# Polymer-Induced Flip-Flop in Biomembranes

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**ABSTRACT**

This Account describes the ability of amphiphilic polymers (e.g., EO/PO/EO block copolymers) and polycations [e.g., quaternized poly(4-vinylpyridine)] to accelerate translocation from the inside leaflet to the outside leaflet ("flip-flop") within vesicle bilayer membranes. Driving forces and mechanisms of flip-flop catalyzed by the nonionic and cationic polymers are different. The nonionics are bound to the biological membrane via incorporation of their hydrophobic blocks into the inner part of the lipid bilayer occupied by the hydrocarbon chains. The resulting scrambling of lipid molecules is favored by the overall hydrophobicity of the copolymer and the volume of its hydrophobic block. External binding of the cationic polymers, on the other hand, is driven by electrostatic interactions between the positively charged polymer units and the negatively charged lipid headgroups within the outside leaflet. Electrostatic binding favors both the flip-flop of anionic lipid from the inner to outer leaflet and the formation of anionic domains in the outer leaflet. When it is considered that less than 1% of the liposome surface is occupied by certain bound polymers, their effect upon membrane dynamics, as will be described herein, is considerable. A distinct correlation has been found between the "flippase" activity of the polymers and their ability to mediate drug permeation through biomembranes.

## 1. Introduction

Molecular-biology studies during past decades have revealed that a cell membrane represents a concerted ensemble of lipids and proteins.[1] This "micromachine" controls incoming and outgoing transmembrane fluxes while translating chemical signals into a cell-understandable language. For proper membrane operation, a specific arrangement of lipid molecules in the molecular bilayer, both within each of the two component leaflets and between them, is required. Interleaflet transfer (flip-flop) is an important mechanism for achieving such proper lipid ordering.

Flip-flop of lipid molecules has been quantified by using artificial bilayer membranes. The approaches and experimental details were summarized in reviews.[2] The characteristic half-times of flip-flop were found to lie within wide limits: from milliseconds for simple fatty acids in nondissociated (uncharged) forms up to several hours for zwitterionic phosphatidylcholine.[1,2] Rates of the overall process depend upon the net charge and size of polar headgroups as well as the length of hydrophobic tails of the mobile lipid molecules.[3] In cell membranes, flip-flop proceeds more rapidly; even charged lipids have half-times equal to a few seconds.[4]

Transmembrane asymmetry (i.e., unequal leaflet composition) is common among cells. Such asymmetry is promoted by specific membrane enzymes, the so-called flippases. The physiological role of transmembrane asymmetry in biological membranes, generated and regulated by flippases, has been reviewed[4,5] but is not completely understood. Given below are two examples demonstrating the primary importance of asymmetrical structure in cell membranes. Phosphatidylserine (PS), located primarily on the inner leaflet, ensures close proximity of the erythrocyte plasma membrane with the protein cytoskeleton. Flippase-induced transfer of PS to the outer leaflet is accompanied by conversion of initially spherical erythrocytes into "discocytes".[6] In the case of platelets, PS exposure triggers cell activation and their interaction with prothrombin.[7]

Not only specific enzymes but also low-molecular-weight and polymeric substances are capable of activating flip-flop. Diverse structures that demonstrate flippase-like behavior are usually referred to as "synthetic flippases". Flippase activity was mimicked, for example, with tris(2-aminoethyl)amine derivatives having appended urea and sulfonamide groups[8] as well as with ureidyl derivatives of steroids.[9]

Amphiphilic polymers (i.e., polymers with both hydrophilic and hydrophobic fragments) can embed into a biological membrane and fluidize it, causing a significant acceleration of the interleaflet lipid exchange.[10,11] Externally bound cationic polymers, on the other hand, accelerate membrane charge asymmetry and the subsequent extrusion of anionic lipids from the inner to outer membrane leaflet.[12] In the present Account, we describe how and why neutral amphiphilic polymers and polycations can influence membrane flip-flop in model liposome (vesicle) systems.

## 2. Amphiphilic Nonionic Polymers

In our research, four types of nonionic amphiphilic polymers were coupled to small bilayer lipid liposomes

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Fredric M. Menger received his Ph.D. long ago ("fire had yet to be domesticated", he claims), and he is now professor of chemistry at Emory University. His collaboration with Russian scientists has been a great pleasure and education for him.

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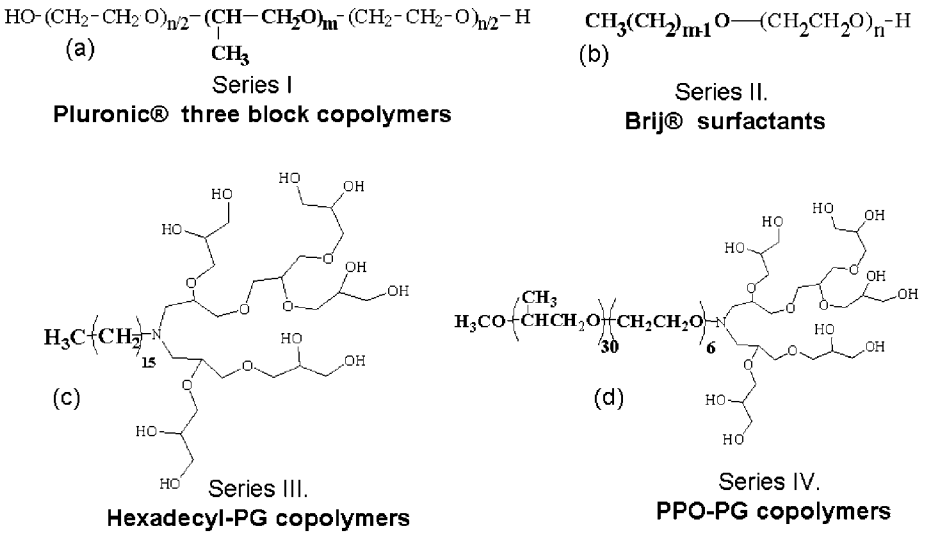


**FIGURE 1.** Chemical structures of amphiphilic polymers.

composed of electrically neutral egg yolk lecithin (EL), a phospholipid mixture that is always in the fluid or "liquid crystalline" state. Possible effects of the polymers on the lipid flip-flop were then examined. The first type of polymer was the commercially available ethylene oxide/propylene oxide ($EO_n/PO_m/EO_n$) three-block copolymers, i.e., the Pluronics (series I in Figure 1). The second was a series of nonionic surfactants with poly(ethylene oxide) (PEO) headgroups tied to long chains, i.e., the Brijs (series II in Figure 1). In the third and fourth types, hyperbranched polyglycerol (PG) fragments were attached to alkyl chains and to $EO_nPO_m$ blocks, respectively (series III and IV in Figure 1).

Polymer-catalyzed flip-flop is obviously mediated by the initial binding of the polymer to the liposomal membranes. Therefore, the starting point of our research was to study polymer−liposome complexation. The interaction of an amphiphilic compound with a lipid membrane is usually quantitatively modeled as a partition coefficient between water and membrane $K_P$.[13,14] A literature search provided us with information on the water/membrane partition of conventional nonionic oligomeric surfactants from the Brij family (series II).[14] In contrast, studies of amphiphilic polymers binding to biological membranes have been more descriptive than quantitative. In view of this uncertainty, we exploited equilibrium dialysis to obtain discrete binding data. For these experiments, Pluronic L61 ($EO_2PO_{30}EO_2$) was selected (a polymer able to induce significant disturbances in artificial lipid bilayers[15−17] while being active toward tumor cells as well).[18] With the aid of tritium-labeled Pluronic L61, obtained from the hot atom bombardment technique, the membrane/water partition coefficient was found to be 45 ± 7,[16] a value 2 orders of magnitude smaller than that for Brij surfactants.[14] However, as discussed later, flippase activities of the Brij surfactants were much less than those of the Pluronics despite the much stronger binding of the former. Thus, the affinity of amphiphilic compounds to the lipid membranes is not the main factor controlling flippase activity in our liposome systems.

How might Pluronic surfactants arrange themselves in the bilayer lipid membranes? These macromolecules are composed of alkylene oxide blocks, whose chemical structures seem, at first glance, to resemble each other (series I in Figure 1). However, the thermodynamic behavior of these blocks in water is different. For example, PEO has a high water solubility, while PPO is insoluble in water at room temperature.[19,20] Hence, it is reasonable to expect that Pluronics interact with lipid membranes via incorporation of rather hydrophobic PPO blocks into the interior region of the bilayer, whereas the hydrophilic PEO blocks are exposed to the surrounding bulk water. This model for the Pluronic−lipid bilayer binding was originally hypothesized by Topchieva et al.[21] and further confirmed by Firestone et al.[22]

After the efficiency of Pluronic complexation to liposomes had been evaluated, we investigated possible Pluronic effects on lipid flip-flop. Up to now, a number of approaches have been developed to study flip-flop in bilayer lipid vesicles, with all of them based on asymmetrical liposomes having the translocating lipids originally present in only one of the membrane leaflets. Kinetics of the lipid appearance on the opposite leaflet obviously reflects the lipid flip-flop rate under the given conditions (liposome composition, specific structure of translocating lipid, temperature, etc.)[23] and is the subject of a recent review.[24]

We prepared asymmetric lecithin liposomes with a fluorescently labeled lipid, *N*-[(7-nitrobenz-2-oxy-1,3-diazol-4yl)dipalmitoyl]phosphatidylethanolamine (NBD-PE), originally located only in the internal membrane leaflet as described in ref 25. The overall procedure is schematically represented in Figure 2. Outward migration of NBD-PE was assessed by treating the liposomes with sodium dithionite that irreversibly destroys NBD fluorescence only in the external leaflet. The kinetics of spontaneous NBD-PE flip-flop, corresponding to this fluorescence loss, was found to obey a first-order law with an effective rate constant of about 0.2 $h^{-1}$ (curve 1 in Figure 3),[10] consistent with earlier published data.[25] As would be expected, the

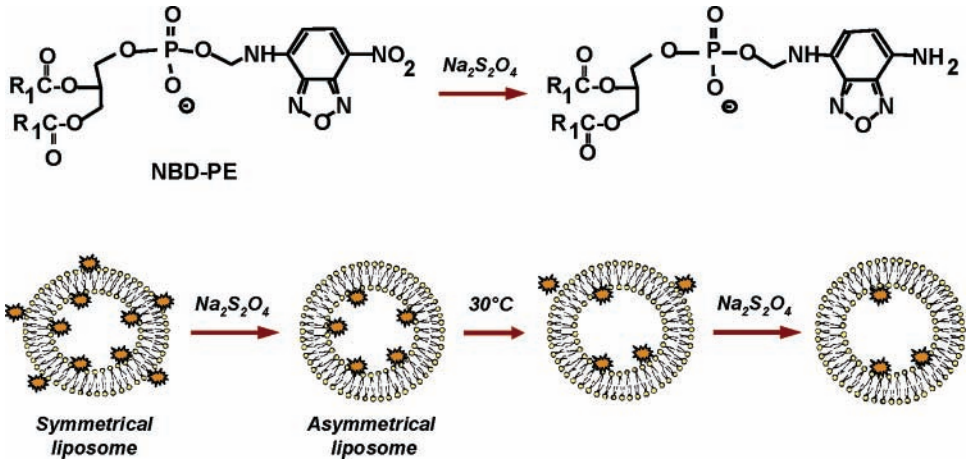


**FIGURE 2.** Evaluation of transmembrane asymmetry in liposomes using the dithionite technique (schematic representation).

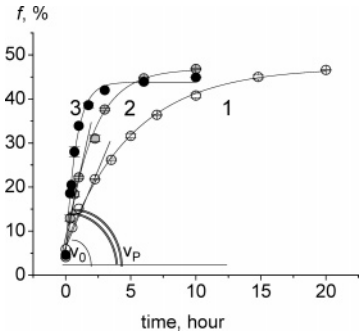


**FIGURE 3.** Kinetics of NBD-PE flip-flop in the absence (1) and in the presence of $10^{-2}$ M (2) and $2.5 \times 10^{-2}$ M (3) Pluronic L61. NBD-PE was initially located on the inner leaflet of egg lecithin (EL) liposomes. Total lipid concentration, 0.15 mg/mL; buffer solution, $10^{-2}$ M Tris, $1.5 \times 10^{-1}$ M choline chloride, and $10^{-3}$ M EDTA at pH 7.0 and 25 °C. Lines in this figure and all other figures are included as visual guides only.

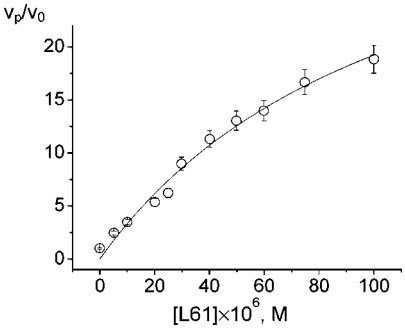


**FIGURE 4.** Effect of the Pluronic L61 concentration on NBD-PE flip-flop in egg lecithin (EL) liposomes. See the experimental details in the caption to Figure 3.

fraction of NBD-PE migrating from the inner to outer leaflet approaches 50%.

The addition of Pluronic L61 to the asymmetrically labeled liposomes resulted in a considerable acceleration of NBD-PE flip-flop (plots 2 and 3 relative to plot 1 in Figure 3). Thus, the effective rate constant increased to 0.51 $h^{-1}$ for $5 \times 10^{-6}$ M and 1.28 $h^{-1}$ for $20 \times 10^{-6}$ M Pluronic solutions.[16] The amount of migrated lipid at infinity with and without Pluronic addition was nearly the same. This means that Pluronic did not alter the transmembrane lipid distribution but only the rate at which the equilibrium state is achieved.

Pluronic-induced acceleration of the NBD-PE flip-flop was quantified as a ratio of the initial flip-flop rates in the presence ($v_p$) and absence of the copolymer ($v_0$). As follows from the data of Figure 4, the Pluronic effect (expressed as the $v_p/v_0$ ratio) increases with rising copolymer concentrations, reaching 6-fold at $20 \times 10^{-6}$ M Pluronic and corresponding to adsorption of about 20 polymer molecules to the surface of each 100 nm diameter liposome.[16] Simple calculations show that only about 0.5% of the liposome surface was covered by the adsorbed Pluronic. In other words, a negligible portion of vesicular lipids had direct contact with the copolymer.

It was useful to develop a quantitative parameter for the flip-flop-inducing activities of Pluronics and other membrane-active amphiphiles independent of their concentrations. This allowed a simple comparison of the activities for different amphiphilic polymers. To accomplish this, we described the efficacy of the polymer with an empirical linear eq 1

$$\frac{v_p}{v_0} = 1 + \beta_{f-f}[P]_0 \qquad (1)$$

where $[P]_0$ is the amphiphile concentration. The coefficient $\beta_{f-f}$ in this equation, referred to as the polymer "flippase activity", is a measure of the inherent ability of amphiphilic copolymers to accelerate lipid flip-flop.

To reveal a relationship between the structures of amphiphilic polymers and their flippase activity, we studied 19 compounds with different relative contents of hydrophobic and hydrophilic blocks. Hydrophobic blocks were represented by PPO or aliphatic chains, and the hydrophilic blocks were represented by PEO or PG (Figure 1).[26] All of the compounds were tested for their effects on the flip-flop rate as represented by their particular $\beta_{f-f}$ value. To assess how the flippase activity correlates with the changes in structures of amphiphilic polymers, the

**Table 1. Physicochemical Characteristics of Amphiphilic Polymers (According to Ref 8)**

| copolymer (trade name in parentheses) | molecular weight | $\beta_{f-f}$ ($\mu M^{-1}$) | log $K_{P\ water/hexane}$ | $V_{hydrophobic}$ ($nm^3$) |
|---|---|---|---|---|
| series I | | | | |
| $EO_2PO_{30}EO_2$ (L61) | 2090 | 0.16 ± 0.01 | −0.24 ± 0.037 | 8.38 |
| $EO_{13}PO_{30}EO_{13}$ (L64) | 2900 | 0.07 ± 0.01 | −1.83 ± 0.27 | 8.38 |
| $EO_{76}PO_{30}EO_{76}$ (F68) | 8400 | 0.01 ± 0.05 | −3.5 ± 0.53 | 8.38 |
| $EO_3PO_{40}EO_3$ (L81) | 2750 | 0.56 ± 0.045 | −0.11 ± 0.02 | 11.2 |
| $EO_{26}PO_{40}EO_{26}$ (P85) | 4500 | 0.22 ± 0.06 | −2.65 ± 0.4 | 11.2 |
| $EO_{61}PO_{40}EO_{61}$ (F87) | 7700 | 0.06 ± 0.01 | −3.19 ± 0.48 | 11.2 |
| $EO_5PO_{59}EO_5$ (L101) | 3800 | 1.35 ± 0.13 | 0.11 ± 0.017 | 16.4 |
| $EO_{37}PO_{56}EO_{37}$ (P105) | 6500 | 0.21 ± 0.03 | −2.6 ± 0.4 | 15.6 |
| $EO_{132}PO_{50}EO_{132}$ (F108) | 14 600 | 0.11 ± 0.02 | −3.65 ± 0.54 | 13.9 |
| series II | | | | |
| $C_{12}H_{25}EO_4$ (Brij 30) | 345 | 0.0035 ± 0.0012 | −1.79 ± 0.06.4 | 0.74 |
| $C_{12}H_{25}EO_{24}$ (Brij 35) | 1225 | 0.01 ± 0.003 | 3.20 ± 0.61 | 0.74 |
| $C_{16}H_{33}EO_{10}$ (Brij 56) | 700 | 0.028 ± 0.003 | 2.32 ± 0.52 | 0.89 |
| series III | | | | |
| $C_{16}H_{33}G_{10}$ | 981 | 0.03 ± 0.01 | −2.20 ± 0.58 | 0.89 |
| $C_{16}H_{33}G_{64}$ | 5000 | 0.01 ± 0.005 | −2.92 ± 0.11 | 0.89 |
| series IV | | | | |
| $PO_{30}EO_6G_2$ | 2267 | 0.565 ± 0.07 | −0.51 ± 0.02 | 8.38 |
| $PO_{30}EO_6G_{30}$ | 3601 | 0.19 ± 0.02 | −2.45 ± 0.07 | 8.38 |
| $PO_{30}EO_6G_{76}$ | 7760 | 0.11 ± 0.02 | −4.07 ± 0.16 | 8.38 |

latter are arranged into four series as shown in Table 1 (corresponding to the four compound types depicted in Figure 1).

Within each series, the flippase activity diminishes with an elongation of the polymer hydrophilic blocks, i.e., with a decrease in the hydrophobic/hydrophilic ratio. Because the interaction of amphiphilic polymers with the lipid membranes is contributed mainly by hydrophobic interactions, we estimated a total hydrophobicity of polymers by measuring their partition coefficients in a biphasic water/hexane mixture. In this system, hexane serves as a model for the hydrophobic membrane interior. The results are shown in Table 1 (column 4) as log $K_{P\ water/hexane}$. In this way, a good correlation was obtained between the flippase activity and total polymer hydrophobicity for Pluronics and PG−Pluronic conjugates. At the same time, the behavior of the Pluronic polymers was different from the behavior of surfactants in which hydrophilic blocks were attached only to single hydrocarbon chains. For example, as mentioned, Brij surfactants (series II) exhibited extremely high affinities for lipid membranes while having flippase activities much smaller than those of Pluronics with a weaker membrane binding.

The structures of Pluronics and Brijs differ in the size of their hydrophobic fragments. Because hydrophobic PPO blocks of Pluronic macromolecules are much larger than the alkyl chains of Brij surfactants, the former should have more substantial effects upon the lipid bilayer organization. This, in turn, might be a reason for differences between the flippase activities of Pluronics and Brijs. We calculated van der Waals volumes of hydrophobic fragments for all studied compounds, $V_{hydrophobic}$ (column 5 in Table 1), and found an obvious interconnection between these parameters and $\beta_{f-f}$ values. This, as seen in the next paragraph, allowed an explanation for the differences between the flippase activities of Pluronics and Brijs.

The best correlation between the flippase activities and the abilities of amphiphilic compounds to modulate the membrane characteristics was achieved with a linear combination of the total hydrophobicity and volumes of hydrophobic fragments (eq 2)

$$\log \beta_{f-f} = 0.182 \log K_{P\ water/hexane} + 0.155 V_{hydrophobic} \quad (2)$$

The results are shown in Figure 5 as two parallel separate lines, with the lower referring to series I and II copolymers and the upper referring to series III and IV derivatives.[10]

The numerical coefficients in eq 2 resemble each other, showing nearly equal contributions of both factors, namely, the total hydrophobicity and the hydrophobic fragment volume, to the flippase activity of the amphiphiles. Moreover, Pluronics with their extended hydrophobic PPO blocks and Brij surfactants with their hydrocarbon tails are both described by the key $K_{P\ water/hexane}$ and $V_{hydrophobic}$ parameters. This indicates that flippase activity is mainly determined by the balance between these parameters, while the exact chemical nature of hydrophobic fragments apparently plays an auxiliary role.

In completing this section, we would like to emphasize that a pronounced catalytic effect by amphiphilic polymers toward lipid flip-flop manifests itself when only a few macromolecules are bound to each liposome. This

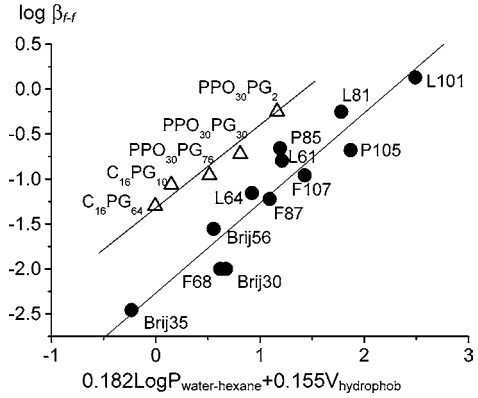


**FIGURE 5.** Correlation between the flippase activities of amphiphilic (co)polymers and their hydrophobicities. See the explanation in the text.

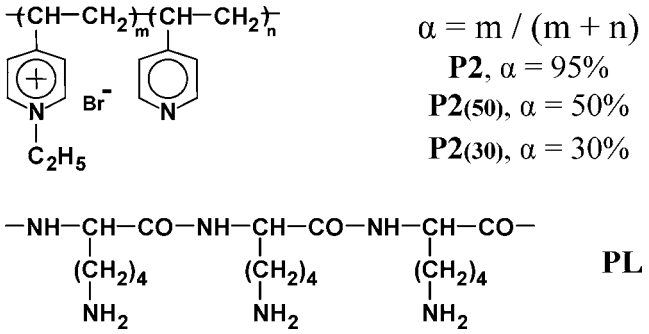


**FIGURE 6.** Chemical structures of cationic polymers.

corresponds to 0.5% of the liposome surface occupied by the adsorbed polymer. Three currently indistinguishable reasons for such a sensitive acceleration can be postulated: (a) The adsorbed polymer may perturb the cooperative packing of lipid molecules throughout the bilayer. Accordingly, flip-flop takes place uniformly across the bilayer. If this is true, then for small liposomes, 60−80 nm in diameter, as many as 1000 lipid molecules may be involved in coordinated structural rearrangements induced by a single disruptive macromolecule. (b) It is possible that a polymer/lipid complex is formed that "shuttles" lipid from one side of the bilayer to the other. (c) Finally and perhaps most likely, flip-flop may occur only in close proximity to adsorbed macromolecules. In other words, lipid translocation results from polymer-induced "edge defects" in the lipid bilayer. If this is the source of the catalysis, the observed acceleration, e.g., 6-fold with $20 \times 10^{-6}$ M Pluronic (Figure 4), must be multiplied by a factor equal to the ratio of the total to polymer-occupied membrane surfaces. Thus, the accelerating effect, normalized to the membrane surface area occupied by the adsorbed polymer, is 3−4 orders of magnitude.

## 3. Cationic Polymers

Binding of cationic polymers to anionic liposomes required a totally different approach. When poly(4-vinylpyridine) was quaternized with differing amounts of ethyl bromide, polycations with a varying content of positive units in the macromolecules were obtained. Thus, chemical structures of the polycations with cation content equal to 95, 50, and 30% are represented by P2, P2(50), and P2-(30), respectively (Figure 6). A commercially available cationic polypeptide, poly-L-lysine (PL), was also used.

In the first experiments, liposomes (50−80 nm) were constructed from a mixture of neutral zwitterionic dipalmitoylphosphatidylcholine (DPPC) and cardiolipin ($CL^{-2}$), a lipid bearing two negative headgroups and four alkyl tails. The molar content of the negative headgroups was equal to 10%.[27] Calorimetric data, obtained on this system by differential scanning calorimetry, showed a single symmetrical peak, indicating a nearly uniform distribution of negative $CL^{-2}$ molecules throughout the DPPC bilayer.[28]

Binding of a cationic polymer, P2, to DPPC/$CL^{-2}$ liposomes was accompanied by neutralization of the

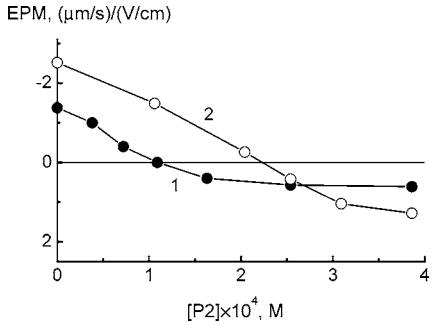


**FIGURE 7.** Effect of the P2 concentration on the electrophoretic mobility (EPM) of solid (1) and liquid (2) DPPC-$CL^{-2}$ ($\nu$ = 10%) vesicles. Lipid concentration, 1 mg/mL; buffer solution, $10^{-2}$ M borate at pH 9.2 and 20 °C (1) and 55 °C (2).

liposome surface charge, as measured by the electrophoretic mobility (EPM) of polycation/liposome complexes (Figure 7). It is instructive to explain certain details about the EPM method as applied to our system. Consider a liposome that, like ours, is negatively charged owing to the presence of anionic lipid. Now anionic lipid within the inner leaflet is charge-neutralized by an equivalent amount of counterions (bound or otherwise). Because these counterions cannot escape when the liposome migrates in the applied field, the inner leaflet plays no role in the mobility measurements. This is not true for the outer leaflet with its "loose" counterions, which are free to migrate oppositely to the liposome itself. However, when a cationic polymer sticks to the anionic liposome, the liposome will cease to migrate only when there is a precise charge-neutralization. Thus, a plot of EPM versus the polymer concentration (using a polymer of known charge content) reveals, at zero mobility, the exact anionic charge content of the outer leaflet. It is as if the outer liposomal charge has been titrated. This is a highly useful piece of information. We can tell, for example, if the anionic lipid originally in the inner leaflet remains at that site or whether it manages to flip-flop to the outer leaflet under the influence of a bound cationic polymer.

One other fact needs to be explained to understand our EPM data: Membrane bilayers are characterized by a transition temperature, $T_m$. Below $T_m$ , the membrane is "solid" (a rigid gel with little chain mobility). Above $T_m$ , the membrane is "liquid" (a liquid crystal with considerable chain disorder). As we will see, flip-flop is critically dependent upon the phase status of the membrane.

As follows from the data of Figure 7, the amount of P2, required for complete neutralization of the solid liposome surface charge, was half that of the liquid liposome. In calculating the fraction of $CL^{-2}$ involved in electrostatic complexation with P2, the following observations were taken into account. (a) At EPM = 0, all added P2 was found to be bound to liposomes (for both solid and liquid); no polycation was found in solution. This was proven by measuring (via spectrophotometry) the amounts of unbound P2 in the supernatants after centrifugation of P2−liposome complexes.[27,29] (b) By potentiometric titration with a $Br^-$-selective electrode of a P2−liposome complex

suspension, the fraction of quaternized P2 units, involved in electrostatic binding with liposomes, was found to be about 90%.[27,29] (c) Liposomes retained their integrity when contacting polycation as shown by a lack of leakage from internalized NaCl.[30] These findings allowed for the evaluation of the amounts of polycation needed to neutralize the anionic vesicles and, therefore, the fraction of total $CL^{-2}$ on the outer surface of the liposomes: 50% for the solid DPPC/$CL^{-2}$ liposomes and 100% for the liquid DPPD/$CL^{-2}$ liposomes. In other words, the polycation electrostatically interacted with only half of the $CL^{-2}$ molecules when bound to solid liposomes but with all of the $CL^{-2}$ molecules when the polycation was bound to the liquid liposomes. The latter can only happen if there is fast transmembrane migration of $CL^{-2}$ ions from the inner to outer leaflet of the liquid liposomal membrane. In summary, polycation adsorption induced a $CL^{-2}$ flip-flop in liquid but not in solid membranes.[12,27,31]

Polymer binding also resulted in aggregation of the liposomes.[27−31] As measured by quasi-elastic light scattering, the largest particle sizes were observed at EPM = 0. The neutral particles here are not now electrostatically inhibited from growing, and they can do so either with or without polymer bridging. A further increase of the polycation concentration caused the particle size to decrease. In this latter polycation concentration range, polycation−liposome complex particles were stabilized against aggregation by the abundant positive charge imparted by adsorbed polycation.

Polycation adsorption also promoted lateral lipid segregation within the outer leaflet of a mixed negative/neutral lipid membrane. We assessed this P2-induced lateral segregation in the DPPC/$CL^{-2}$ membrane calorimetrically.[28,32] Pure DPPC membranes have a narrow endothermic peak, corresponding to its $T_m$, at 41.5 °C.[33] On the other hand, $CL^{-2}$ is a mixture of natural lipids with a $T_m$ around 10 °C,[34] which is outside the temperature range investigated. Our DPPC/$CL^{-2}$ mixture displayed a single broad melting peak at 39 °C. As the concentration of P2 added to the liposomal dispersion increased, the melting peak gradually narrowed and shifted to higher temperatures, finally superimposing itself upon the melting peak of the single-component DPPC liposomes at 41.5 °C. Thus, P2 had "cleansed" the binary mixture of $CL^{-2}$ to create large domains of pure DPPC.

As a result of these structural rearrangements, the initial liquid membrane divided into two microphases: one enriched with neutral DPPC molecules and other enriched with $CL^{-2}$ lipid. These rearrangements caused abnormal asymmetry in the charge distribution: all negative lipid molecules were concentrated and clustered on the outer leaflet of the membrane, thereby providing a maximum number of ionic contacts with the polycation. An equal number of neutral zwitterionic DPPC molecules apparently transferred in the opposite direction, namely, from the outer to inner membrane leaflet. Such synchronous movements of lipid molecules enabled the membrane of liquid DPPC/$CL^{-2}$ liposomes to keep its bilayer structure intact.

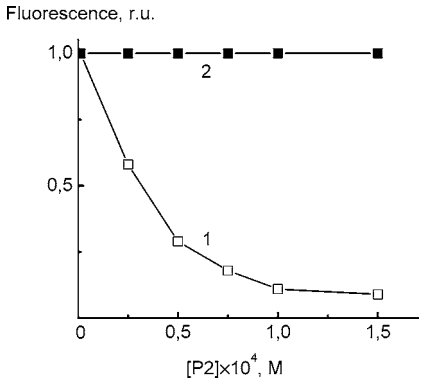


**FIGURE 8.** Changing of the relative fluorescence intensity of labeled EL/$CL^{-2}$ vesicles ($\nu$ = 20%) after complexation with P2 (1) followed by the addition of a PAA excess (2). Lipid concentration, 1 mg/mL; buffer solution, $10^{-2}$ M borate at pH 9.2.

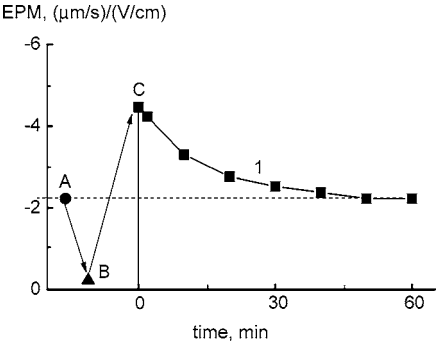


**FIGURE 9.** EPM of original liquid DPPC/$CL^{-2}$ liposomes (A) and complexed with P2 (B). In 1 min after the addition of a 3-fold PAA excess to the P2−liposome complex, the EPM of particles rises to C and then decreases following curve 1. Total lipid concentration, 1 mg/mL; buffer solution, $10^{-2}$ M borate at pH 9.2 and 55 °C.

To control a possible removal of polycation from the liposomal membrane, a fluorescence approach was used. P2 is a strong fluorescence quencher. Therefore, to follow its adsorption/desorption on the fluid membrane, a suspension of liquid lecithin liposomes incorporating a small amount (approximately 0.5 mol %) of a fluorescent-labeled lipid, *N*-fluorescein-iso-thiocyanyldipalmitoylphosphatidyl-ethanolamine (FITC-DPPE), was mixed with a P2 solution. Adsorption of P2 on the surface of the labeled liposomes resulted in the quenching of FITC fluorescence (plot 1 in Figure 8). Removal of the polycation from the liposomal membrane, if such occurred, should be accompanied by a recovery of fluorescence. It was found in this manner that P2 could be completely removed from the surface of liquid anionic liposomes by a 3-fold excess of poly(acrylate) (plot 2 in Figure 8). Apparently, polymer/polymer binding exceeds in strength polymer/liposome binding. The whole process was completed within a few seconds.[32] These observations are in agreement with reversible binding found previously.[35−38]

What happens to the liposomal membrane after polycation removal? We could answer the question with the help of microelectrophoresis and microcalorimetry.[28,32] Figure 9 shows how EPM of liquid DPPC/$CL^{-2}$ liposomes changed after binding to P2 followed by the removal of the polycation via recomplexation with PAA. Point A in

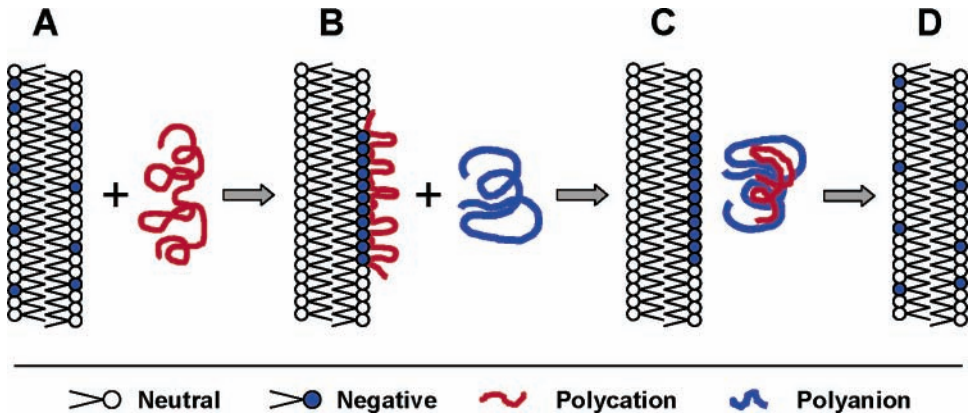


**FIGURE 10.** Schematic representation of reversibility of structural rearrangements in the negative liposomal membrane upon electrostatic P2 adsorption/desorption processes. See the explanation in the text.

the figure corresponds to the EPM of the original liposomes with nearly uniform distribution of $CL^{-2}$ between both leaflets of the membrane. It should be emphasized again that only the outer $CL^{-2}$ molecules (approximately half of the total $CL^{-2}$ molecules) contribute to the surface charge of liposomes and, therefore, determine their EPM value. Point B corresponds to the EPM of liposomes, whose surface charge has been neutralized by adsorbed P2. After 1 min following the addition of PAA at point B to the polycation−liposome complex, negative particles with an EPM value nearly twice as high as the EPM of the initial liposomes were detected in the system (point C). The size of these particles, measured by quasi-elastic light scattering, was equal to that of the original liposomes. When the electrophoretic and light-scattering data as well as the results on the fluorescence study of the P2 removal by PAA were taken into account, we concluded that the EPM at point C corresponded to individual liposomes having been completely cleared from the adsorbed polycation. At that moment, the liposomal membrane had attained maximum asymmetry; all negative $CL^{-2}$ molecules were now located on the outer leaflet of the membrane (having been previously extracted by adsorbed P2). In the course of an hour, half of the $CL^{-2}$ molecules spontaneously transferred from the outer leaflet back to the inner leaflet, decreasing the EPM to its initial value (curve 1 in Figure 9). A calorimetric study, performed in parallel to the EPM measurements, showed that the return $CL^{-2}$ transfer was accompanied by the complete recovery of the original lipid distribution in the lateral direction as well.

The overall cycle of metamorphoses, induced in the liquid anionic lipid membrane by adsorption/desorption of the polycation, is schematically represented in Figure 10. Although polycation-catalyzed flip-flop along with domain formation (A−B) takes place in at most a few seconds, spontaneous flip-flop in the absence of the polycation (C−D) requires, as mentioned, about an hour (curve 1 in Figure 9).

We emphasize that a high linear charge density in the cationic macromolecule is critical for the flip-flop catalysis. This was demonstrated by using quaternized derivatives of poly(4-vinylpyridine) with lower degrees of ethylation and, therefore, with lower positive linear charge densities: P2(50) and P2(30) (see Figure 6). Electrophoretic studies showed that, in contrast to P2, neither of them accelerated lipid flip-flop in liquid lecithin/$CL^{-2}$ liposomes.[31]

An ability of the polycation to catalyze lipid flip-flop was found to depend upon its degree of polymerization (DP). All of the above-described experiments were carried out with a rather long P2 (DP = 1100). This flexible polycation, being adsorbed on the surfaces of colloidal particles including liposomes, forms loops and tails exposed to the surrounding water solution.[39] We found by microelectrophoresis that the polycation-induced flip-flop disappeared below a DP = 30−60. Interestingly, a DP = 30−60 approximates the persistence length for quaternized poly(4-vinylpyridine).[40] A persistence length is a minimum length of the polymer chain necessary for a rigid rod conformation.[41] Such short polymers are obviously incapable of loop-and-tail formation when adsorbed onto the solid interfaces. It was found that cationic polypeptide polylysine (DP = 430) was also unable to promote flip-flop in liquid membranes.[42,43]

These electrophoretic, calorimetric, light scattering, and fluorescence studies allow us to suggest one possible mechanism for the polycation-induced flip-flop (Figure 11).[31,43,44] In solid liposomes, severe packing requirements prevent transmembrane lipid migration. As a result, no flip-flop occurs when polycations complex with the outer negative lipid molecules of solid bilayers. In liquid liposomes, however, the adsorption of a flexible polycation with a high linear charge density, e.g., P2, produces dynamic distortions in the lipid bilayer structure arising from electrostatic repulsion between positive units located in loops of the adsorbed polycation. Such distortions promote a reversible lipid interchange within the bilayer. Negative lipids, shifting from the inner to outer leaflet, become bound to portions of the external polycations. Simultaneously, neutral lipid molecules migrate in the opposite direction, thus maintaining the integrity of the liposomes. Note that transmembrane migration was also observed with smaller negative lipids, e.g., PS and phosphatidic acid,[12] as well as with conventional negative surfactants, e.g., sodium dodecyl sulfate.[45]

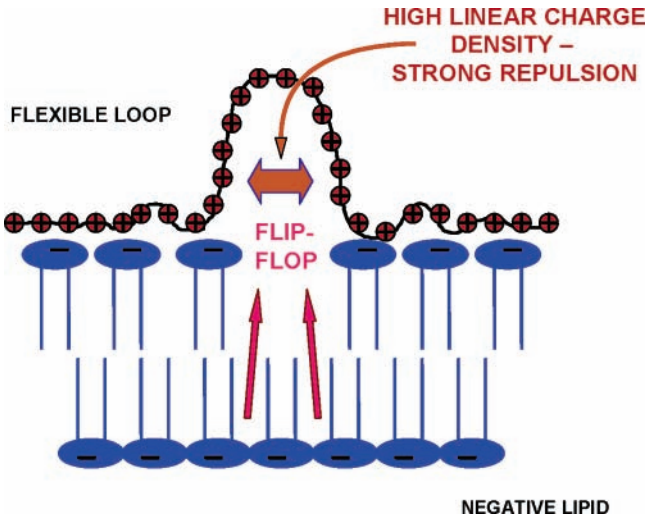


**FIGURE 11.** Schematic representation of the structural deformation of DPPC/$CL^{-2}$ membranes by P2, postulated to give rise to accelerated flip-flop.

Larger anions, however, such as anionic hydrophobicized α-chymotrypsin, were not successfully transmigrated with polycations.[46]

The mechanism in Figure 11 implies polycation adsorption onto the bilayer surface without any direct penetration of the polycation into the bilayer. This latter possibility cannot be excluded, but it seems unlikely for two reasons. First, polycation P2 can be rapidly and totally removed by polyacrylate to reinstate the original size and anionic charge of the liposomes. This suggests a reversible electrostatic binding of the polycation to the liposome surface as opposed to an irreversible polymer strand invasion of the bilayer. Second, the mechanism in Figure 11 is favored by the observation that the liposomal membranes keep their integrity when coupled to P2. The invasion of the polycation would induce significant disorders (defects) in the lipid packing.

## 4. Final Comments

In this Account, we presented our findings concerning an ability of amphiphilic nonionic polymers, e.g., EO/PO/EO block copolymers (Pluronics), and polycations, e.g., quaternized poly(4-vinylpyridine)s, to accelerate flip-flop in liquid phospholipid membranes. Driving forces and mechanisms of the processes induced by amphiphilic copolymers and polycations are different. Nonionic polymers are bound to the biological membrane because of the incorporation of their hydrophobic fragments into the inner part of the lipid bilayer comprised of the lipid chains. The binding of cationic polymers is induced by electrostatic interactions between the positive polymer units and the negative lipid headgroups. In both cases, the polymer association is accompanied by significant distortions of the bilayer packing. Nonionic polymers favor scrambling of lipid molecules within the membrane, with the effect being dependent upon the overall hydrophobicity of the copolymer and the volume of its hydrophobic block. Electrostatic binding of cationic polymers produces dynamic perturbations in the lipid bilayer, favoring translocation of negative lipids from the inner to outer membrane leaflet. The process is controlled by the degree of polymerization, linear charge density, and flexibility of the cationic macromolecule.

Polymer-induced flip-flop can influence, in turn, other biologically important events in the biological membranes. We have found that the catalyzed flip-flop by polymers is closely associated with the ability of the polymers to increase the permeability of the membranes toward biologically active compounds, e.g., an antitumor drug doxorubicin (Dox). This substance, containing an amino group with a $pK_a$ = 8.6, is able to permeate in its uncharged form through the lipid membrane via a partition-diffusion mechanism. When bound to lipsosomes, both Pluronics and polycations catalyze a pronounced acceleration of the transmembrane Dox permeation.[10,16,43,46] Polymer adsorption favors Dox diffusion across the hydrophobic region of the membrane but does not alter the amount of drug finally accumulated within the liposomes. For Pluronics, a distinct correlation between the flippase activity and their ability to accelerate the Dox permeation has been revealed.[10] As for cationic polymers, a Dox-permeating effect has also been found for polylysine and P2.[43,46]

The great influence of transbilayer migration and transbilayer asymmetry in cell membranes has been briefly mentioned in the Introduction. These processes, regulated by specific enzymes (flippases), mediate cell responses and control cell functioning. At present, we do not know if nonionic and cationic polymers are able to affect flip-flop in biological systems. This area should be further developed, especially considering the growing biomedical applications of synthetic polymers. Nevertheless, in some cases, a relationship between cell biology and flip-flop-inducing activities of polymers was already observed. For example, adsorption of Pluronics on the membranes of multidrug-resistant tumor cells results in the inhibition of *P*-glycoprotein, responsible for drug efflux, thereby promoting the accumulation of drugs in the cells.[18] As reported in the literature, this might be related to the fluidization of lipids in the vicinity of the membrane proteins.[47] Additional examples of polymer-induced effects in membrane biology will, no doubt, be forthcoming.

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