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Lipid composition determines interaction of liposome membranes with Pluronic L61

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Abstract

Triblock copolymers of ethylene oxide (EO) and propylene oxide (PO) of $EO_{n/2}PO_mEO_{n/2}$ type (Pluronics) demonstrate a variety of biological effects that are mainly due to their interaction with cell membranes. Previously, we have shown that Pluronics can bind to artificial lipid membranes and enhance accumulation of the anti-tumor drug doxorubicin (DOX) inside the pH-gradient liposomes and transmembrane migration (flip-flop) of NBD-labeled phosphatidylethanolamine in the liposomes composed from one component—lecithin. Here, we describe the effects caused by insertion of other natural lipids in lecithin liposomes and the significance of the lipid composition for interaction of Pluronic L61 with the membrane. We used binary liposomes consisting of lecithin and one of the following lipids: cholesterol, phosphatidylethanolamine, ganglioside GM1, sphingomyelin, cardiolipin or phosphatidic acid. The influence of the additives on (1) membrane microviscosity; (2) binding of Pluronic L61; (3) the copolymer effect on lipid flip-flop and membrane permeability towards DOX was studied. The results showed that insertion of sphingomyelin and cardiolipin did not influence membrane microviscosity and effects of Pluronic on the membrane permeability. Addition of phosphatidic acid led to a decrease in microviscosity of the bilayer and provoked its destabilization by the copolymer. On the contrary, cholesterol increased microviscosity of the membrane and decreased binding of Pluronic and its capacity to enhance flip-flop and DOX accumulation. Analogous tendencies were revealed upon incorporation of egg phosphatidylethanolamine or bovine brain ganglioside GM1. Thus, a reverse dependence between the microviscosity of membranes and their sensitivity to Pluronic effects was demonstrated. The described data may be relevant to mechanisms of Pluronic L61 interaction with normal and tumor cells.

Keywords: Liposome; Pluronic; Flip-flop; Doxorubicin; Permeability; Lipid composition

1. Introduction

Triblock copolymers of ethylene oxide (EO) and propylene oxide (PO) of a common formula $EO_{n/2}PO_mEO_{n/2}$ (Pluronics) represent a large family of non-ionic surfactants of 2–20 kDa differing in length of hydrophilic EO and hydrophobic PO blocks [1,2]. As all water-soluble surfactants, these polymers are able to decrease interfacial tension, emulsify water-insoluble substances, and form supramolecular associates (micelles or vesicles) in water solutions that can trap various compounds. A characteristic feature of these synthetic polymers is a relatively low toxicity and biological compatibility. These properties assured their wide application in pharmacology for the therapy of wounds and burns [3–5], as cryoprotectants for low-temperature preservation of plant and mammalian tissues [6,7], as emulsifiers of drugs [8–10] and perfluorocarbons in the formulations of artificial blood [11,12] and as adjuvants in vaccine formulations [13,14]. Recently, it has been shown that Pluronics increase drug permeation through blood–brain barrier [8,15–19] and accumulation in multi-drug resistant (MDR) tumor cells expressing P-glycoprotein [20–24], an ATP-dependent pump responsible for drug efflux. A combined application of Pluronics with an anti-tumor drug enhances accumulation of the latter in malignant cells and improves the therapeutic effect. The pharmacological formulation SP1049C based on anti-tumor drug doxorubicin (DOX) and two Pluronic copolymers has undergone phase I clinical

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trials and was found to be efficient in patients with advanced resistant solid tumors [25].

Pluronics–cell interaction depends on the cell type. Mouse myeloma SP2/0 cells accumulated approximately 3 times more Pluronics L61 and P85 than normal murine splenocytes. Consequently, the microviscosity of membranes of SP2/0 cells decreased by a factor of two upon Pluronic binding, while the membranes of normal cells were less responsive [26]. A distinct difference between MDR and drug sensitive tumor cells was also observed. Thus, Pluronic L61 induced permeabilization of acidic endocytosis vesicles in MDR cells, whereas the vesicles of sensitive cells were less sensitive [27].

These distinctions may be attributed to difference in composition of cell membranes. It is well established that the membranes of leukemic cells are characterized by a lowered cholesterol content [28,29], a fact of medical importance for diagnostics of myeloid leukemia [29]. The membranes of tumor cells with higher metastatic potential have a lesser cholesterol/phospholipid ratio and a larger content of unsaturated fatty acids, both effects diminishing the membrane microviscosity [30,31]. Recently, it has been found that cholesterol content is down-regulated by activation of Ras/ERK oncogenes signaling pathway in MCF-7 cells [32]. All these data suggest that interaction of Pluronic with cell membranes is determined at least partially by their lipid composition.

Manifold pharmacologically important effects induced by Pluronics in biological systems justified interest to their interaction with model membranes. Ability of Pluronics to induce disturbances in membranes was shown in a number of artificial systems. In particular, interaction of Pluronic L61 with dipalmitoylphosphatidylcholine (DPPC) liposomes resulted in a 2-fold decrease in the membrane microviscosity [33] and a 3-5-fold increase in membrane permeability towards weak acids and bases [34]. Insertion of 2–8 mol% of hydrophilic Pluronics into lipid membrane induced significant alterations in the vesicles size and even shape [35]. Binding of Pluronic L61 to the liposomes composed of egg-yolk lecithin resulted in acceleration of flip-flop [36] and transport of DOX into the vesicles [33,34,36]. The copolymer-induced changes in the rates of both processes were found to be closely related and proportional to the amount of the bound copolymer [36,37].

The relationship between the lipid composition of membranes and their ability to bind amphiphilic copolymers was studied in artificial systems. Thus, Johnsson et al. have found that insertion of cholesterol into small unilamellar phosphatidylcholine liposomes altered their ability to interact with hydrophilic Pluronics. In the absence of cholesterol, the Pluronics with large poly(oxyethylene) (PEO) blocks, such as F127, F108, and F87, induced formation of bilayer disks, whereas those with relatively short PEO blocks, P105 and P85, tended to promote a decrease in the size of liposomes. These effects of Pluronics were highly reduced or even abolished in some cases upon insertion of 25% cholesterol in the liposomal preparations [35]. These results are in accordance with the theoretical predictions made by R. Lipowsky ten years ago [38]. The effect of other types of lipids on the ability of liposomes to interact with Pluronics has not been studied yet.

The regularities governing interactions of amphiphilic copolymers with model and biological membranes are the main topic of our investigations. Our recent publication was focused on the role of the copolymer structure [37]. The present paper concerns chemical structure of the second partner in this interaction, the lipid bilayer. Namely, we studied interactions of Pluronic with membranes composed of different types of lipids. Since the lipid–protein structure of cell plasma membranes is too complex, the studies were performed on liposomes prepared from binary lipid mixtures. Lecithin, the major lipid of cell plasma membrane, was the basic component of liposomes while the additive was either cholesterol, or a neutral or negatively charged glycerophospholipid, or sphingolipid. Pluronic L61 ($EO_2PO_{30}EO_2$, HLB=3) was chosen as a representative of relatively hydrophobic copolymers because it showed a high chemosensitizing activity in respect to MDR cells [22] and flippase activity in model lipid membranes [36,37].

2. Materials and methods

2.1. Materials

The following substances were used in the experiments: block copolymer of ethylene oxide and propylene oxide Pluronic L61 (Serva, Germany), egg yolk lecithin and phosphatidylethanolamine, bovine brain sphingomyelin and ganglioside GM1, and synthetic dipalmitoylphosphatidic acid (Sigma, USA), cholesterol (Serva, Germany), 1,6-diphenyl-1,3,5-hexatriene (DPH) (Reanal, Hungary). Doxorubicin hydrochloride was purchased from the Russian Antibiotic Institute. Buffer components and organic solvents were obtained from "Reakhim" and "PanEco" (Russia). ^{3}H-labeled Pluronic L61 with specific radioactivity 0.2 Ci/mmol was prepared as described previously [26].

2.2. Preparation of two-component (binary) liposomes

Ethanol/chloroform solutions of lecithin and the required lipid, 10 mg in total, were mixed and the solvents were carefully evaporated under vacuum using Heidolph Rotavapor (120 min^{-1}, 40 °C). Thin film of the lipid mixture was dispersed in 0.3 M citrate–Tris buffer, pH 4.0, and vortexed until complete dissolution of the film. The emulsion was subjected to 3 repeated freeze–thawing cycles (−196 °C/+40 °C) and sonicated using Cole Parmer 4700 ultrasonic generator (4×200 s, 22 kHz, 30 W) with water-cooling under the atmosphere of nitrogen. Thus obtained liposomes were separated from titanium dust by centrifugation (Beckman microcentrifuge, 10 min, 9400g) and used immediately. The vesicles were 70–120 nm in diameter as measured by quasi-elastic light scattering with Autosizer 2c (Malvern, UK).

2.3. pH-gradient liposomes

1 ml of liposomes prepared as described above was applied to a Sepharose CL-4B column (1.1×18 cm) equilibrated with 20 mM HEPES–Tris buffer, pH 7.0, supplemented with 0.6 M sucrose to avoid osmotic swelling of the vesicles. The liposomes were eluted with the same buffer at 15 ml/h. Their concentration in the eluate was calculated from dilution factor that was estimated as the ratio of UV absorbance at 250 nm of the pre- and post-column samples. Thus prepared liposomes had pH 4 inside and pH 7 in the surrounding medium.

2.4. Kinetics of DOX transmembrane permeation

The kinetics of DOX accumulation in the liposomes was studied as described by Harrigan et al. [39]. 1 ml of pH-gradient liposomes was added to 30 μl of DOX- Pluronic L61 mixture and incubated at 30 °C. The final

concentrations were 1.2 mg/ml liposomes, 50 μM DOX and 20 μM Pluronic. The fluorescence of samples was checked immediately after mixing of the reagents and was followed until a stationary level was achieved using a Hitachi F-4000 (Japan) spectrofluorimeter at λ_{em}=557 nm, λ_{ex}=490 nm. The temperature was maintained about 30 °C during the records.

2.5. Flip-flop measurements

Spontaneous and Pluronic-induced flip-flop of lipids in vesicular membranes was evaluated on the basis of migration of a fluorescent lipid N-[(7-nitrobenz-2-oxy-1,3-diazol-4yl) dipalmitoyl] phosphatidylethanolamine (NBD-PE) from the inner to the outer leaflet of the liposomes. Egg yolk lecithin (EL) vesicles containing NBD-PE were prepared according to McIntyre and Sleight [40]. Briefly, ethanol solutions of EL and NBD-PE (0.995/0.005 w/w) were mixed and the solvent was evaporated under vacuum. A thin lipid layer was dispersed in 10 mM Tris–HCl buffer, pH 7, supplemented with 150 mM choline chloride and 1 mM EDTA. NBD-PE containing liposomes were prepared from this lipid dispersion following the procedure described above (see Preparation of two-component (binary) liposomes). The NBD-PE liposomes were 80–100 nm in diameter. According to the previously reported data [40], the NBD-PE species are uniformly distributed between both membrane leaflets. So, such vesicles were denoted as symmetrically labeled. To make the vesicles applicable for flip-flop kinetics measurements, they were converted into asymmetrically labeled. To this end, an appropriate amount of a 0.25 M freshly prepared sodium dithionite solution in 20 mM Tris buffer, pH 10, was added to 1.5 mg/ml suspension of the symmetrically labeled vesicles to achieve the final concentration of sodium dithionite 2 mM. The mixture was incubated for 6 min at room temperature (18–22 °C). Dithionite reduces NBD nitro groups to form non-fluorescent amino groups [40]. NBD fluorescence was measured at λ_{em}=530 nm, λ_{ex}=450 nm with a Hitachi F-4000 spectrofluorimeter. Treatment of symmetrically labeled vesicles with dithionite usually decreased the NBD fluorescence by 46–48% indicating approx. 92–94% content of unilamellar vesicles in the original symmetrically labeled EL/NBD-PE vesicles. The reduced EL/NBD-PE vesicles were immediately separated from the excess of sodium dithionite by gel-filtration on Sephadex G-50 column (1.2×12 cm) equilibrated with 10 mM Tris–HCl, 150 mM choline chloride, 1 mM EDTA buffer, pH 7.0 (TCE buffer). Since sodium dithionite is unable to penetrate through the lipid membrane [40], it reduced the label only in the outer membrane leaflet. Thus treated liposomes contained NBD–PE only in the inner leaflet and so were denoted as asymmetrically labeled. The completeness of the reduction of NBD-PE in the outer leaflet of the membrane was checked by a control treatment of the asymmetrically labeled vesicles with a fresh portion of dithionite. Usually this verifying test gave only a minor decay (1–1.5%) of NBD fluorescence.

To measure flip-flop kinetics, the asymmetrically labeled vesicles, 0.15 mg/ml in TCE buffer, were incubated at 30 °C. At given points in time 1 ml aliquots of the sample were treated with 20 μl of freshly prepared 0.5 M sodium dithionite solution, pH 10, at 20 °C, and the kinetics of the fluorescence decay was recorded within 3–4 min until a new stationary fluorescence level, I_t^{asym}, was achieved. The decay was due to reduction of NBD that had migrated from the inner to the outer leaflet (flip-flop) during incubation, thus becoming susceptible to reduction by dithionite.

The fraction, f, of fluorescent NBD-PE species that had migrated from the inner to the outer membrane leaflet was calculated using equation:

$$f(\%) = \frac{\Delta I_t^{asym}}{I_0^{asym} - (1-\alpha)\times I^{sym}} \times 100\%, \quad (1)$$

where I^{sym} and I_0^{asym} are the fluorescence intensities of the symmetrically and asymmetrically labeled liposomes, respectively. $\alpha = 2\left(\frac{I^{sym}-I_0^{asym}}{I^{sym}}\right)$ is the content of unilamellar vesicles in the NBD-labeled vesicle sample. This value was checked in each experiment and usually was in 0.92–0.96 range. The f values checked at different incubation times showed the kinetics of spontaneous NBD-PE transmembrane migration (flip-flop).

2.6. Microviscosity measurements

The average microviscosity of liposomes was tested by measuring the fluorescence of DPH incorporated in the bilayer [33,41,42]. Stock solution of DPH (acetone, 2×10^{-5} M) was diluted 100-fold with 20 mM HEPES–Tris buffer, pH 7.0, supplemented with 0.6 M sucrose, and vigorously stirred for approx. 2 h at room temperature until complete removal of acetone. Then a liposome suspension, 2 mg/ml, was added to an equal volume of DPH solution and the mixture was stirred for 1 h to achieve DPH incorporation in the bilayer. The DPH fluorescence with parallel (I_1) or crossed (I_2) polarizers was measured at λ_{ex}=366 nm, λ_{em}=433 nm. The anisotropy of fluorescence r was calculated as:

$$r = \frac{I_1 - I_2}{I_1 + 2I_2}. \quad (2)$$

Absolute values of microviscosity η were obtained according to Perren equation:

$$\frac{r_0}{r} = 1 + C(r)\frac{T\times\tau}{\eta}, \quad (3)$$

where r_0=0.362, $C(r)$=8.6×10^5 Poise/s·K, T=305 K, $\tau=6\times10^{-9}$ s [41,42]. To exclude light scattering contribution into the observed microviscosity values, they were extrapolated to zero liposome concentration [43].

2.7. Binding measurements

Binding of Pluronic L61 to vesicles was measured using the equilibrium dialysis technique [26]. 0.4 ml of liposomes, 40 mg/ml, in 20 mM HEPES, 5 mM Tris buffer, pH 7.0, were placed in a small dialysis sack (12–14 kDa cut-off) and dialyzed against 1 ml of ^{3}H-Pluronic L61 in the same buffer (20 μM, 0.2 Ci/mmol) under argon for 72 h at 30 °C in a 2 ml Eppendorf tube. After dialysis, the radioactivity inside the sack and in the outer solution was measured. 0.3 ml of each sample was mixed with 3 ml of the toluene scintillator containing 30% Triton X-100 and counted in a Delta-400 (USA) scintillation counter. The amount of the bound copolymer was evaluated as a difference between the counts inside and outside the sack, divided by ^{3}H-Pluronic L61 specific radioactivity (0.2 Ci/mmol) and sample volume.

3. Results

In this paper, the relationship between the lipid composition of a membrane and its ability to interact with Pluronic L61 was studied. The experiments were performed on two-component liposomes composed of egg-yolk lecithin and one of the following lipids: cholesterol, phosphatidylethanolamine, ganglioside GM1, sphingomyelin, cardiolipin or phosphatidic acid. The additives were tested in a concentration range that matched their content in biological membranes [44,45]. All lipids, except for phosphatidic acid, were of natural origin and contained about 50% of unsaturated fatty acids with 1–2 double bonds according to the manufacturer's data. To prepare liposomes, the lipid under study and lecithin were taken in different proportions that guaranteed their complete mixing. Interaction of Pluronic L61 with binary liposomes was evaluated by three parameters: (i) the polymer binding, (ii) its influence on the rate of lipid flip-flop and (iii) on membrane permeability towards doxorubicin.

3.1. Influence of cholesterol on binding of Pluronic L61 with lipid membranes

Binding of Pluronic L61 with liposomes was checked by equilibrium dialysis technique using tritium labeled copolymer.

A rather low molecular weight of Pluronic L61 (about 2.09 kDa) assured its penetration through the dialysis membrane (cut-off 14 kDa) inside the bag filled with liposomes. Equilibrium concentrations of Pluronic were determined outside and inside the bag and their difference showed the amount of the polymer bound to the liposomes. It was found that at high liposome concentration (35 mg/ml) about half of the added polymer bound to the membranes (about 9 μM from 20 μM, Fig. 1, left axis). Insertion of cholesterol in the liposome membrane lowered the copolymer binding inversely proportional to the cholesterol content. When the cholesterol mole fraction reached 0.55, binding of the copolymer decreased more than 2-fold (Fig. 1).

3.2. Influence of cholesterol on the Pluronic-induced acceleration of lipid flip-flop

The rate of lipid flip-flop was evaluated on the basis of NBD-PE transfer from the inner to the outer membrane leaflet. In the liposomes made only of lecithin without any additives, the first order rate constant of flip-flop in the absence of Pluronic L61 (k_{EL}^{-L61}) was equal to 0.21 h^1 (Fig. 2A, curve 1). Addition of Pluronic L61 to these liposomes induced a pronounced acceleration of lipids mobility (Fig. 2A, curve 2). The first-order rate constant of flip-flop in the presence of 20 μM Pluronic L61 (k_{EL}^{+L61}) augmented 6-fold up to 1.28 h^{-1}.

Incorporation of 0.1 mole fraction of cholesterol in the liposomes hindered flip-flop for 1.5 times ($k_{EL/Chol}^{-L61}$ = 0.14 h^{-1}) and drastically diminished the effect of the copolymer. Addition of 20 μM Pluronic L61 increased the rate of flip-flop only 1.5-fold ($k_{EL/Chol}^{+L61}$ = 0.21 h^{-1}) (Fig. 2B).

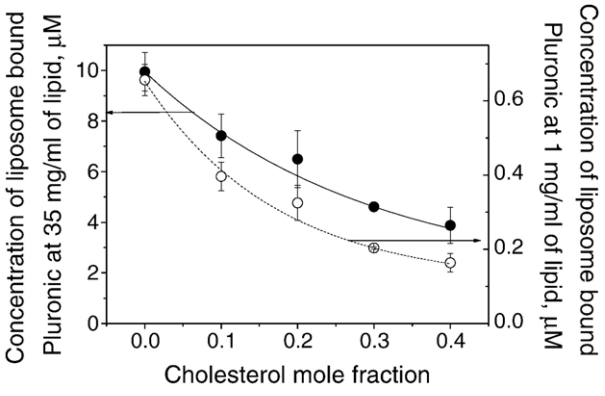

Fig. 1. Influence of cholesterol on the copolymer binding with lipid vesicles at liposomes concentration 35 mg/ml (1, left axis and black circles) and 1.2 mg/ml (2, right axis and open circles). Curve 1 was obtained by the direct measurement of the copolymer binding using equilibrium dialysis technique (see experimental section), at 30 °C, in 10 mM NaH_2PO_4 buffer, pH 7.4, containing 150 mM NaCl. Curve 2 (corresponded to kinetic studies) was recalculated from curve 1 using the equation arising from proportionality (5): $[P]_b^{(2)} = \frac{[P]_0[P]_b^{(1)}[L]_0^{(2)}}{[L]_0^{(2)}[P]_b^{(1)} + [L]_0^{(1)}\left([P]_0 - [P]_b^{(1)}\right)}$, where $[P]_0$ denotes the total concentration of Pluronic (20 μM), $[L]_0^{(i)}$—the total concentration of liposomes, and $[P]_b^{(i)}$—the concentration of the copolymer bound to liposomes, upper index in brackets (i) is referred to the number of the curve.

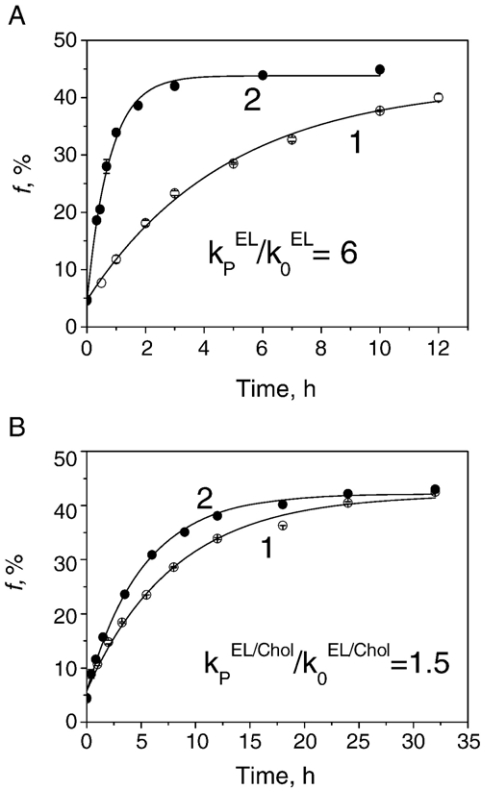

Fig. 2. Kinetics of NBD-PE flip-flop in lecithin (A) and lecithin-cholesterol 9:1 (B) vesicles in the absence (1) and in the presence of 20 μM Pluronic L61 (2). f—fraction of fluorescent NBD-PE molecules that migrated from the inner to the outer leaflet of the liposomes. Lipid concentration 0.15 mg/ml; 10 mM Tris, 150 mM choline chloride, 1 mM EDTA, pH 7.0; 25 °C.

3.3. Effect of cholesterol on the Pluronic L61-induced acceleration of DOX accumulation in liposomes

To induce DOX transport through lipid membrane, we used so-called pH-gradient liposomes that were filled with an acidic buffer pH 4, while the surrounding medium had pH 7.0. Since the amino group of DOX has pK_a 8.6, most DOX molecules in the external buffer are positively charged. However, about 2.5% of drug molecules remain neutral. Such molecules can permeate through lipid bilayer via partition-diffusion mechanism. In the internal lumen filled with pH 4 buffer, practically all DOX molecules acquire a positive charge and cannot go out from the liposomes. As a result, DOX accumulates in the vesicles, its local concentration increasing by 2–3 orders of magnitude compared to that in the external solution. When DOX concentration exceeds 50 μM, its fluorescence decreases because of self-quenching [39]. Measuring the decay of DOX fluorescence in the whole system allows following the kinetics of DOX permeation (Fig. 3A, curve 1).

The kinetics of DOX fluorescence decay meets the first-order law [39]. Therefore, it was characterized by an effective rate constant. In the case of lecithin liposomes (Fig. 3A, curve 1), the first order rate constant, k_0, was equal to $2{\cdot}10^{-2}$ s^{-1}. If Pluronic L61 was added in the external medium, the fluorescence decay proceeded much quicker (Fig. 3A, curve 2) indicating a higher rate of DOX accumulation in the

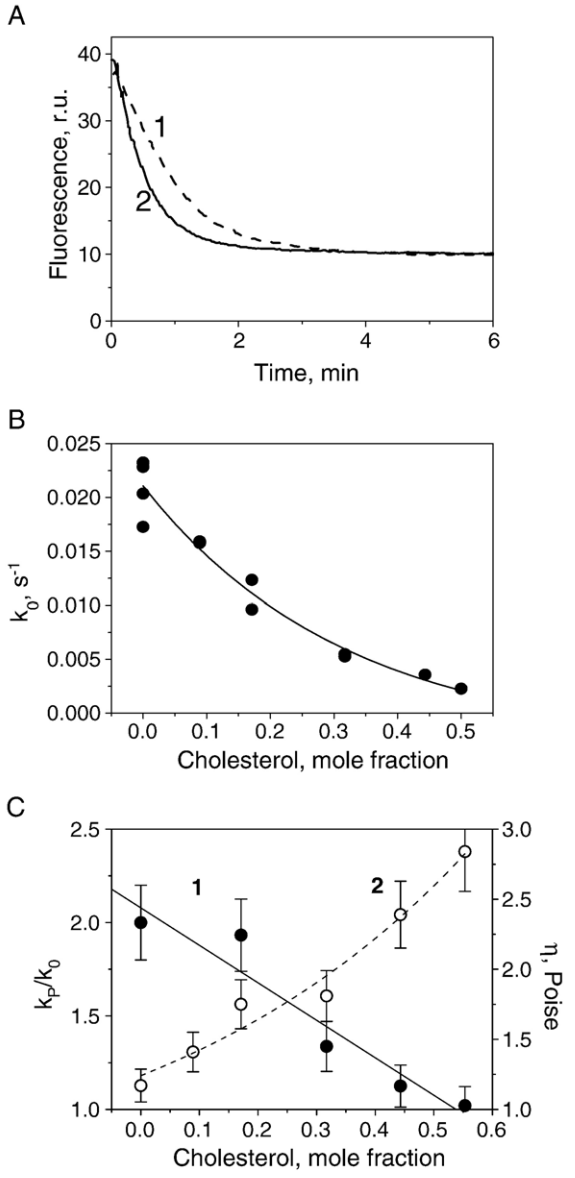

Fig. 3. (A) Kinetics of DOX accumulation in lecithin liposomes in the absence (1) and in the presence of 20 ìM Pluronic L61 (2). Influence of cholesterol incorporated in pH-gradient binary liposomes (B) on the rate constant (k_0) of DOX accumulation, (C) on the L61-induced acceleration of DOX transport (curve 1) and microviscosity of the lipid bilayer (curve 2). The external buffer: 20 mM HEPES–Tris, pH 7.0, containing 0.6 M sucrose, the internal buffer: 0.3 M citrate–Tris, pH 4.0, liposomes concentration 1.2 mg/ml. The latter data represent mean values of 3 independent experiments.

liposomes. Indeed, the rate constant in the presence of 20 μM Pluronic, k_P, increased to 3.4×10^{-2} s^{-1}. Previously, we have shown that the rate of DOX accumulation increases nearly proportionally to the copolymer concentration in solution and to the amount of the copolymer bound to lecithin liposomes [36].

In the present work, similar experiments were performed on binary liposomes containing different amounts of cholesterol. In accordance with the previously published data [39], insertion of cholesterol was accompanied by a decrease in membrane permeability towards DOX (Fig. 3B) and an increase in membrane microviscosity (Fig. 3C, curve 2). To evaluate the Pluronic's ability to enhance DOX permeation in such liposomes, the ratio of rate constants in the presence of the copolymer and in its absence (k_P/k_0) was calculated. The effect caused by Pluronic L61 on the rate of DOX permeation gradually decreased as the content of cholesterol increased (Fig. 3C, curve 1). At equimolar amounts of lecithin and cholesterol the influence of the copolymer was negligible. So, incorporation of cholesterol in the lipid bilayer decreases binding of Pluronic to the liposomes and diminishes its effect on lipid flip-flop and DOX permeation.

The decrease in the efficiency of Pluronic in this case could be due to either a reduced binding of the copolymer, or, alternatively, to a decreased ability of the bound Pluronic to disturb the lipid bilayer tightly packed by inserted cholesterol molecules. To discern the two mechanisms, we calculated the effects caused by 1 μM of the bound copolymer.

The apparent acceleration of DOX transport (k_P/k_0) was previously reported to be proportional to the copolymer bulk concentration $[P]_0$ with the coefficient β_{DOX} [36]:

$$\frac{k_p}{k_0} = 1 + \beta_{DOX}[P]_0. \quad (4)$$

On other hand, the concentration of the bound polymer $[P]_b$ is proportional to its bulk concentration:

$$[P]_b = [P]_0 \frac{[L]_0}{[L]_0 + \frac{\rho}{K_P}}, \quad (5)$$

where $[L]_0$—is the total concentration of lipid (mg/ml), ρ is the density of the lipid bilayer (g/l) and K_P is the partition coefficient of Pluronic between water and lipid phase. Therefore, we can establish proportionality:

$$\frac{k_P}{k_0} = 1 + \gamma_{DOX}[P]_b, \quad (6)$$

where γ_{DOX} characterizes the ability of bound macromolecules to influence the DOX transport. Using the data of kinetic (Fig. 3C) and binding experiments (Fig. 1, curve 2), we calculated γ_{DOX} values according to Eq. (6). We found that the effect caused by 1 μM of the bound copolymer did not depend on the content of cholesterol up to its mole fraction about 0.4 (Fig. 4). This indicates that the extent of disturbance induced by each bound macromolecule did not depend on the content of cholesterol. So, changes in membrane responsiveness to Pluronic were solely due to a decreased binding of the copolymer.

3.4. Influence of glycerophospholipids and sphingolipids on Pluronic L61-induced increase in the rate of DOX accumulation in liposomes

Besides cholesterol, we tested the effect of incorporation of other natural lipids, viz. ganglioside GM1, phosphatidylethanolamine (PE), sphingomyelin (SM), cardiolipin (CL) and phosphatidic acid (PA). It turned out that these lipids could be divided into three groups according to their influence on Pluronic–membrane interaction.

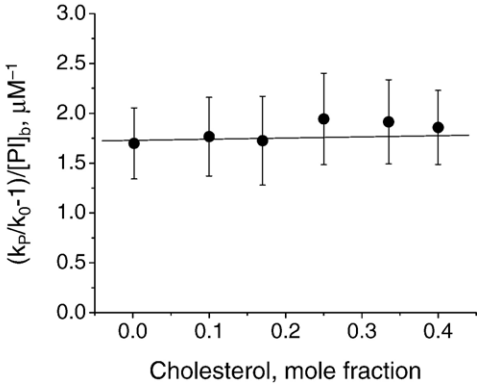

Fig. 4. Influence of cholesterol mole fraction in EL bilayer on the effect caused by 1 μM of copolymer bound to the membrane. The data refer to the liposome concentration 1.2 mg/ml and Pluronic L61 concentration 20 μM. The plotted values are calculated from the data presented in Fig. 3C, curve 1 (k_P/k_0 values) and Fig. 1, curve 2 ($[P]_b$). Standard error is 15–20%.

Lipids of the first group, bovine brain ganglioside GM1 and egg yolk PE affected membrane properties in a way qualitatively similar to that of cholesterol. Both lipids increased membrane microviscosity (Fig. 5, curve 2), decelerated transport of DOX in the absence of Pluronic, and weakened the Pluronic's effect on the membrane permeability towards DOX (Fig. 5, curve 1). However, GM1 and PE displayed these properties to a different extent.

GM1 produced only a mild effect on microviscosity. This lipid, mole fraction 0.25 in the bilayer, increased the membrane microviscosity only by 0.2 Poise (Fig. 5A, curve 2), while incorporation of the same amount of PE in the bilayer caused a more pronounced effect on the membrane increasing its microviscosity by 0.5 Poise (Fig. 5B, curve 2), the effect being comparable with that of cholesterol (cf. Figs. 3C and 5B, curves 2).

A reverse situation was observed when membrane permeability towards DOX was tested. The ability of Pluronic L61 to accelerate DOX permeation considerably decreased upon incorporation of GM1 (Fig. 5A, curve 1). Thus, GM1, mole fraction 0.23, decreased the k_p/k_0 value by 50%, which was quite similar to the cholesterol effect. In contrast, PE influence on this parameter was far less pronounced. Insertion of PE, mole fraction 0.26, in lecithin liposomes resulted in approximately 10% decrease in k_p/k_0 value (Fig. 5B, curve 1).

The second group of lipids consisted of sphingomyelin and cardiolipin that did not cause any detectable changes in the membrane microviscosity and the Pluronic ability to accelerate DOX permeation (Fig. 6). Meanwhile, the influence of these lipids on DOX transport in the absence of Pluronic was different. Insertion of SM in the whole range of its concentrations caused no effect on DOX transport, while insertion of CL resulted in a considerable decrease in the membrane permeability towards DOX. This fact was observed previously

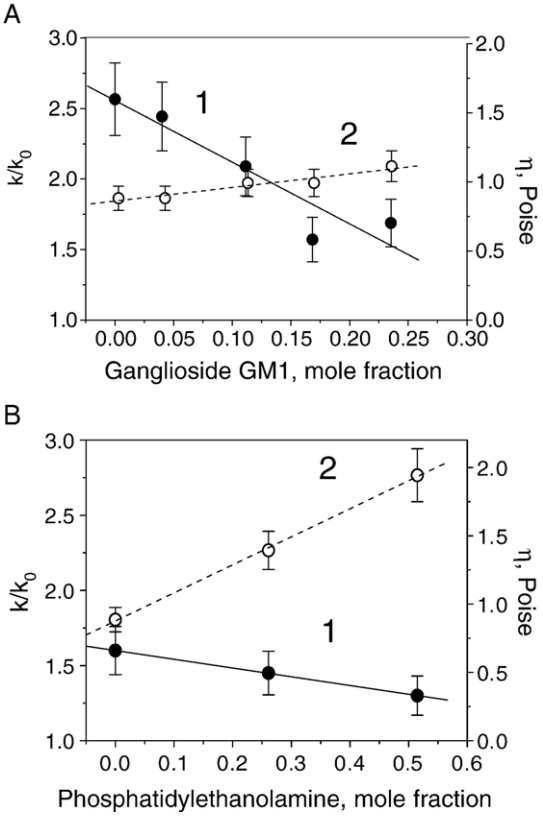

Fig. 5. Effect of ganglioside GM1 (A) and egg yolk phosphatidylethanolamine (PE) (B) on the L61-induced acceleration of DOX transport (curve 1, left axes, closed symbols) and the membrane microviscosity (curve 2, right axes, open symbols). The conditions of the experiments are specified in the legend to Fig. 3. The data represent mean values of 3 (A) and 2 (B) independent experiments.

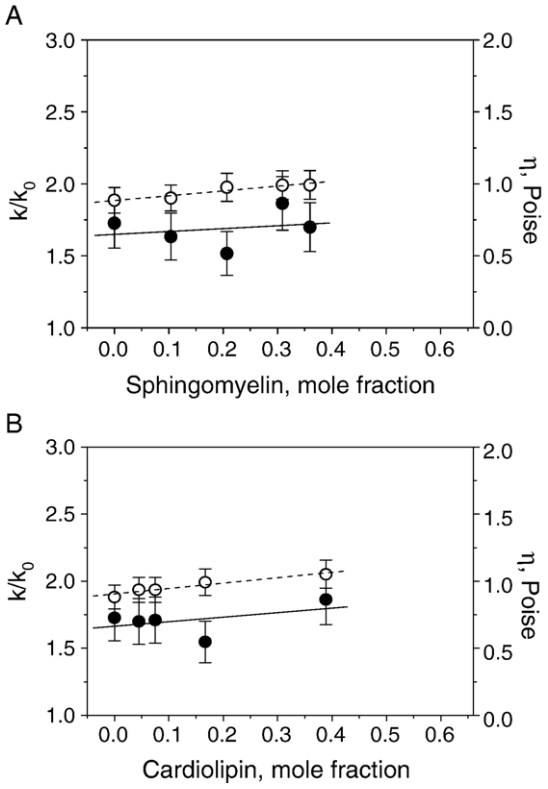

Fig. 6. Effect of sphingomyelin (A) and cardiolipin (B) on the lecithin membrane microviscosity (right axes and open dots) and the L61-induced acceleration of DOX transport (left axes and black dots). The conditions of the experiments are specified in the legend to Fig. 3. Mean values of 3 experiments are shown.

[46,47]. It was suggested to be due to a decrease in the drug concentration in solution caused by its electrostatic binding to the membrane surface. Comparison of the data on CL and cholesterol effects on the membrane permeability and Pluronic action shows that both lipids decelerated DOX permeation, but the former had no effect on Pluronic-induced increase of DOX permeation, while the latter decreased Pluronic-induced acceleration of DOX transport. These data show that the effect of add-lipids on the Pluronic-induced increase in the membrane permeability (k/k_0) does not necessarily correlate with the lipid influence on DOX transport in the absence of Pluronic (k_0).

Phosphatidic acid was singled out as a separate group because of its strikingly different influence on membrane properties. In contrast to the lipids described above, incorporation of phosphatidic acid decreased microviscosity of the membrane (Fig. 7A). Similar to other negatively charged lipids, cardiolipin and ganglioside, incorporation of phosphatidic acid decelerated DOX transport. When phosphatidic acid content in the liposomes was increased from 9% to 25%, a distinct decrease in the rate constant of DOX permeation (k_0) from 0.0081 s^{-1} to 0.0022 s^{-1} was observed (Fig. 7B). A comparison of the kinetic curves 1 and 3 in Fig. 7B shows that higher amounts of phosphatidic acid in the liposomes increased the infinite level of fluorescence decay indicating a lesser amount of DOX accumulated in the vesicles. Probably, this phenomenon arises from reduction of DOX effective concentration in the external buffer due to electrostatic binding of DOX with phosphatidic acid that is negatively charged at neutral pH.

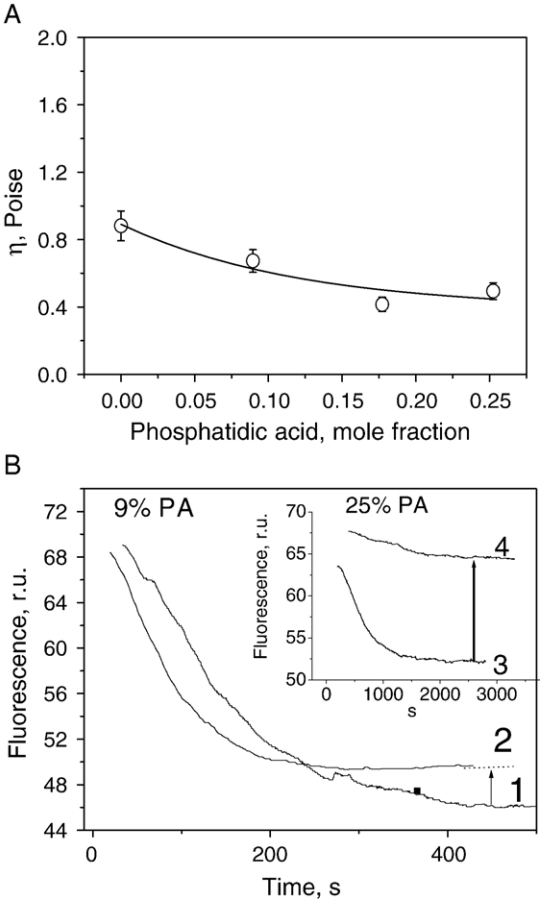

Fig. 7. (A) Effect of phosphatidic acid (PA) incorporated into lecithin liposomes on membrane microviscosity. (B) Kinetics of DOX permeation into the liposomes containing 9% of PA (curves 1, 2) and 25% of PA (curves 3, 4) either in the presence of 20 μM Pluronic (curves 2, 4) or in its absence (curves 1, 3). Mean values of 2 experiments are shown.

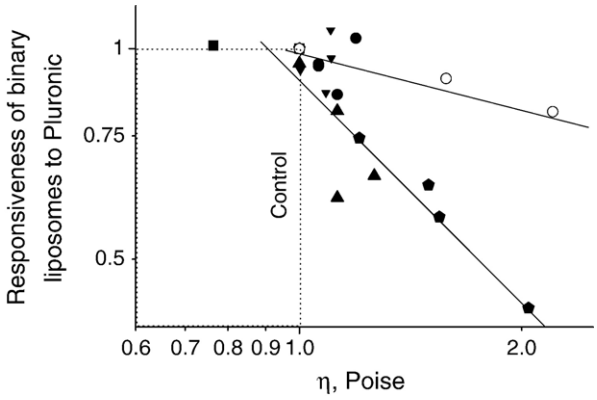

Fig. 8. Correlation between the microviscosity of two-component liposome membrane (η) and its responsiveness towards Pluronic L61. Responsiveness was calculated as a ratio of DOX transport constant in the presence (k_p) and in the absence (k_0) of the polymer. This ratio was specified to a corresponding k_p/k_0 value of EL bilayer determined at the same concentration of polymer. *Axes* are logarithmic. Points related to cholesterol contained liposomes are marked as pentagons, PA—squares, SM—black circles, PE—open circles, GM—triangles, CL—reverse triangles.

Addition of Pluronic to EL/PA liposomes reinforced these tendencies (Fig. 7B, curves 2 and 4). Previously we have shown that an increase in the infinite levels of the kinetic curves and sometimes a decrease in the apparent rate constant in the presence of Pluronic arise from smoothing of pH-gradient due to formation of very small pores permeable for buffer components [40]. The results obtained on EL/PA liposomes permitted to suggest that Pluronic caused additional disturbances in the membranes already destabilized by phosphatidic acid and provoked permeabilization of liposomes. This phenomenon was specific for phosphatidic acid. It was not observed either in liposomes composed of pure EL, or in the binary vesicles containing any other lipid described above. Pluronic did not affect this parameter even in the case of cardiolipin and ganglioside GM1 that decelerated DOX permeation and elevated the infinite level of the fluorescence decay.

The results described herein showed that Pluronic's effect on the permeability of lipid bilayer depended strongly on the membrane composition. The lipids that elevated membrane microviscosity decreased Pluronic-induced changes in the membrane permeability. The lipids, which did not affect the membrane microviscosity, had no effect on Pluronic–membrane interactions either. Insertion of PA that decreased the membrane microviscosity enhanced the Pluronic-induced membrane destabilization.

These observations indicated some relationship between the changes in microviscosity induced by admixture of a lipid under consideration and the Pluronic's effects. To test this hypothesis we compared the responsiveness of binary liposomes to Pluronic action with the microviscosity of their

membranes. The responsiveness was estimated as a logarithm of the ratio of the rate constant of DOX permeation in the presence of 20 μM Pluronic L61 to that in its absence. As shown in Fig. 8, the increase in membrane microviscosity was accompanied by a decrease in the bilayer responsiveness to Pluronic. This inverse correlation held true for all binary mixtures tested, except for the liposomes containing phosphatidylethanolamine. Its insertion into the vesicles resulted in a strong increase in microviscosity, while the responsiveness of the bilayer decreased less than in the case of cholesterol or GM1. The correlation coefficient of the linear fit of all studied binary systems (N= 18) except for PE was 0.9, indicating that microviscosity is an important factor that controls the membrane responsiveness to Pluronic.

4. Discussion

In the present work, interaction of Pluronic L61 with two-component lipid vesicles made of different lipids was investigated. This study was motivated by at least two groups of well established data, which point to the differences in, first, Pluronic-induced effects in the cells of different types [26,27], and second, in the lipid composition of plasma membranes of various cells [28–32]. These facts stimulated our interest in the relationship between the lipid composition of a membrane and its ability to interact with Pluronics. The possibility of such relationship was predicted for amphiphilic copolymers by theoretical calculations [38]. However, the experimental data on this item dealt only with cholesterol, while the effect of other lipids has not been studied yet. To fill this gap, in addition to cholesterol, we studied the influence of five other lipids that represented all main classes of naturally occurring lipids: sterols, phospholipids and sphingolipids.

The main lipid components of cell plasma membranes are phosphatidylcholine and phosphatidylethanolamine. They constitute 35–60% and 10–40% of all lipids, respectively [44,45]. Therefore, we chose phosphatidylcholine as a basic component of binary liposomes. The influence of other lipid additives on the membrane responsiveness to Pluronic action was examined by measuring Pluronic-induced acceleration of DOX permeation through liposome membranes.

To begin with, we studied the influence of cholesterol that makes up to 10–40% of membrane lipids [44,45] and is incorporated into the cavities in the fatty acid region of the bilayer formed by unsaturated acid chains. Such arrangement of cholesterol molecules determines its influence on the membranes microviscosity [41], permeability [39,48], bending modulus [49], and rates of lateral [50,51] and transbilayer [52] diffusion of lipids. We found that insertion of cholesterol in liposomes led to a decrease in their ability to bind Pluronic L61 (Fig. 1). The effect was accompanied by a decrease in the Pluronic effect on the rate of lipid flip-flop (Fig. 2) and on membrane transport of DOX (Fig. 3). It is noteworthy that incorporation of only 10% of cholesterol led to a considerable (about 4-fold) suppression of Pluronic-induced acceleration of lipid flip-flop, while the Pluronic-induced acceleration of DOX permeation was only marginally (by about 20%) affected under these conditions. The reasons for this phenomenon remain unclear until now, however it may be hypothesized that it is due to different intrinsic activation energies of flip-flop and DOX translocation [36].

The evaluation of the efficacy of the bound copolymer at various cholesterol concentrations gave an unexpected result. It was found that the ability of Pluronic to favor DOX permeation depended exclusively on the amount of the bound polymer molecules. But being bound, Pluronic accelerates DOX transport independently on the density of lipid packing, at least at naturally occurring cholesterol content (Fig. 4). This observation poses a question about the mechanism of Pluronic effect on DOX permeation through a lipid membrane.

The literature data concerning the influence of cholesterol on the partitioning of other hydrophobic or amphiphilic solutes into lipid bilayer is rather ambiguous and shows that the cholesterol effect depends strongly upon the fatty acid composition of the phospholipid component of the membrane and chemical structure of the solute. Thus, it has been shown that partition coefficients of hexane and benzene between water and saturated phosphatidylcholine-cholesterol bilayers reduced by 2- to 3-fold as cholesterol portion increased up to 40% [53]. On the contrary, the partition coefficient of *tert*-octyl-phenyl poly(ethylene oxide) (Triton X-100) between water and egg lecithin liposomes was close to that observed between water and the liposomes prepared from lipids of *stratum corneum*, containing about 25% of cholesterol [54]. It may be supposed that the main reason for the reduction of Pluronic binding on liposomes containing cholesterol is the decrease in the mobility of lipid components of the membrane, which in turn impedes formation of a cavity in the bilayer sufficient to accommodate a Pluronic chain. These data are in accordance with the previously reported squeeze-out of Poloxamer 188 (equivalent to Pluronic F68) from lipid monolayers at high lateral pressures. This fact indicates that lipid ordering is unfavorable for Pluronic–lipid interactions [55].

Such a decrease in the mobility of membrane components is expressed quantitatively by the value of membrane microviscosity, which is drastically increased as cholesterol content is elevated (Fig. 3C). It may be supposed that the previously reported insignificant effect of the water/lipid partition coefficient of Triton X-100 on the membrane composition [54] is due to the small size of its hydrophobic block.

Another natural membrane component, whose effect on the decrease in the membrane fluidity is well documented, is ganglioside [56,57]. This lipid makes up about 10% of all lipids in plasma membranes of neuronal and some tumor cells [44,45]. Although the reasons for GM1-mediated increase in the membrane ordering seem to be not as clear as in the case of cholesterol, it is commonly suggested that the stiffness of highly branched carbohydrate headgroup of this lipid mainly contributes to the decrease in the membrane microviscosity. We found that the incorporation of ganglioside GM1 induced changes in membrane properties qualitatively similar to those caused by cholesterol: the microviscosity of the membrane increased and Pluronic effect decreased upon addition of the ganglioside (Fig. 5A).

The incorporation of egg phosphatidylethanolamine into lecithin bilayer also increased the membrane microviscosity measured by DPH fluorescence polarization and decreased the membrane responsiveness to Pluronic. Such effect of PE in lecithin-based bilayer can be ascribed to the well-known cone-shaped geometry (negative spontaneous curvature) of this lipid that may cause a condensation of lipid packing in the headgroup region resulting in the decrease in the membrane fluidity. Nevertheless, in this case the membrane responsiveness to Pluronic reduced to a lesser extent than in the case of other add-lipids, which increased the membrane microviscosity—cholesterol and ganglioside (Figs. 5B and 8). A pronounced difference between the effects exerted by PE on the microviscosity and Pluronic action is unclear. However, a possible reason may lie in the method of measuring the microviscosity based on DPH fluorescence polarization. Firestone showed that Pluronics are located in the hydrophobic core region [58]. We observed that PE caused a subtle effect on Pluronic efficacy, indicating that the insertion of PE into the mixed bilayer altered the packing in the hydrophobic core region only slightly. At the same time, it is known that DPH is located in the hydrophobic region of the membrane but stays in contact with glycerol residues due to high length of its molecule [59]. Obviously, in this case, the mobility of DPH appeared to be more sensitive to the changes in the membrane structure than Pluronic ability to accelerate DOX permeation.

The insertion of lipids whose cylindrical geometry and nearly zero spontaneous curvature was close to that of EL (CL and SM) [60,61] did not alter membrane microviscosity and its responsiveness to Pluronic (Fig. 6). This fact is worth noticing in connection with Pluronic-induced chemosensitization of tumor cells. As Kabanov et al. suggested one of the ways by which Pluronic suppresses the activity of P-glycoprotein is the penetration of the polymer into the cells and its interaction with the mitochondrial membranes resulted in uncoupling of oxidative phosphorylation [19,24]. It is known that, as a rule, there are negligible quantities of cardiolipin (about 1%) in the plasma membrane. This lipid is located mainly in the mitochondria membranes where its content amounts to 20% [45]. Our present data demonstrated that such a high content of CL in lipid bilayer did not diminish the effect caused by Pluronic on the membrane permeability, indirectly supporting thus a possibility of effective polymer interaction with mitochondrial membrane.

The insertion of phosphatidic acid that contains a small and highly charged polar head group should result in the mutual repulsion of these molecules within the monolayer. In fact, an increase in the area per polar head group by 30–40% was observed upon insertion of PA into lipid monolayer [62,63]. This may destabilize the membrane and decrease its microviscosity. These effects were indeed observed in the present work: increasing the mole fraction of PA to 0.25 led to a decrease of the membrane microviscosity from 0.9 Poise in the absence of PA to 0.5 Poise. It may be supposed that destabilization of membrane packing by PA may be a biological reason for the low content of this lipid in cell membranes. On the other hand, this fluidization of the membrane favors Pluronic insertion into the bilayer and may be suggested as a reason for the increase in the membrane responsiveness to Pluronic. In mammalian cells, PA is located mainly in the membranes of organelles (sarcoplasmic reticulum, Golgi apparatus, nuclear membrane), where its content may reach about 10% [44]. The fact that the presence of this lipid favors incorporation of Pluronic into the membranes suggests the preferred interaction of the copolymer with the membranes of intracellular organelles enriched with this lipid (e.g. nuclear or Golgi membranes).

So, in the present work, we have shown that interaction of Pluronics with membranes depends on their lipid composition. Insertion of cholesterol, ganglioside or phosphatidylethanolamine into lipid bilayer reduces the membrane responsiveness to Pluronic. On the contrary, insertion of phosphatidic acid increases the membrane sensitivity to Pluronic. The effect of lipids on the membrane responsiveness to Pluronic is determined by their influence on the physical state of lipid bilayer, the latter factor being measured by the membrane microviscosity. This factor influences the ability of the copolymer to insert into bilayer and does not affect the disturbing activity of the copolymer molecules incorporated into the membrane.

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