# Pluronic L61 Accelerates Flip – Flop and Transbilayer Doxorubicin Permeation[+]

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**Abstract:** It has recently been found that Pluronics (block copolymers of ethylene oxide, EO, and propylene oxide, PO) favor the permeability and accumulation of anthracycline antibiotics, for example doxorubicin (Dox), in tumor cells. In an effort to understand these results, the interaction of $EO_2/PO_{32}/EO_2$ (Pluronic L61) with unilamellar egg yolk vesicles (80 – 100 nm in diameter) was examined. A partition coefficient $K_p = [Pl]_{membrane}/[Pl]_{water} = 45$ was determined. This corresponds to adsorption of about 20 polymer molecules to the surface of each vesicle in a 20 μM polymer solution. Despite this rather weak adsorption, Pluronic has a substantial effect upon the transmembrane permeation rate of Dox and upon the phospholipid flip – flop rate within the bilayers. Thus, the Dox permeation rate increases threefold and the flip – flop rate increases sixfold in 20 μM Pluronic. The two rates increase linearly with the amount of adsorbed polymer. The obvious ability of Pluronics to increase the mobility of membrane components may have important biomedical consequences.

**Keywords:** flip – flop · lipids · liposomes · membrane permeability · membranes · Pluronic

## Introduction

Ethylene oxide (EO) and propylene oxide (PO) block copolymers (also referred to as Pluronics) are nowadays used in pharmacy and medicine as immunoadjuvants[1] and components of artificial blood.[2] They were shown to inhibit thrombosis[3, 4] and modulate neutrophile activity.[5] It has recently been found that Pluronics favor accumulation of anthracycline antibiotics, for example doxorubicin (Dox), in tumor cells[6] and demonstrate promising results in therapy of multidrug resistant tumors.[7–9] In order to study the mechanism of Pluronic-induced transmembrane drug permeation, native and malignant cells, as well as lipid vesicles as cell-mimetic objects, have been used. It has been shown in particular that Pluronics significantly increase the permeability towards anthracycline antitumor drugs[10] and other bioactive compounds.[11] The permeabilizing activity of Pluronics depends on the copolymer composition: the molecular mass of polyPO block and molar content of EO units.[12–13]

We have demonstrated earlier that binding of Pluronics to mammalian tumor cells decreases the membrane microviscosity,[14] which reflects a rise in mobility of lipids and other membrane components. In the present work we continue to investigate the mechanism of Pluronic/biomembrane interaction and in doing so, have disclosed a correlation between the amount of adsorbed Pluronic and both the Pluronic-mediated transmembrane migration of lipids and the permeation of Dox. This seems to be useful for interpreting biological effects of water-soluble synthetic polymers. To perform the research, membrane active $EO_2/PO_{32}/EO_2$ three-block copolymer (Pluronic L61) and egg yolk lecithin (EL) vesicles, as cell mimetic species, were used.

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[+] **Abbreviations**: Dox: doxorubicin (adriamycin); EO: ethylene oxide; PO: propylene oxide; EL: egg yolk lecithin; Pl: Pluronic L61; NBD-PE: *N*-[(7-nitrobenz-2-oxy-1,3-diazol-4yl) dipalmitoyl] phosphatidylethanolamine; Tris: tris-hydroxymethylaminomethane; EDTA: ethylenediaminetetraacetate disodium; MES: 2-(*N*-morpholino)ethanesulfonic acid; HEPES: *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonate free acid; S.R.: specific radioactivity.



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## Results and Discussion

**Binding of Pluronic L61 to lipid vesicles**: The starting point of the work was to estimate the efficiency of Pluronic binding to EL vesicles, that is how Pluronic molecules are distributed between the vesicle surface and the surrounding solution. This was accomplished by means of the equilibrium dialysis technique. The dialysis cell included two compartments: internal and external, separated from each other by a cellulose dialysis membrane (see Experimental Section for details). Before the actual Pluronic binding measurements, diffusion of Pluronic molecules through the dialysis membrane was tested and the equilibration time of the process was estimated. For this, the internal compartment was filled with 0.4 mL of 20 mM HEPES-Tris buffer solution (pH 7.0), the external one with 1 mL of 20 μM Pluronic $^3$H-L61 solution in the same buffer. The changes of copolymer concentrations in both compartments in the course of time, controlled by a radiocounting method, are represented in Figure 1a. It can be seen that the copolymer concentration increases in the internal compartment (curve 1) and decreases in the external (curve 2), achieving an identical value in both compartments after about 50 hours. This means that the Pluronic molecules were able to pass through the dialysis membrane, which results in a homogeneous distribution of Pluronic over the entire dialysis cell. Finally, a possible loss of Pluronic due to its binding to the dialysis membrane was taken into account and quantified as follows. Figure 1a shows that the equilibrated Pluronic concentration was 14 μM. Multiplying it by the overall volume of dialysis cell (1.4 mL), the total amount of equilibrated Pluronic in the dialysis was found to be 19.6 nmol. Comparison of this value with the initial amount of Pluronic injected into the external compartment (1 mL × 20 μM = 20 nmol) indicates only negligible binding of Pluronic to the dialysis membrane.

Subsequently, the equilibrium dialysis experiment was repeated except that a 30 mg mL$^{-1}$ EL vesicle solution was added to the internal compartment of the dialysis cell. The results are shown in Figure 1b. An increase of the Pluronic concentration in the internal compartment (curve 1) and its decrease in the external compartment (curve 2) were also observed. However, in contrast to the vesicle-free experiment, the ultimate Pluronic concentration in the internal compartment, $[Pl]^{int}$, appeared to be higher then that in the external, $[Pl]^{ext}$. Such an accumulation of Pluronic in the internal compartment could only be due to its binding to EL vesicles. A difference between these values obviously corresponded to a concentration of Pluronic bound to the EL vesicles: $[Pl]^{int} - [Pl]^{ext} = [Pl]_b$. By measuring $[Pl]_b$ values for different initial bulk concentrations of Pluronic, $[Pl]_o$, the linear isotherm for Pluronic L61 binding to EL vesicles was obtained (Figure 2a). Saturation was unachievable in this experiment because of a large excess of the vesicle concentration over that of Pluronic. At the same time, the increase in vesicle concentration in the internal compartment (at a fixed Pluronic external concentration) resulted in a progressive elevation in the amount of the vesicle-bound Pluronic, the $[Pl]_b - [EL]$ dependence being described by a hyperbolic curve (Figure 2b).

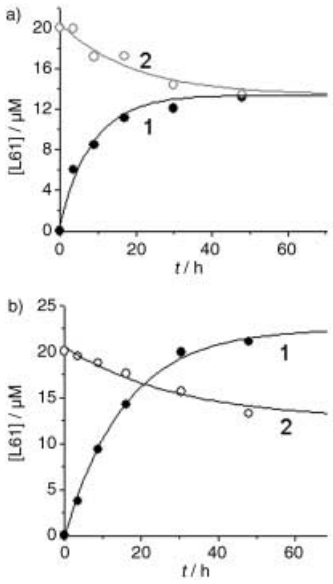


Figure 1. Time-dependence of the Pluronic concentration in the internal and external compartments of a dialysis cell at 30 °C. The internal compartment contained 0.4 mL of 20 mM HEPES/5 mM Tris buffer solution, pH 7.0 (curve 1, A), and 0.4 mL of 30 mg mL$^{-1}$ EL vesicle suspension in the buffer (curve 1, B); the external: 1 mL of 20 μM Pluronic $^3$H-L61 solution in the buffer (curves 2, A and B).

The interaction of amphiphilic compounds with bilayer lipid membranes is usually treated as a partition of a solute between water and the vesicular membrane.[15] Following this approach, the Pluronic-vesicle interaction can be described by an equilibrium:

$$Pl_{water} \rightleftarrows Pl_{membrane} \quad (1)$$

with a partition coefficient of Pluronic between both phases:

$$K_p = \frac{[Pl]_m}{[Pl]_w} \quad (2)$$

where $[Pl]_m$ and $[Pl]_w$ are the concentrations of Pluronic in the membrane and water phases, respectively. $[Pl]_m$ and $[Pl]_w$ were expressed via concentration of vesicle-bound copolymer,

**Abstract in Russian:**

Недавно было показано, что блок-сополимеры этиленоксида (EO) и пропиленоксида (PO) (плюроники) способствуют проникновению антибиотиков антрациклинового ряда, например, доксорубицина, в опухолевые клетки. Для выяснения механизма активирующего действия сополимеров в настоящей работе исследовано взаимодействие плюроника L61 ($EO_2/PO_{32}/EO_2$) с малыми моноламеллярными везикулами, сформированными из яичного лецитина. Рассчитан коэффициент распределения плюроника между липидной мембраной и водным раствором $K_p = [Pl]_{мембрана}/[Pl]_{вода} = 45$. В 20 μM растворе полимера на поверхности каждой везикулы адсорбируется лишь около 20 молекул плюроника. Однако даже столь слабое связывание вызывает 3-кратное ускорение проникновения доксорубицина и 6-кратное ускорение трансмембранной миграции липидных молекул. Эффект плюроника на оба процесса возрастает пропорционально количеству адсорбированного полимера. Способность плюроника L61 повышать подвижность мембранных компонентов представляет интерес для интерпретации эффектов, вызываемых в клеточной мембране адсорбированными полимерами.

  

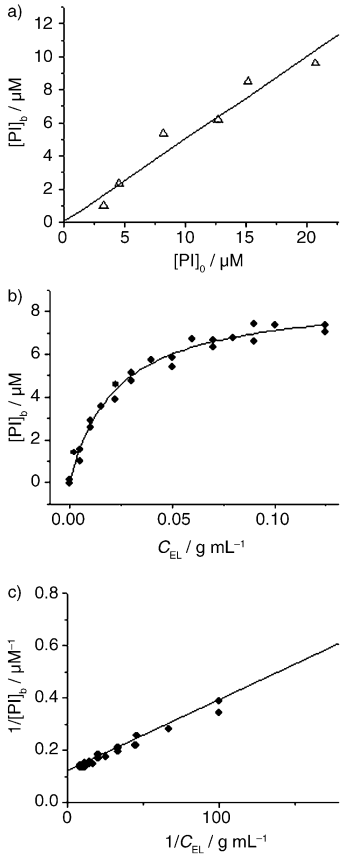


Figure 2. Dependence of concentration of Pluronic $^3$H-L61 bound to EL vesicles on the total concentration of A) Pluronic and B) vesicles, and C) inverse vesicle concentration. EL concentration 30 mg mL$^{-1}$ (A), $[Pl]_0 =$ 7.1 μM (B and C). See other conditions in the legend of Figure 1.

$[Pl]_b$, total (internal + external) volume of water phase, $V_0$, and volume of the membrane microphase, $V_m$:

$$[Pl]_m = [Pl]_b \frac{V_0}{V_m} \quad (3a)$$

$$[Pl]_w = ([Pl]_0 - [Pl]_b) \frac{V_0}{V_0 - V_m} \quad (3b)$$

$V_m$ was calculated from Equation (4):

$$V_m = \frac{EL_{conc} V_0}{\rho} \quad (4)$$

where $EL_{conc}$ is the weight EL concentration and $\rho$ is the density of bilayer lipid membrane equal to 1.0135 g mL$^{-1}$.[16] By substituting Equations (3a), (3b) and (4) in Equation (2), as well as taking into account $V_m \ll V_0$, Equation (5) was obtained:

$$K_p = \frac{[Pl]_b \rho}{EL_{conc} ([Pl]_0 - [Pl]_b)} \quad (5)$$

which was then reduced to a linear form:

$$\frac{1}{[Pl]_b} = \frac{\rho}{[Pl]_0 K_p} \frac{1}{EL_{conc}} + \frac{1}{[Pl]_0} \quad (6)$$

In order to quantify $K_p$ value, the dependence of Pluronic binding on EL concentration from Figure 2b was rearranged as $1/EL_{conc} - 1/[Pl]_b$ plot (Figure 2c). The best fit for the experimental data was observed for a curve with $K_p = 45 \pm 8$, which indicates an extremely low affinity of Pluronic to the lipid membrane.

Based on the data of Figure 2a, a dependence of average number of Pluronic macromolecules bound to one vesicle, $N_p$, on copolymer concentration was calculated using a simple relationship:

$$N_p = \frac{2\pi D^2 [Pl]_b}{[EL] S} \quad (7)$$

where $D$ is a hydrodynamic diameter of EL vesicles and $S$ is an area per one EL headgroup, 0.75 nm$^2$.[17] As follows from Figure 3, addition of a 20 μM Pluronic solution to the vesicle suspension was accompanied by adsorption of about 20 macromolecules on the surface of each vesicle.

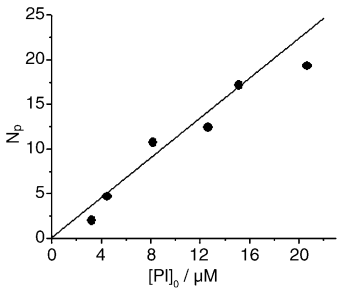


Figure 3. Dependence of average number of Pluronic $^3$H-L61 macromolecules bound to one EL vesicle ($N_p$) on the total Pluronic concentration. Recalculated from the data of Figure 2a by using Equation (8).

Hydrophobic interactions are known to play a key role in the binding of amphiphilic compounds to biological and artificial lipid membranes. In the case of conventional membrane active compounds, their hydrophobic residues are free to incorporate into the lipid bilayer. Such anchoring is highly effective: the $K_p$ values for aliphatic alcohols and local anesthetics lie in 700 – 1000 range.[15] The same mechanism has been also proposed for Pluronic binding to the membranes.[18] However, as shown above, this interaction is characterized by a much lower efficiency, apparently because of a strong difference in structures of hydrophobic bulky PPO blocks and highly ordered layer represented by alkyl residues of lipid molecules, and the presence of polar ether groups in incorporating PPO fragments.

**Pluronic-induced acceleration of transmembrane doxorubicin permeation**: To study the transmembrane permeation of noncharged form of Dox via partition – diffusion mechanism, the technique, based on the pH-induced uptake of Dox into vesicles, was used. Following the previously reported technique,[19] a suspension of EL vesicles was prepared with neutral aqueous buffer solution outside (pH 7) and an acidic one inside (pH 4). Addition of a strong fluorophore Dox to these suspensions resulted in pH gradient induced uptake of noncharged Dox molecules inside vesicles and their protonation. Within the first 5 s of the process, the local concentration of Dox inside vesicles is sufficient to cause self-quenching due to an internal filter effect, liposome entrapped drug being invisible by fluorescence technique. Therefore

Dox located in the external solution mainly determines the sample fluorescence. Decrease in the concentration of this form of Dox resulted in a monoexponential decay of the sample fluorescence that was monitored using fluorescence spectroscopy. This process, following a first order kinetics, reflected the transmembrane Dox permeation.

The kinetics of spontaneous Dox permeation through the membrane of EL vesicles is described by curve 1 in Figure 4a with the first-order constant $k_0$ equal to 0.33 $min^{-1}$. Addition of Pluronic L61 solution to this system resulted in an acceleration of the Dox uptake (curve 2). As shown in our recent paper,[10] binding of Pluronic L61 to the vesicular membrane was not accompanied by formation of hydrated pores or other transient channels permeable for Dox. In other words, both in the presence and absence of Pluronic, the permeation of Dox through the membrane developed via a partition–diffusion pathway.

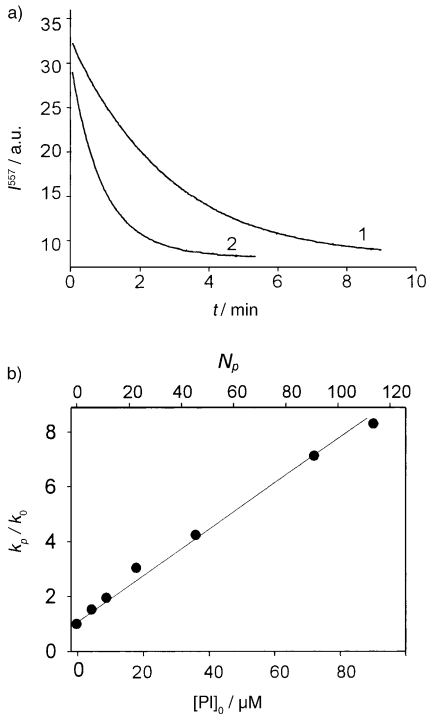


Figure 4. Kinetics of Dox permeation in the absence (1) and in the presence of 20 μM Pluronic L61 (2) (A); and the effect of Pluronic on DOX permeation as a function of Pluronic concentration (bottom axis) and $N_p$ value (top axis) (B). External buffer: 20 mM HEPES/5 mM Tris, pH 7.0, supplied with 0.3 M sucrose; internal buffer: 0.3 M citrate-Tris, pH 4.0; 30 °C.

Figure 4b demonstrates the effect of Pluronic on the transmembrane Dox permeation, quantified as a $k_p/k_0$ ratio, where $k_p$ and $k_0$ are the rate constant values in the presence and in the absence of Pluronic, on the total Pluronic concentration (bottom $x$ axis) and $N_p$ value (upper $x$ axis). As follows from the figure, addition of 20 μM Pluronic solution to the vesicle suspension caused a threefold acceleration of the transmembrane Dox permeation. Importantly, this effect actually resulted from binding of only 20 macromolecules of Pluronic (in average) to each EL vesicle.

**Pluronic-induced acceleration of lipid flip–flop**: To investigate the Pluronic effect on the mobility of membrane components, the transmembrane migration of lipid molecules (flip–flop) was examined. Up to now, a number of methods for controlling flip–flop have been developed, including ESR and NMR spectroscopy,[20] measurements of transmembrane potential[21] and resonance energy transfer fluorescence spectroscopy.[22] In the present work a method originally proposed by McIntyre and Sleight[23] was used. For this, EL vesicles with fluorescent NBD-PE incorporated only into the inner leaflet of the membrane were prepared (see Experimental Section). Addition of a fresh solution of sodium dithionite to the newly made asymmetrically labeled vesicles resulted in a minor decay of NBD fluorescence (curve 1 in Figure 5). Since sodium dithionite was shown to be unable to

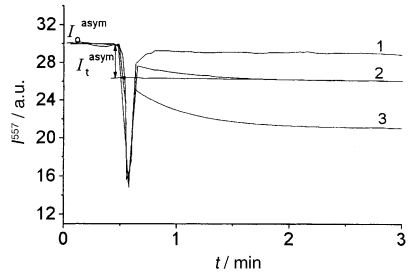


Figure 5. Kinetics of sodium dithionite-induced decay in fluorescence of EL vesicles asymmetrically labeled with NBD-PE. Sodium dithionite solution was added to the vesicles just after their preparation (1) and after keeping them in the absence (2) and in the presence of 25 μM Pluronic L61 (3) for 1 hour at 25 °C. EL concentration 0.15 mg $mL^{-1}$, 10 mM Tris-HCl/150 mM choline chloride/1 mM EDTA buffer, pH 7.0.

penetrate through the lipid membrane,[23, 24] a decrease in the NBD fluorescence intensity obviously reflects the kinetics of reduction of NBD-PE transferred from the inner to outer membrane leaflet for the time of the kinetics record (7 min). Addition of sodium dithionite to the same vesicles but kept for an hour at 25 °C was accompanied by a time-dependent decrease in the NBD fluorescence intensity with a lower ultimate fluorescence level (curve 2 in Figure 5). This curve described a reduction of NBD-PE species already transferred to the outer membrane leaflet for one hour. A fraction of fluorescent NBD-PE species migrated from the inner to outer membrane leaflet, $f$, was calculated using Equation (8):

$$f(\%) = \frac{I_0^{asym} - I_t^{asym}}{I_0^{asym} - (1-\alpha) \times I^{sym}} \times 100\,\% \quad (8)$$

where

$$\alpha = 2\left(\frac{I^{sym} - I_0^{asym}}{I^{sym}}\right) \quad (8a)$$

Equation (8a) is the content of unilamellar vesicles in the NBD-labelled vesicle sample. This value was detected in each experiment and commonly was in 0.92–0.96 range. By measuring $f$ for different incubation time values, the kinetics of spontaneous NBD-PE transmembrane migration (flip–flop) was obtained (Figure 6, curve 1).

The experiment was then repeated but a solution of Pluronic L61 was added to the suspension of asymmetrically

  

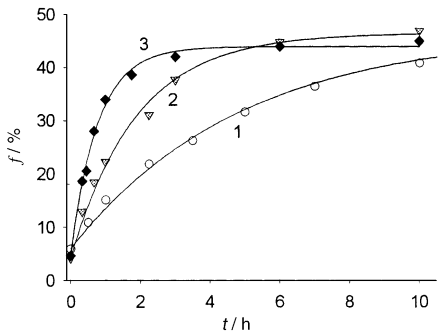


Figure 6. Kinetics of NBD-PE flip–flop in the asymmetrically labeled vesicles in the absence (1) and in the presence of 5 μM (2) and 20 μM Pluronic L61 (3). See other conditions in the legend of Figure 5.

labeled vesicles. The kinetics of decrease in the NBD fluorescence intensity for the Pluronic/vesicle mixture, kept for one hour at 25 °C, after treating it by sodium dithionite is described by curve 3 (Figure 5). Importantly, addition of Pluronic had no effect on the slope of the stationary segment of the kinetic curve indicating no copolymer effect on the permeability of lipid membranes towards dithionite ions. This is in agreement with the earlier results[10] where no formation of pores or other transient defects in the membrane in the presence of Pluronics was reported. At the same time, amplitude of the dithionite-induced fluorescence decay was higher in the presence of Pluronic L61 than in its absence (cf. curves 2 and 3 in Figure 6). This means that Pluronic induced the transfer of an additional amount of NBD-PE species from the inner to outer leaflet of the vesicular membrane over and above the amount ensured by the spontaneous NBD-PE flip–flop. The kinetics of NBD-PE flip–flop in the presence of Pluronic L61 for two copolymer concentrations is represented by curves 2 and 3 in Figure 6. The effect of Pluronic L61 on NBD-PE flip–flop was quantified as $k_p^f/k_0^f$ ratio, where $k_p^f$ and $k_0^f$ are the rate constant values in the presence and in the absence of Pluronic L61, respectively. The dependence of $k_p^f/k_0^f$ value on Pluronic concentration is shown in Figure 7. It is observed that the Pluronic effect on flip–flop rate increased continuously with rising copolymer concentration, reaching tenfold increase at 30 μM Pluronic concentration.

Thus, we demonstrated that Pluronics were able to accelerate the flip–flop of lipid molecules and transmembrane Dox permeation. It is well known that both processes can be

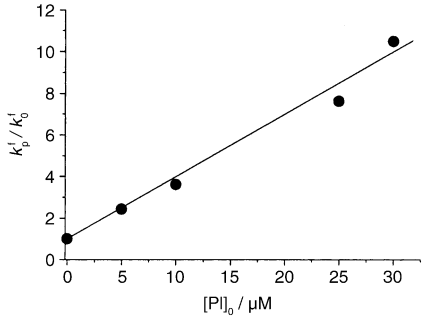


Figure 7. Effect of Pluronic L61 on NBD-PE flip–flop in the asymmetrically labeled vesicles as a function Pluronic concentration. See other conditions in the legend of Figure 5.

also intensified by an increase in temperature.[19, 25] Thus, it would be interesting to compare quantitatively the effects induced in the lipid bilayer by Pluronic and temperature. As follows from the data of Figure 7, the binding of 22 Pluronic macromolecules to mixed EL/NBD-PE vesicles induces a sixfold acceleration of NBD-PE flip–flop. According to the work of Homan and Pownall,[25] where the temperature dependence of spontaneous flip–flop in bilayer lipid vesicles has been examined, a sixfold flip–flop acceleration also results from an increase in the temperature by nearly 10 °C. Thus, the binding of 22 Pluronic macromolecules per each vesicle results in the increase of NBD-PE flip–flop rate that can be also achieved by heating of the membrane by about 10 °C. At the same time, a 10 °C increase in temperature is accompanied by a threefold acceleration of Dox accumulation in EL vesicles.[19] It is this effect that is observed when binding about 20 Pluronic macromolecules to each EL vesicle according to the data of Figure 4b. An excellent correlation between the Pluronic effects on lipid flip–flop and the permeation of Dox was thus shown. Each of them, in turn, resulted from structural disarrangement (fluidization) in the lipid bilayer due to incorporation of hydrophobic PPO blocks of Pluronic macromolecules.

The strong effect on the mobility of the membrane components, which is caused by adsorption of a few Pluronic molecules, may be explained either by cooperative influence on the membrane structure or by formation of small zones on the vesicle surface in which the mobility of membrane components is increased drastically. The techniques applied in the present work do not allow a definite comparison between these explanations. Nevertheless it seems likely, that any cooperative influence on liposome structure should affect the size distribution of liposomes, cause their aggregation or fusion. Measurement of photon-correlation spectra shown that in the presence of Pluronic L61 size distribution of lecithin vesicles remained practically unchanged, no aggregation or fusion being detected. Therefore, it is most likely that adsorbed copolymer forms "defective" areas on the liposome membrane where the mobility of lipid molecules and permeability of the bilayer is highly increased. We suppose that it is these areas that are responsible for the increase of the flux of Dox through the membrane and acceleration of transmembrane movement of lipid.

The low affinity of Pluronics seems to be important for diminishing side effects and toxicity of the copolymer in comparison with other synthetic polymers of biomedical application. Recently, a few other synthetic compounds ("chemical flipases") that is alcohols, diols, local anesthetics, amides and sulfonamides were also reported to accelerate a lipid flip–flop.[26] Based on the Pluronic-mediated structural effects in the lipid membrane, one could expect that lipid flip–flop acceleration, induced by the chemical flipases, will be probably accompanied by increase in the membrane permeability towards Dox. Attaching "chemical flipase" moieties to a polymer chain might additionally enhance their permeabilizing effect.

Certainly, the data obtained in the present study using a simple model system cannot be simply extrapolated to the mechanism of Pluronic action in biological systems. The

apparent rate constant of Dox permeation to liposomes is about 0.3 $min^{-1}$ (see Figure 4), while the transport of the drug into living cells is usually much slower ($k_{app} \sim 0.03\,min^{-1}$);[28] this indicates that the transmembrane permeation is not a rate-limiting step in the drug accumulation in cells. Therefore it is unlikely that the acceleration of this process can result in considerable changes in the amount of drug accumulated in living cells. Nevertheless, Pluronic induced acceleration of Dox permeation through model lipid membranes and activation of flip–flop processes both indicate a considerable disturbance of the lipid bilayer that agrees with our previously reported data concerning fluidization of tumour cells membranes caused by Pluronics L61 and P85.[14] It can be assumed that such disturbance is the main reason for Pluronic effects on cell functioning. At the same time, it should be emphasized that the biomedical importance of the reported results needs further confirmation.

## Experimental Section

**Materials**: The three-block copolymer $EO_2/PO_{32}/EO_2$ (Pluronic L61) was from Serva (Germany), *N*-[(7-nitrobenz-2-oxy-1,3-diazol-4yl) dipalmitoyl] phosphatidylethanolamine (NBD-PE) was purchased from Avanti Polar Lipids (USA), Dox from the Russian Institute of Antibiotics (Russia). Egg yolk lecithin (EL) ethanol solution, sodium dithionite and buffer components: tris(hydroxymethyl) aminomethane (Tris), ethylenediaminetetraacetate disodium (EDTA), 2-(*N*-morpholino)ethanesulfonic acid (MES), *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonate free acid (HEPES) and choline chloride, from Sigma-Aldrich (USA) were purchased and used as received.

To prepare a Triton X-100 containing toluene scintillation solution, 2,5-diphenyloxazole (3.66 g; Serva, Germany), 1,4-bis[2-(5-phenyl)-oxazolyl]-benzene (0.24 g; Serva, Germany), concentrated ammonia solution (4.4 mL; Reakhim, Russia) and Triton X-100 (300 mL; scintillation grade, Sigma, USA) were dissolved in toluene (600 mL; Reakhim, Russia).

**Unilamellar vesicles**: To prepare unilamellar EL vesicles, we followed the procedure described by Yaroslavov et al.[27] First, EL ethanol solution was added into a flask and the solvent was carefully evaporated under vacuum. A thin lipid layer was vortexed in 20 mM HEPES, 5 mM Tris buffer with pH 7.0 and then sonicated with a 4700 ultrasonic homogenizer (Cole-Parmer, USA) at 22 kHz ($4 \times 200$ s) and 4 °C. Vesicles samples thus obtained were separated from titanium dust by centrifugation. The diameter of vesicles, measured by photon correlation spectroscopy with Autosizer 2c (Malvern, UK), was in 80–100 nm range.

**Tritium-labeled Pluronic L61**: Tritium atoms were incorporated into Pluronic C-H groups by atomic bombardment technique according to the procedure we reported previously.[14] The preparation was purified from exchangeable tritium by several cycles of dissolution in ethanol and further solvent evaporation. Then the labeled Pluronic $^3$H-L61 was separated from low molecular radioactive by-products by gel-permeation chromatography on Sephadex LH-20 using ethanol as eluent. The specific radioactivity of thus prepared copolymer was 0.2 Ci $mmol^{-1}$.

**Binding of Pluronic L61 to EL vesicles**: Pluronic binding to the vesicles was measured using the equilibrium dialysis technique. For this, a small dialysis sack with 0.4 mL of EL vesicle suspension inside was placed into 2 mL Eppendorf tube and 1 mL of Pluronic $^3$H-L61 solution in 20 mM HEPES, 5 mM Tris, pH 7.0 buffer were added. The solution was purged with argon for 3 min to remove any oxygen and the tube was then closed. The tube was incubated for 72 hours at 30 °C. Subsequently the sack was extracted from the tube, and 0.3 mL of solution from the sack (inner solution) and 0.3 mL of solution remaining in the tube (outer solution) were taken and mixed each with 3 mL of the Triton X-100-containing toluene scintillation solution. Radioactivity of both samples was measured using Delta-400 (USA) scintillation counter. Concentrations of Pluronic $^3$H-L61 were calculated with S.R. = 0.2 Ci $mmol^{-1}$.

**Kinetics of transmembrane doxorubicin permeation**: The kinetics of transmembrane permeation of Dox, characterized by a strong fluorescence, were investigated using the procedure described by Harrigan et al.[19] A Dox molecule contains amino group with p$K_a$ 8.6, so in neutral or slightly alkaline solutions part of the Dox molecules is noncharged and can incorporate into the vesicular membrane. If the internal vesicle cavity is loaded with an acidic buffer, Dox desorbs from the membrane and accumulates inside vesicles that finally results in self-quenching of Dox fluorescence. This allowed us to follow the transmembrane Dox permeation by measuring the fluorescence intensity in the system at $\lambda_{em} = 557$ nm ($\lambda_{ex} = 490$ nm) using an F-4000 spectrofluorometer (Hitachi, Japan).

According to this scheme, in the present work EL vesicles were prepared in 0.3 M Tris-citrate buffer solution with pH 4.0, using the sonication procedure described above, and then passed through a Sepharose CL-4B column, equilibrated with 20 mM HEPES-Tris buffer with pH 7.0, additionally containing 0.3 M sucrose for osmotic pressure compensation. The suspension of pH-gradient EL vesicles with pH 4.0 inside vesicles and pH 7.0 in surrounding solution was thus prepared. Hydrodynamic diameter of such vesicles, measured by photon correlation spectroscopy, was in 100–115 nm range.

Dox was added to the pH-gradient vesicle suspension at 50 μM concentration, which corresponded to the maximum Dox fluorescence intensity. When concentration of Dox inside vesicles exceeded 50 μM, the fluorescence intensity in the system began decreasing due to the self-quenching effect, the process following the first order kinetics.

**Flip–flop measurements**: To control spontaneous and Pluronic-induced lipid flip–flop in the vesicular membrane, a fluorescence approach was applied. For this, EL vesicles with NBD-PE incorporated into the bilayer were prepared as it has been recently described.[24] Mixed EL/NBD-PE (0.995/0.005 *w*/*w*) ethanol solution was put in a flask 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. The lipid dispersion obtained was subjected five times to the freeze-thawing procedure and finally passed 35 times through polycarbonate filter with 100 nm pore diameter using an Avanti miniextruder. Hydrodynamic diameter of the resulting vesicles was in 90–95 nm range as determined by photon correlation spectroscopy. According to the previously reported data,[24] the NBD-PE species are uniformly distributed between both membrane leaflets (symmetrically labeled vesicles). To make the vesicles applicable for flip–flop kinetics measurements, they were converted to asymmetrically labeled by the following procedure. An appropriate amount of a 0.25 M freshly prepared sodium dithionite solution with pH 10 was added to the 1.5 mg $mL^{-1}$ suspension of the symmetrically labeled vesicles so that 2 mM sodium dithionite concentration was achieved. The mixture was incubated for 6 min at room temperature (18–22 °C) for completing sodium dithionite-induced reduction of NBD-PE nitro groups located only in the outer membrane leaflet. This was accompanied by approx. 46 % decrease in the NBD fluorescence intensity, apparently indicating approx. 92 % content of unilamellar vesicles in the original symmetrically labeled EL/NBD-PE vesicles. The reduced EL/NBD-PE vesicles (asymmetrically labeled) were immediately separated from an excess of sodium dithionite by gel-filtration on Sephadex G-50 column equilibrated with Tris-HCl buffer.

The kinetics of migration of intact NBD-PE species from the inner to outer leaflet of the asymmetrically labeled vesicles in the absence and in the presence of Pluronic L61 was studied as follows. 1 mL of 0.15 mg $mL^{-1}$ suspension of the asymmetrically labeled vesicles was prepared and its fluorescence intensity, $I_0^{asym}$, was measured. From this moment, the flip–flop of NBD-PE species began to be measured, a temperature of 25 °C being held. After period of time chosen, *t*, the sample was cooled down to 20 °C in order to suppress the NBD-PE flip–flop and minimize spontaneous sodium dithionite decomposition and 20 μL of freshly prepared 0.5 M sodium dithionite solution, pH 10 was then added. The kinetics of fluorescence decay was recorded until a new stationary fluorescence level, $I_t^{asym}$, achieved. A fraction of intact NBD-PE species migrated from the inner to the outer membrane leaflet for *t* moment, ***f***, was calculated according to Equation (8). By using different samples from the same asymmetrically labeled vesicle batch and calculating *f* for different *t* values, the kinetics of spontaneous NBD-PE flip–flop was obtained. NBD fluorescence intensity was measured using Hitachi F-4000 spectrofluorimeter with $\lambda_{em} = 530$ nm ($\lambda_{ex} = 450$ nm).

  

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