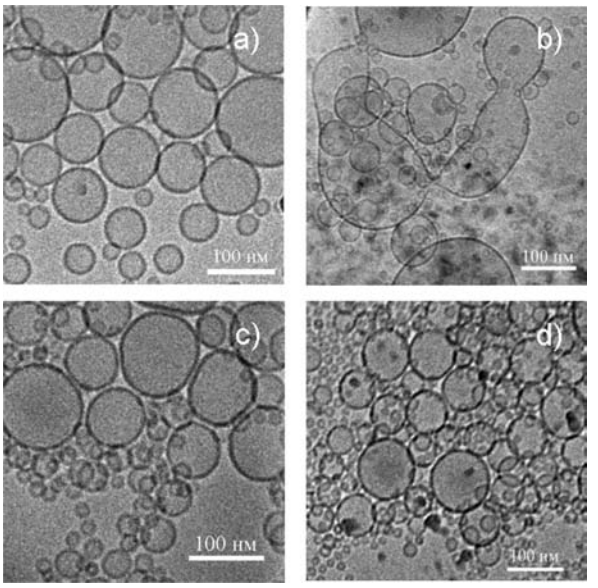


**Figure 1.** Cryo-TEM images of EL/$CL^{2-}$ liposomes in the presence of cationic polymers. (a, b) Liposomes + polylysine after (a) 5 min and (b) 2 h following mixing. (c, d) Liposomes + PEVP after (c) 5 min and (d) 2 h following mixing. The total lipid concentration was 1 mg/mL.

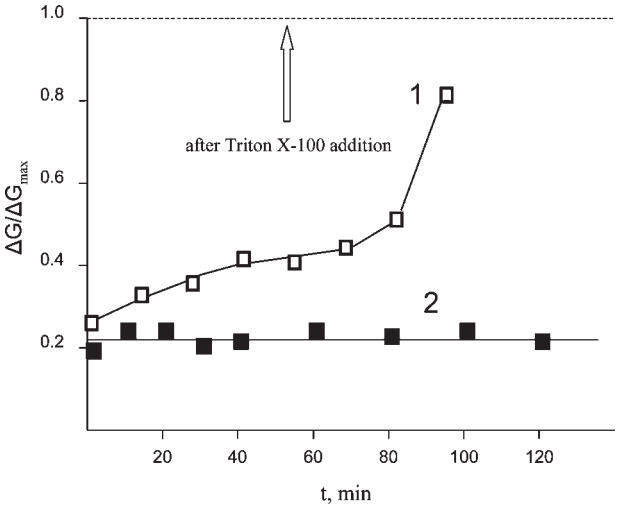


**Figure 2.** Time dependence of the relative conductivity of EL/$CL^{2-}$ liposome suspensions initially loaded with 1 M NaCl after addition of 0.06 mM (1) polylysine or (2) PEVP. The total lipid concentration was 1 mg/mL; $\Delta G = G_{lipo+polym} - G_{lipo}$ and $\Delta G_{max} = G_{Triton+lipo} - G_{lipo}$, where $G$ represents the measured conductivity.

Liposome fusion is usually accompanied by leakage of a solution from the inner liposome cavity into the environment.[16] With this in mind, we prepared a suspension of EL/$CL^{2-}$ liposomes encapsulating a 1 M NaCl solution as described in ref 17 and then mixed the preparation with a $0.6 \times 10^{-4}$ M polylysine solution. The subsequent NaCl leakage into the surrounding solution was monitored conductometrically (Figure 2).[17] A sharp rise in conductivity was observed 1 h after addition of polylysine (curve 1). In contrast, injection of PEVP solution into a suspension of NaCl-loaded liposomes had no effect on the conductivity within a 2 h interval (curve 2). Thus, binding of polylysine to the liposomes promoted NaCl release, whereas PEVP-covered liposomes displayed no leakage that would have indicated liposome fusion and disruption. This agrees with the cryo-TEM results and renders unlikely the possibility that the cryo-TEM data reflect some sort of artifact from sample preparation.

What could be the reason that polylysine catalyzes liposome fusion and promotes membrane leakage but PEVP does not? Upon first consideration, the behaviors of the two polycations seem to resemble each other in that they both demonstrate effective electrostatic complexation with anionic EL/$CL^{2-}$ liposomes. Both polycations cause a progressive increase in the size of the individual liposomes as charge is neutralized, and both induce lateral segregation of $CL^{2-}$ into $CL^{2-}$-rich domains.[18] To explain the difference in fusion properties, we hypothesized that the thickness of the polymer coating might play a role in the fusion process. Thick adsorbed layers would prevent direct liposome−liposome contact and impede their fusion.

In order to test our hypothesis, we measured the thickness of the interfacial polycation layer using dynamic light scattering (DLS).[19] In these experiments, the polycations were taken in 5-fold charge excess relative to the $CL^{2-}$ charge in order to impart a maximum positive charge to the polycation-covered liposomes. By this means, electrostatic repulsion prevented liposome aggregation that would have complicated the DLS analysis. Although the liposome surface charge and degree of surface coverage were different from those in the fusion experiments, the information on the relative layer thicknesses provided by polylysine and PEVP coatings should still be relevant.

The thickness of the polycation layer was calculated as the difference between the hydrodynamic radii of liposomes with and without the polymer coating: $\Delta R = R_{comp} - R_{lipo}$. Size homogeneity of the liposome population was essential for this experiment. In order to achieve minimum polydispersity, the lipid/buffer mixture was sonicated for $3 \times 200$ s and additionally extruded (30 times) through a polycarbonate filter with 100 nm pores using an Avanti mini-extruder. This allowed the preparation of an EL/$CL^{2-}$ liposome suspension with a hydrodynamic radius of 30 nm and a size distribution (polydispersity index = 0.005) that was sufficiently narrow for the DLS experiments. After the polycations were adsorbed on the liposomes, the hydrodynamic radii of the liposome/polycation complexes were found to be 35 nm in the presence of polylysine and 65 nm in the presence of PEVP. Thus, the radii increased by 5 and 35 nm for polylysine and PEVP interfacial layers, respectively. The radius increase caused by polylysine was understandably small because according to circular dichroism data, polylysine transforms from a random coil into a planar $\beta$-sheet conformation when adsorbed on anionic lipid bilayers.[20] Adsorption of PEVP, on the other hand, would greatly expand the liposome radius if the polymer were packed loosely on the membrane surface, perhaps as a result of looping and/or generalized disorganization. Thus, the DLS data provide an interconnection between the fusogenic ability of cationic polymers and the structure of interfacial polymer layers on the liposome surface. Thickness seems to be the key difference between PEVP and polylysine layers adsorbed onto the outer surfaces of liposomes.

There are at least three consequences of binding of polylysine to the liposomes. (a) The polylysine layer reduces the electrostatic repulsion among the anionic liposomes and promotes their aggregation. (b) The layers are thin enough to allow intimate contact between neighboring liposomal membranes, as schematically represented in Figure 3a. (c) The liposomes become leaky

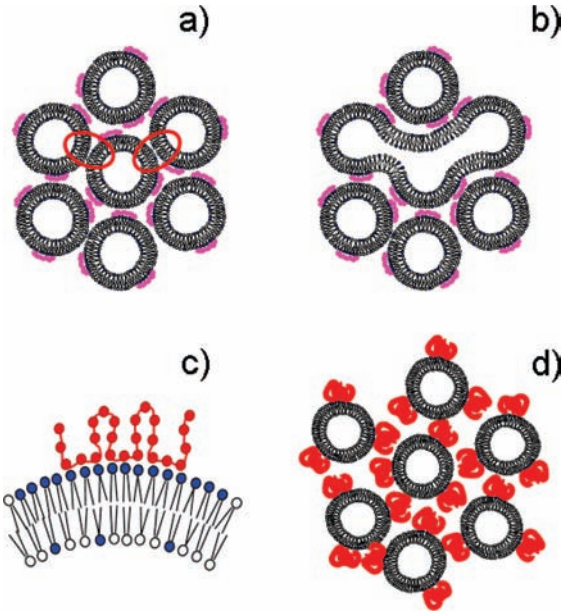


**Figure 3.** (a, b) Structures of polylysine/liposome complexes (a) before and (b) after fusion. The red ovals in (a) show points of contact between bilayers. (c) Schematic illustration of looping in a PEVP/liposome complex, showing domain formation involving PEVP (red circles) and $CL^{2-}$ (blue circles) embedded in phosphatidylcholine (white circles). (d) Schematic showing how PEVP physically retards the fusion process.

to water, as is necessary to accommodate volume changes during major morphological modifications. As a result, fusion of aggregated liposomes can take place (Figure 3b) according to a stalk—pore mechanism.[21] PEVP macromolecules also reduce the interliposomal repulsion, but at the same time they create a physical barrier that inhibits fusion, as depicted in Figure 3c,d.

In summary, thickness has been shown to be a key element in determining the ability of polycations to induce membrane fusion. Thus, fusion is favored by the ability of charge-neutralizing macromolecules to self-assemble into thin, planar arrays (e.g., $\beta$-sheets) at the liposome surfaces. In contrast, polymers that form looped configurations or engage in "brush-heap" stacking on the membrane surface physically retard fusion despite the favorable reduction in interliposomal electrostatic repulsion. This study therefore provides new evidence relating fusogenic ability to adsorbent conformation. The same principle is likely to apply to important polycation-induced fusion of cell membranes.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Experimental details on TEM and liposome preparations. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

**Corresponding Author**
menger@emory.edu

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