Relationship between the Structure of Amphiphilic Copolymers and Their Ability To Disturb Lipid Bilayers†

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ABSTRACT: Nonionic amphiphiles and particularly block copolymers of ethylene oxide and propylene oxide (Pluronics) cause pronounced chemosensitization of tumor cells that exhibit multiple resistance to antineoplastic drugs. This effect is due to inhibition of P-glycoprotein (P-gp) responsible for drug efflux. It was suggested that the inhibition of P-gp might be due to changes in its lipid surrounding. Indeed, high dependence of P-gp activity on the membrane microviscosity was demonstrated [Regev et al. (1999) *Eur. J. Biochem. 259*, 18−24], suggesting that the ability of Pluronics to affect the P-gp activity is mediated by their effect on the membrane structure. We have found recently that adsorption of Pluronics on lipid bilayers induced considerable disturbance of the lipid packing [Krylova et al. (2003) *Chemistry 9*, 3930−3936]. In the present paper, we studied 19 amphiphilic copolymers, including newly synthesized hyperbranched polyglycerols, Pluronic and Brij surfactants, for their ability to accelerate flip-flop and permeation of antitumor drug doxorubicin (DOX) in liposomes. It was found that not only bulk hydrophobicity but also the chemical microstructure of the copolymer determines its membrane disturbing ability. Copolymers containing polypropylene oxide caused higher acceleration of flip-flop and DOX permeation than polysurfactants containing aliphatic chains. The effects of copolymers containing hyperbranched polyglycerol "corona" were more pronounced, as compared to the copolymers with linear poly(ethylene oxide) chains, indicating that a bulky hydrophilic block induces additional disturbances in the lipid bilayer. A good correlation between the copolymer flippase activity and a linear combination of copolymer bulk hydrophobicity and the van der Waals volume of its hydrophobic block was found. The relationship between the structure of a copolymer and its ability to disturb lipid membranes presented in this paper may be useful for the design of novel amphiphilic copolymers capable of affecting the activity of membrane transporters in living cells.

Amphiphiles are compounds containing topologically separate hydrophobic and hydrophilic parts. Such architecture ensures their ability to interact with cell membrane and change its properties. An addition of amphiphiles to cultured cells was shown to stimulate vesiculation processes (*1*), change electrical properties of biological membranes (*2*), and change functional activity of membrane proteins (*3*−*8*). Very important applications of surfactants in pharmacy are determined by their ability to inhibit drug efflux systems in the liver (*3*), intestine (*4*), and tumor cells resistant to chemotherapeutics (*6*−*8*). These studies were mainly focused on the surfactants containing hydrocarbon substituents in their hydrophobic part. However, these surfactants were effective only at relatively high (5−20%) amphiphile/lipid ratios (*9*). In contrast, Pluronics [amphiphilic block copolymers of ethylene oxide (EO)[1] and propylene oxide (PO)] could induce substantial changes in cell membrane fluidity when adsorbed on the membrane in very low amounts, less than 0.1% with respect to lipids (*10*). The high efficiency of Pluronics and their relatively low toxicity explain their broad application in various fields of medicine and pharmacology (*11*−*14*). A pharmacological formulation SP1049C based on doxorubicin and two Pluronic copolymers has been recently reported to undergo phase I clinical trial and was found to be efficient in patients with advanced resistant solid tumors (*15*).

Multiple drug resistance of tumors is frequently induced by chemotherapy and in many cases is caused by integral membrane proteins that are responsible for the ATP-dependent efflux of drugs from cell cytoplasm (*16*). Pluronics can inhibit at least two of these proteins: P-glycoprotein (P-

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[1] Abbreviations: CL, cardiolipin; DMF, *N*,*N*-dimethylformamide; DOX, doxorubicin; DP, degree of polymerization; EDTA, ethylenediaminetetraacetate disodium salt; EL, egg yolk lecithin; EO, ethylene oxide; G, glycerol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; NBD-PE, *N*-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl]phosphatidylethanolamine; PEO, poly(ethylene oxide); PG, hyperbranched polyglycerol; PO, propylene oxide, PPO, polypropylene oxide; SM, sphingomyelin; THF, tetrahydrofuran; TMP, 1,1,1-tris(hydroxymethyl)propane; Tris, tris(hydroxymethyl)aminomethane.

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gp) (*17*−*21*) and the multidrug resistance associated protein (MRP1) (*22*). At least two different mechanisms of Pluronic action were proposed by A. Kabanov et al.

First, Pluronics induce ATP depletion of cells presumably by uncoupling of oxidative phosphorylation and thus lead to a decrease of functional activity of ATP-dependent drug efflux proteins (P-glycoprotein and MRP1) (*19*−*22*). This hypothesis was confirmed by the fact that FITC-labeled Pluronic P85 was able to permeate into the cells and was located in mitochondria (*18*).

Another explanation for the Pluronic-induced ATP depletion of MDR cells has been proposed by Pohl and Krylova. They demonstrated the ionophoric activity of Pluronic L61 with cationic selectivity (*23*) and suggested that Pluronic-induced ionic fluxes in plasma membrane could cause compensatory ATP consumption by Na^+,K^+-ATPase, thus decreasing the intracellular ATP concentration (*23*).

Second, it is believed that the inhibition of P-gp ATPase activity by Pluronics, presumably through nonspecific changes in lipid mobility and protein conformation, has a major contribution to the inhibition of P-gp efflux function (*14*). This hypothesis is supported by two sets of data. On one hand, P-gp activity is highly dependent on the physical state and microviscosity of the surrounding lipid bilayer. Changes in the membrane microviscosity inhibited the transporter activity (*24*, *25*). On the other hand, adsorption of Pluronics results in a decrease of cell membrane microviscosity, which may be an alternative reason for inhibition of P-gp activity (*10*, *17*, *25*).

Biological membranes are structurally heterogeneous and too complex to reveal the factors essential for the copolymer interactions with membranes. Therefore, many studies were performed on artificial membranes. Of course, the results obtained on model systems cannot be strictly extended to biological systems, but they might help in elucidating the first step of Pluronic interaction with a cell membrane. Using model lipid bilayers, it was shown that addition of amphiphiles led to solubilization of the membranes (*26*) and formation of transient pores (*27*). The molecular mechanisms of these processes have been widely discussed during the last two decades. It has been proposed that, upon incorporation into a lipid bilayer, the amphiphilic molecules may alter the membrane thickness (*28*), change the hydration state of the headgroup region (*29*), and affect the lipid packing due to mismatch between their hydrophobic segments and lipid fatty acid region (*28*, *30*−*32*). P. Cullis et al. showed that the insertion of PEGylated lipids into the membrane composed of HII-phase-forming lipids promoted formation of a bilayer, i.e., induced HII-L_α transition (*33*). Similar events were induced by other amphiphilic polymers with randomly distributed hydrophobic tags adsorbed on liposomes (*34*). Formation of disk micelles was observed in the work of K. Edwards et al. (*35*), in which lipid molecules conjugated with PEG oligomers were introduced into liposome membranes. Incorporation of amphiphilic pNIPAM copolymers into the bilayer at a temperature exceeding a lower critical solution temperature (LCST) resulted in acceleration of flip-flop in liposomes (*36*). These results are consistent with the theoretical evaluations made by R. Lipowsky (*37*), who predicted that the insertion of amphiphilic copolymers into a lipid bilayer should induce its deformation due to repulsive interactions with hydrophilic corona of the copolymer.

Studies of the effects of Pluronics on artificial lipid membranes showed that these copolymers were highly potent in disturbing lipid membranes. The interaction of Pluronics with liposomes and planar bilayers resulted in a 2-fold decrease in the membrane microviscosity (*38*), increase in the rate of flip-flop (*39*), and membrane permeability toward carboxyfluorescein (*40*, *41*) and various weak acids or bases including doxorubicin (*42*). Insertion of high quantities of hydrophilic Pluronics into a lipid membrane induced significant alterations in the vesicle's size and even shape (*40*).

Numerous studies searched for correlations between diverse effects of amphiphiles and their structure. Most of these studies were devoted to amphiphiles containing hydrocarbon radicals as hydrophobic blocks. Heerklotz and Seelig investigated the relationship between the CMC of low molecular weight neutral amphiphiles (Brij, Triton, alkyl glucoside, alkyl maltoside series, and several ionic surfactants) and their ability to bind to lipid membranes (*43*). De la Maza et al. studied the lytic properties of a number of series of surfactants and correlated it with their structure (*31*). Pantaler and Kamp studied the relationship between the structure of Brij and Triton surfactants and their ability to accelerate flip-flop in erythrocyte membranes. It was found that an increase in the volume of hydrophilic parts of these detergents increased their flippase activity (*9*). On the contrary, a comparison of Pluronics whose hydrophobic block is formed by PPO revealed the leading role of hydrophobicity in the copolymer-mediated effects. The increase in the length of the PPO block enhanced their ability to inhibit P-gp mediated efflux of Rhodamine-123 from the cells exhibiting MDR phenotype. Pluronics with intermediate lengths (30−40) of PO chains and relatively short EO segments (2−25) had the highest efficacy in MDR cells (*19*−*21*). The decrease of the efficacy of Pluronics with high hydrophobicity was obviously due to the increasing tendency of these copolymers to form micelles, which were less effective than single Pluronic molecules (unimers) (*19*). Thus, the reports cited above demonstrate that large diversity of the effects induced by amphiphiles was obviously predicted by their chemical structure.

This work presents an attempt to reveal the parameters of the polymers, which correlate with their ability to interact with a lipid membrane. To this end, we studied the structure−function relationship in seven series of copolymers containing different hydrophobic and hydrophilic blocks. The significance of the hydrophobic block was examined by comparing the effects of copolymers containing either polypropylene oxide (PPO) or aliphatic radicals as a hydrophobic part. The role of the hydrophilic block was studied with amphiphilic copolymers containing either poly(ethylene oxide) (PEO) or branched hydrophilic polyglycerol (PG) (*44*−*47*). All 19 copolymers differing in chemical structure and length of their hydrophobic and hydrophilic parts were tested for their effects on the rate of lipid flip-flop and transmembrane permeation of an antitumor drug doxorubicin. The relationship between these effects and the copolymer properties allowed discovering structural factors that determine the disturbing ability of the amphiphiles. We believe that the quantitative structure−property relationships established for amphiphilic copolymers may clarify molecular mechanisms of their interaction with lipids and be helpful for the design

and synthesis of novel copolymers for pharmacological applications.

EXPERIMENTAL PROCEDURES

Materials. The macroinitiators TMP (Fluka), hexadecylamine ($C_{16}H_{33}NH_2$; Aldrich) and Jeffamine M2005 (PO_{30}-EO_6NH_2; Huntsman) were used without further purification. Glycidol (Degussa) was distilled prior to polymerization. Tetrahydrofuran (THF; Aldrich) and diglyme (Aldrich) were stored over sodium and distilled; methanol and acetone (Sigma) were used as received. The three-block copolymers of EO and PO (Pluronics, Table 1, series I−III) were a generous gift of BASF Corp. Their polydispersity (M_w/M_n) was guaranteed by the supplier to be 1.2−1.4 that was confirmed for P85 and L61 preparations by size-exclusion chromatography using a Waters GPC system equipped with Styragel HR columns, which was calibrated with Waters polyethylene oxide standards. The amount of peroxides in Pluronic preparations checked with the potassium iodide technique (*48*, *49*) was found to be negligible. Dodecyl and hexadecyl ethers of polyethylene oxide (Brij 30, 35, 56) were from Serva, and NBD-PE was from Avanti Polar Lipids. Doxorubicin hydrochloride was purchased from the Institute of Antibiotics (Moscow). Cardiolipin (Biolek) was 99% pure as analyzed by TLC. A chloroform solution of egg yolk lecithin (EL) (purity ≥99%, analyzed by TLC), SM, sodium dithionite, Tris-HCl, EDTA, MES, HEPES, and choline chloride were from Sigma-Aldrich and used without further purification.

Syntheses. The synthesis of PG homopolymers was based on controlled anionic ring-opening polymerization of glycidol using TMP as initiator as described previously (*46*). This way of synthesis of such copolymers allows preparation of well-defined structures with rather low polydispersity ($M_w/M_n \sim 1.3-1.4$) (*44*−*47*).

To synthesize amphiphilic copolymers of PG, two types of initiator-cores, $C_{16}H_{33}G_2$ and PPO-G_2, were prepared by dropwise addition of a stoichiometric amount of glycidol to hexadecylamine and Jeffamine M2005, respectively. The reaction was run at 120 °C for 30 min until no excess of glycidol could be detected. Dissolution in warm ethyl acetate and precipitation upon cooling were repeated twice, yielding the bisglycidolized, tetrahydroxyfunctional macroinitiators. Thus obtained functionalized macroinitiator cores were then used instead of TMP for the synthesis of PG copolymers as described previously (*46*).

TMP-G_x: ^{1}H NMR spectrum (300 MHz, MeOH-d_4) δ 4.7 (s × 1H OH), 3.9, 3.2 (m × 5H PG scaffold), 1.41 (m, 2H, CH_2- TMP), 0.93 (m, 3H, $-CH_3$ TMP); ^{13}C NMR spectrum (300 MHz, MeOH-d_4) δ 82−81, 80.0−79.5, 74.5−73.5, 73.5−72.0, 72.0−70.5, 65.0−64.0, 63.5−62.0 (PG scaffold), 45.46 (C_q TMP), 24.39 ($-CH_2-$ TMP), 8.86 ($-CH_3$ TMP).

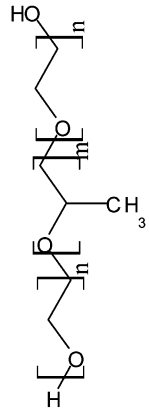
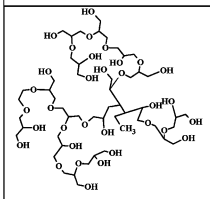
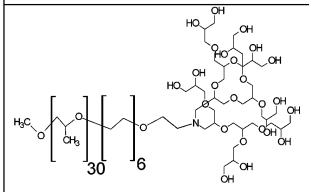
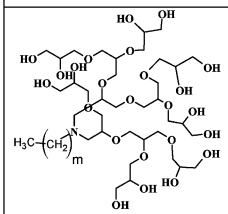
$C_{16}H_{33}$-G_x: ^{1}H NMR spectrum (300 MHz, MeOH-d_4) δ 4.77 (s × 1H OH), 3.9, 3.3 (m × 5H PG scaffold), 2.6−2.4 (m, 6H, $-CH_2-N$), 1.23 (m, 28H, $-CH_2-$), 0.84 (m, 3H, $-CH_3$); ^{13}C NMR spectrum (300 MHz, MeOH-d_4) δ 81.3, 79.5, 73.7, 72.7−71.7, 71.2−70.3, 64.1, 62.5 (PG scaffold), 32.8 ($-CH_2-CH_2-CH_3$), 30.7−30.19 (m, $-CH_2-$), 23.46 ($-CH_2-CH_3$), 14.25 ($-CH_3$).

PPO-G_x: ^{1}H NMR spectrum (300 MHz, MeOH-d_4) δ 4.71 (s, OH), 3.9−3.2 (m, PG scaffold), 1.09 ($-CH_3$ PPO); ^{13}C NMR spectrum (300 MHz, MeOH-d_4) δ 79.79, 78.73, 72.49, 71.23−70.77, 69.23, 62.94, 60.96, 57.75 (PG/PPO scaffold), 17.22 ($-CH_3$ PPO).

Characterization. ^{1}H NMR and ^{13}C NMR spectra were recorded in methanol-d_4 at copolymer concentrations of 100 g/L on a Bruker ARX 300 spectrometer, operating at 300 and 75.4 MHz, respectively. SEC measurements were carried out in DMF at concentrations of about 3 g/L. Measurements were performed with a Knauer microgel set C11 using DMF as an eluent at 45 °C and a Polymer Laboratories evaporative mass detector EMD 960 operating at 110 °C. Polystyrene standards were used for calibration.

Flip-Flop Measurements. Spontaneous and Pluronic-induced lipid flip-flop in vesicular membranes was measured using a fluorescence approach (*39*, *50*, *51*) based on the kinetics of migration of a fluorescent lipid NBD-PE from the inner to the outer leaflet of liposomes. EL vesicles were prepared with NBD-PE incorporated into the bilayer according to ref *51*. Briefly, ethanol solutions of EL and NBD-PE (0.995/0.005 w/w) were mixed 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 was subjected to five repeated freeze−thawing cycles and sonicated using a 4700 Cole-Parmer ultrasonic generator (4 × 200 s, 22 kHz, 30 W). The obtained liposomes were separated from titanium dust by centrifugation using a Beckman microcentrifuge (10 min, 9400*g*). The vesicles were 80−100 nm in diameter as measured by photon correlation spectroscopy with Autosizer 2c (Malvern). According to the previously reported data (*51*), the NBD-PE species were uniformly distributed between both membrane leaflets. Consequently, such vesicles were denoted as symmetrically labeled. To make the vesicles applicable for flip-flop kinetics measurements, they were converted to asymmetrically labeled. To this end, a respective amount of a 0.25 M freshly prepared sodium dithionite solution in 20 mM Tris-HCl buffer, pH 10, was added to a 1.5 mg/mL suspension of the symmetrically labeled vesicles to the final concentration of 2 mM sodium dithionite. The mixture was incubated for 6 min at room temperature (18−22 °C). Dithionite reduces NBD nitro groups to form nonfluorescent amino groups. Sodium dithionite is unable to penetrate through the lipid membrane (*51*). Therefore, it reduced NBD only in the outer membrane leaflet. The fluorescence decay of NBD was measured using a Hitachi F-4000 spectrofluorometer at $\lambda_{em} = 530$ nm and $\lambda_{ex} = 450$ nm. The dithionite treatment of the symmetrically labeled vesicles resulted usually in approximately 46−47% decrease in the NBD fluorescence intensity, apparently indicating approximately 92−94% 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 the excess of sodium dithionite by gel filtration on a Sephadex G-25 column equilibrated with 10 mM Tris-HCl, 150 mM choline chloride, and 1 mM EDTA buffer, pH 7.0 (TCE buffer). The fluorescence of liposomes prepared in this fashion was denoted as I_0^{asym}. The completeness of the reduction of NBD-PE in the outer leaflet of the membrane was checked by a control treatment of the asymmetrically

Table 1: Structures, Composition, and Critical Micellization Concentrations[a] of the Studied Copolymers

Polymer series	Composition (trade name or short name)	Number of hydrophobic repeat units, m	Number of hydrophilic repeat units, n	Molecular weight	CMC[a], mM	Reference
Pluronics 	Series I					
	$EO_2PO_{30}EO_2$ (L61)	30	4	2090	0.11	13
	$EO_{13}PO_{30}EO_{13}$ (L64)	30	26	2900	0.48	13
	$EO_{76}PO_{30}EO_{76}$ (F68)	30	152	8400	0.48	13
	Series II					
	$EO_3PO_{40}EO_3$ (L81)	40	6	2750	0.023	13
	$EO_{26}PO_{40}EO_{26}$ (P85)	40	52	4500	0.065	13
	$EO_{61}PO_{40}EO_{61}$ (F87)	40	122	7700	0.091	13
	Series III					
	$EO_5PO_{59}EO_5$ (L101)	59	9	3800	0.002	13
	$EO_{37}PO_{56}EO_{37}$ (P105)	56	74	6500	0.008	13
	$EO_{132}PO_{50}EO_{132}$ (F108)	50	265	14600	0.02	13
$CH_3—(CH_2)_{m-1}—O—(CH_2CH_2O)_n—H$ (Alkyl-PEO surfactants)	Series IV					
	$C_{12}H_{25}EO_4$ (Brij 30)	12	4	345	0.115	56
	$C_{12}H_{25}EO_{24}$ (Brij 35)	12	24	1225	0.008	56
	$C_{16}H_{33}EO_{10}$ (Brij 56)	16	10	700	0.002	59
 (PG homopolymers)	Series V					
	G_{27}	0	27	2000	N/A	N/A
	G_{81}	0	81	6000		
 (PPO-PG copolymers)	Series VI					
	$PO_{30}EO_6G_2$	30	2	2267	0.1	47
	$PO_{30}EO_6G_{30}$	30	20	3601	N/A	N/A
	$PO_{30}EO_6G_{76}$	30	76	7760	1	47
 (Hexadecyl-PG copolymers)	Series VII					
	$C_{16}H_{33}$-G_{10}	1	10	981	1	47
	$C_{16}H_{33}$-G_{64}	1	64	5000	1	47

[a] CMC values are determined by the pyrene solubilization technique at 37 °C.

labeled vesicles with a fresh portion of dithionite. Usually this verification test gave only a minor decay (1−1.5%) of NBD fluorescence (Figure 1, curve 1).

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

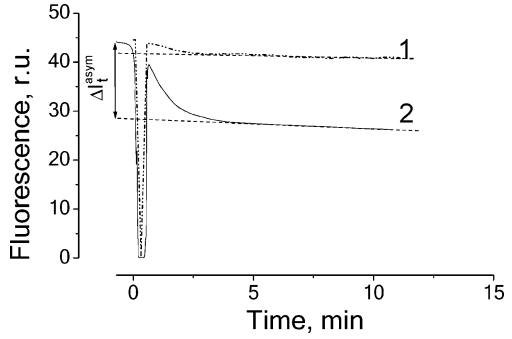


FIGURE 1: Typical curves of sodium dithionite-induced fluorescence decay of EL vesicles asymmetrically labeled with 0.5 wt % NBD-PE. Sodium dithionite solution was added to the vesicles either immediately after their preparation (1) or after incubation for 1 h at 30 °C (2). The two-edged arrow shows the ΔI_t^{asym} value.

freshly prepared 0.5 M sodium dithionite solution, pH 10, at 20 °C, and the kinetics of fluorescence decay was recorded within 3−4 min until a new stationary fluorescence level, I_t^{asym}, was achieved (Figure 1, curve 2). 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 of fluorescent NBD-PE species that had migrated from the inner to the outer membrane leaflet, *f*, was calculated using the equation:

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

where $\alpha = 2[(I^{sym} - I_0^{asym})/I^{sym}]$ is the content of unilamellar vesicles in the NBD-labeled vesicle sample. This value was checked in each experiment and commonly was in the 0.92−0.96 range. *f* values checked at different incubation times showed the kinetics of spontaneous NBD-PE transmembrane migration (flip-flop).

Kinetics of Transmembrane Doxorubicin Permeation. The kinetics of transmembrane permeation of DOX, characterized by strong fluorescence, was investigated using the procedure described by Harrigan et al. (*52*). The DOX molecule contains an amino group with pK_a 8.6. Thus, at pH 7 2.5% of DOX molecules are uncharged and can incorporate into the vesicular membrane. If the internal vesicle cavity is loaded with a buffer with pH 4, DOX desorbs from the membrane and, being charged, accumulates inside the vesicles. When DOX internal concentration exceeds 50 μM, its fluorescence decreases due to the self-quenching effect, thus diminishing the total fluorescence intensity of the sample. This allowed us to follow the transmembrane DOX permeation by measuring the fluorescence of a sample at λ_{em} = 557 nm and λ_{ex} = 490 nm using an F-4000 spectrofluorometer (Hitachi).

EL vesicles were prepared in 0.3 M Tris−citrate buffer solution, pH 4.0, using the sonication procedure described previously (*38*, *39*, *42*) and passed through a Sepharose CL-4B column, 0.8 × 15 cm, equilibrated with 20 mM HEPES−Tris-HCl buffer, pH 7.0, containing 0.3 M sucrose to compensate for the osmotic pressure. Thus prepared EL vesicles had a buffer with pH 4 inside and pH 7.0 in the surrounding medium (pH-gradient vesicles). The hydrodynamic diameter of these vesicles, measured by photon correlation spectroscopy, was 100−115 nm. The pH-gradient vesicles were treated with 50 μM DOX, which is the concentration of maximum DOX fluorescence.

Measurement of Partition Coefficients of the Copolymers between n-Hexane and Water. The bulk hydrophobicity of the polymer was estimated by its partitioning in the biphasic hexane−water mixture, following the approach originally developed by Hansch et al. (*53*). Briefly, 3 mL of the copolymer water solution (0.001−0.004%) in 20 mM HEPES−Tris-HCl buffer, pH 7.0, was shaken intensively for 24 h with 15 mL of hexane in a temperature-controlled chamber at 30 °C. Then, the samples were incubated for at least 2 h to ensure separation of the phases. Both organic and aqueous phases were collected. Hexane solution was evaporated to dryness, and the residue was dissolved in 0.25 mL of water. The polymer concentration was determined in this solution (hexane phase) and in the aqueous phase using the barium/iodine technique as described below, and the apparent partition coefficient *P* was calculated as the ratio of these concentrations. All measurements were performed in triplicate, and standard deviations were calculated.

Determination of the Copolymer Concentration. The barium/iodine method originally proposed for determination of PEO homopolymers in blood samples (*54*, *55*) was found to be applicable for measuring concentrations of any polymer containing ether bonds. PEO, PPO, or PG containing copolymers were capable of forming turbid and strongly colored colloidal complexes with Ba^{2+} and I_3^- ions. The analysis was performed in a 96-well microtitration plate. Two hundred microliters of a copolymer solution in 20 mM HEPES−Tris-HCl buffer was treated with a 25 μL solution of 0.24 M $BaCl_2$ in 1 N HCl and a 25 μL solution of 50 mM I_2 in 0.12 M KI. Three to four minutes later the optical densities of the solutions were measured at 492 nm using a Multiscan 2 Plus multichannel photometer (Titertek) and converted into polymer concentrations using a calibration curve for each polymer in the concentration range 0.00014−0.018%. The calibration curves for all substances were nearly linear up to optical densities of about 0.5. At higher concentrations of the polymer the optical density reached the plateau. Steeper slopes of the calibration curves were observed for polymers possessing higher content of ether groups.

RESULTS

Effect of Amphiphilic Copolymers on the Rate of Flip-Flop. We have shown recently that the adsorption of Pluronic L61 on EL liposome membranes resulted in a drastic acceleration of flip-flop (*39*). In the present work, we investigated changes in the flip-flop rate caused by 19 amphiphilic copolymers of different structure (Table 1). Series I−III consisted of EO and PO block copolymers (Pluronics) differing in the length of their hydrophobic PPO blocks. The effects of Pluronics were compared to those of their analogues containing aliphatic (dodecyl and hexadecyl) chains in their hydrophobic parts (Brij surfactants, series IV). The other three series (V−VII) included hyperbranched polyglycerols (series V) and their block copolymers with PPO (series VI) or hexadecylamine (series VII). The latter are novel AB_n-type copolymers containing one hydrophobic group A and *n* glycerol residues that form a hyperbranched

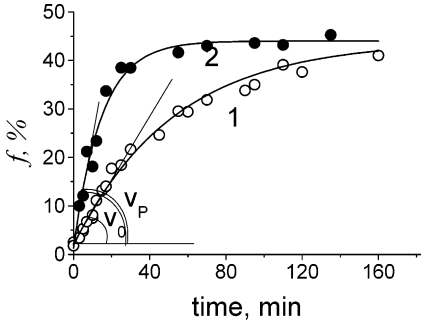


FIGURE 2: Kinetics of NBD-PE flip-flop in lecithin liposomes without any additives (1) and in the presence of 4 mM $PO_{30}EO_6G_2$ (2). The extent of flip-flop (f) was calculated according to eq 1. v_P and v_0 are the initial rates of NBD-PE flip-flop in the presence and in the absence of the polymer. The lines represent pseudo-first-order fits.

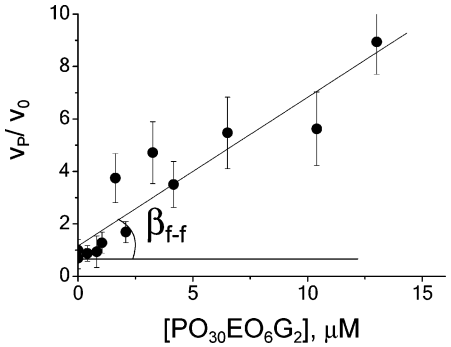


FIGURE 3: Acceleration of NBD-PE flip-flop in EL liposomes, v_P/v_0, as a function of $PO_{30}EO_6G_2$ copolymer concentration.

hydrophilic "corona". In summary, copolymers containing three types of hydrophobic parts (PPO, dodecyl and hexadecyl radicals) and two types of hydrophilic blocks (PEO and PG) were studied for their ability to stimulate flip-flop in lipid membranes.

Flip-flop of NBD-PE is a rather slow process that develops within 2.5 h at 30 °C (Figure 2, curve 1). Addition of 4 mM $PO_{30}EO_6G_2$ resulted in a 2-fold acceleration of flip-flop (Figure 2, curve 2). The effect of the copolymers was quantified as a ratio of the initial rates of flip-flop in the presence of the polymer (v_P) and in its absence (v_0). The effect was proportional to the bulk concentration of the polymer C_0. A representative curve for one of the copolymers studied is shown in Figure 3. This dependence can be fitted by a linear equation:

$$v_P/v_0 = 1 + \beta_{f-f}C_0 \qquad (2)$$

where β_{f-f} (μM^{-1}) is the concentration-independent ability of the copolymer to influence the rate of flip-flop.

The requisite condition for determination of β_{f-f} is the lack of ionic permeability of liposome membranes. If a copolymer was able to promote formation of holes in the membrane, the addition of dithionite would lead to a reduction of NBD on both leaflets of the membrane. The absence of holes was recognized by the fact that, first, the level of flip-flop (f value) at infinite time did not exceed 50% (as demonstrated in Figure 2) and, second, that the fluorescence level after addition of dithionite was constant in time (as shown in Figure 1). The breakage of liposomes was really observed in the case of hexadecylated polyglycerols (series VII) if their concentration exceeded 1 mM. However, when added at lower concentrations, these copolymers did not induce disruption of the liposomes. The β_{f-f} values listed in Table 2, column 2, were determined at the copolymer concentrations that ensured membrane integrity.

To ensure that the copolymer effects on the rate of lipid flip-flop were not due to the presence of organic contaminants, several copolymers (Pluronics L61, P85, polyglycerol $PPO_{30}EO_6G_{30}$, and Brij 35) were purified by size-exclusion chromatography on Sephadex LH-20 using ethanol as eluent. The polymer was isolated from the solution by evaporation of ethanol on a rotor evaporator at 30−35 °C. Polyglycerol $PPO_{30}EO_6G_2$ was purified by three times repeated dissolution in water at 4 °C and precipitation at 70 °C. The bilayer-disturbing abilities of the resulting preparations were found to be indistinguishable from those of the unpurified preparations, indicating that impurities, if present, did not contribute considerably to the effects caused by the copolymers. This fact agrees with the previously reported data on the hemolytic activity of commercial and purified preparations of Pluronic F68 (*49*).

The β_{f-f} values were determined for all copolymers (Table 2, column 2). The set of copolymers allowed differentiating between the contributions of hydrophobic and hydrophilic blocks of the copolymers to their flippase activity. Three series of Pluronics were selected. Each series contained the same number of PO repeat units and a different number of EO repeat units. It is seen in Figure 4A that an increase in the degree of polymerization (DP) of the hydrophilic block (n) was accompanied by a decrease in the copolymer flippase activity within each series. At the same time, the flippase activity of Pluronics increased regularly with the growth of the length of their hydrophobic blocks. The fact that an increase in the length of a hydrophobic block results in greater flippase activity is illustrated in Figure 4A by drawing a straight vertical line at $n = 50$ and comparing the log β_{f-f} values for intercept points of this line with the trends for the series with different m values.

An addition of every 10 PO repeat units to Pluronics resulted in the increase in log β_{f-f} value by 0.36 that corresponds to the $10^{0.36} = 2.3$-fold growth of the copolymer flippase activity (Figure 4). At the same time, for long hydrophilic blocks, a similar increase in the DP of PEO at a constant m resulted only in a minor (about 10%) decrease in the copolymer flippase activity (solid curves in Figure 4A). An increase in the length of the PEO block at low n values resulted in a more pronounced decrease in the flippase activity, indicating a logarithmic dependence of this parameter on DP. Indeed, the same data plotted in double logarithmic coordinates (log β_{f-f} versus log n) were linear in the entire range of n (data not shown).

To reveal the role of the chemical structure of the hydrophobic block, we compared the flippase activity of Pluronics to that of Brij surfactants in which the hydrophobic block is formed by more hydrophobic and compact hydrocarbon substituents. Interestingly, these copolymers caused only negligible effects on lipid flip-flop (Table 2, series IV). This fact cannot be explained by their low affinity to lipid bilayers because the water/lipid bilayer partition coefficients were reported to be 1.9×10^6 for Brij 30 (*56*) and 1.4×10^5 for Brij 56 (*57*), indicating their complete binding to the membrane. Therefore, the inability of these compounds to accelerate flip-flop in contrast to PPO-based amphiphiles

Table 2: Copolymer Effects on Rate of Flip-Flop (β_{f-f}) and DOX Permeation (β_{DOX}), Partition Coefficients in Biphasic Water−Hexane System (log $P_{water-hexane}$), and Literature Data on log *P* Values for Copolymer Partitioning between Water and Lipid Bilayer (log $P_{water-lipid}$)

copolymer	acceleration of flip-flop (β_{f-f}), μM^{-1}	acceleration of DOX transport (β_{DOX}), μM^{-1}	log $P_{water-hexane}$ (measured)	log $P_{water-lipid}$ (lit. data)
series I				
$EO_2PO_{30}EO_2$ (L61)	0.16 ± 0.01	0.04 ± 0.01	−0.24 ± 0.037	1.65 (*39*)
$EO_{13}PO_{30}EO_{13}$ (L64)	0.07 ± 0.01	0.022	−1.83 ± 0.27	N/A[a]
$EO_{76}PO_{30}EO_{76}$ (F68)	0.01 ± 0.05	N/A	−3.5 ± 0.53	N/A
series II				
$EO_3PO_{40}EO_3$ (L81)	0.56 ± 0.045	0.23 ± 0.02	−0.11 ± 0.02	N/A
$EO_{26}PO_{40}EO_{26}$ (P85)	0.22 ± 0.06	0.1 ± 0.01	−2.65 ± 0.4	N/A
$EO_{61}PO_{40}EO_{61}$ (F87)	0.06 ± 0.01	0.02 ± 0.2	−3.19 ± 0.48	N/A
series III				
$EO_5PO_{59}EO_5$ (L101)	1.35 ± 0.13	0.55 ± 0.06	0.11 ± 0.017	N/A
$EO_{26}PO_{56}EO_{26}$ (P105)	0.21 ± 0.03	N/A	−2.6 ± 0.4	N/A
$EO_{132}PO_{50}EO_{132}$ (F108)	0.11 ± 0.02	N/A	−3.65 ± 0.54	N/A
series IV				
$C_{12}H_{25}EO_{24}$ (Brij 35)	0.0035 ± 0.0012	N/A	−1.79 ± 0.06.4	1.31 (*43*)
$C_{12}H_{25}EO_4$ (Brij 30)	0.01 ± 0.003	N/A	3.20 ± 0.61	6.28 (*43*)
$C_{16}H_{33}EO_{10}$ (Brij 56)	0.028 ± 0.003	N/A	2.32 ± 0.52	5.16 (*56*)
series V				
G_{27}	0	0	N/A	N/A
G_{81}	0	0	N/A	N/A
series VI				
$PO_{30}EO_6G_2$	0.565 ± 0.07	0.06 ± 0.01	−0.51 ± 0.02	N/A
$PO_{30}EO_6G_{30}$	0.19 ± 0.02	0.0071 ± 0.001	−2.45 ± 0.07	N/A
$PO_{30}EO_6G_{76}$	0.11 ± 0.02	(8.5 ± 0.9) × 10^{-4}	−4.07 ± 0.16	N/A
series VII				
$C_{16}H_{33}$-G_{10}	0.13 ± 0.02	0.03 ± 0.01	−2.20 ± 0.58	N/A
$C_{16}H_{33}$-G_{64}	0.06 ± 0.01	0.01 ± 0.005	−2.92 ± 0.11	N/A

[a] N/A, not available.

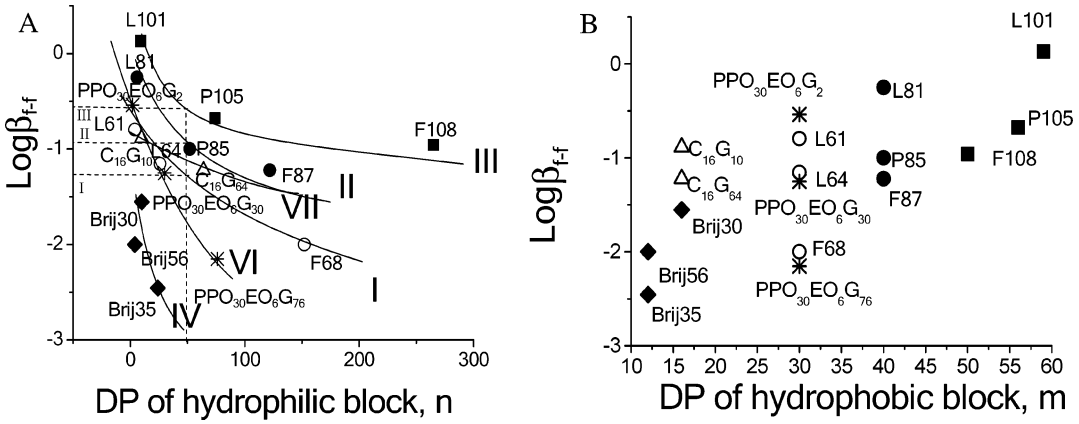


FIGURE 4: Ability of amphiphilic copolymers of series I (○), II (●), III (■), IV (◆), VI (★), and VII (△) to accelerate flip-flop (β_{f-f}) as a function of DP of their hydrophilic (A) and hydrophobic (B) blocks. Solid lines in (A) indicate the traces corresponding to the series with a constant length of hydrophobic blocks. The dashed drop lines in (A) indicate the effects of copolymers with the same DP of their hydrophilic block and different lengths of hydrophobic blocks.

is likely determined by a lower size of their hydrophobic block as compared to the slightly hydrophobic and bulky PPO chain.

Unexpectedly, the copolymer containing 2 glycerol residues, 30 PO repeat units, and 6 EO repeat units ($PO_{30}EO_6G_2$) exhibited nearly 3-fold higher flippase activity in comparison to Pluronic with the same amount of PO repeat units (EO_2-$PO_{30}EO_2$, L61) (Table 2, cf. series VI and I). This fact indicated the importance of the chemical structure and shape of the hydrophilic block. The significance of the chemical structure of the hydrophilic block was also revealed when the flippase activity of hexadecyl surfactants containing hyperbranched polyglycerols (series VII) was compared to that of alkyl-PEO surfactants (Brij, series IV). Hexadecyl-PG containing 10 and 64 glycerol repeat units (series VII) showed β_{f-f} values of 0.13 μM^{-1} and 0.055 μM^{-1}, respectively, which were nearly 1 order of magnitude higher than those of Brij 35 and Brij 30. This fact indicates that the bulky polyglycerol blocks, being attached to the membrane, are somewhat helpful in disturbing it. At the same time, homopolymers of glycerol caused no effect on the rate of flip-flop (Table 2, series V) obviously due to their inability to adsorb on the lipid membrane.

The results lead to the conclusion that PPO is much more effective in the disturbance of the lipid bilayer than hydrocarbon groups. When introduced into amphiphilic molecules, the bulky hyperbranched polyglycerol also promotes disturbances in the lipid bilayer.

Effect of Copolymers on DOX Permeation into Liposomes. The disturbance of the lipid bilayer and acceleration of lipid

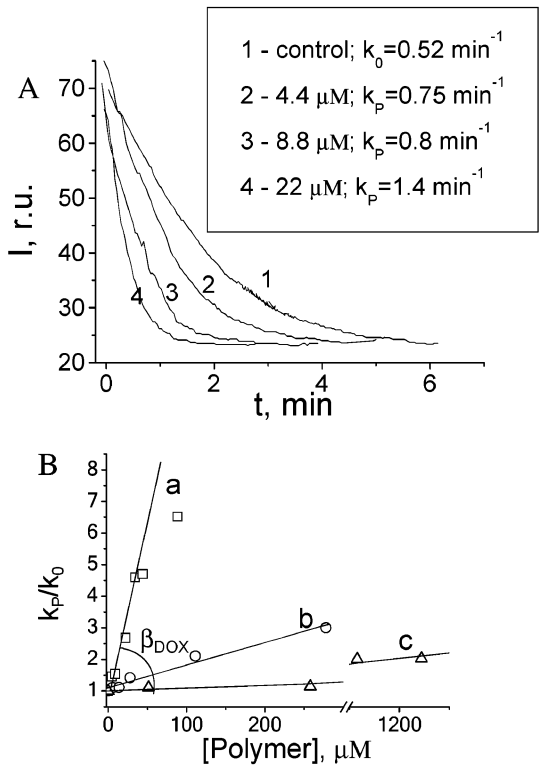


FIGURE 5: Influence of PPO−PG copolymers (series VI) on the rate of DOX permeation through pH-gradient liposome membranes. (A) Kinetics of DOX permeation in the absence of a copolymer (1) and in the presence of 4.4 μM (2), 8.8 μM (3), and 22 μM (4) $PO_{36}EO_6G_2$. Effective rate constants of the corresponding curves are indicated in the plot. (B) Acceleration of DOX permeation (k_P/k_0) caused by $PO_{36}EO_6G_2$ (a), $PO_{36}EO_6G_{20}$ (b), and $PO_{36}EO_6G_{76}$ (c) as a function of the copolymer concentration.

flip-flop caused by polymer adsorption can be expected to increase membrane permeability toward noncharged molecules. To evaluate this effect, we investigated the influence of the polymers on the rate of pH-induced accumulation of DOX in liposomes. DOX accumulated inside the liposomes in response to the pH gradient between the inner cavity (pH 4) and the external solution (pH 7). The kinetics of DOX permeation is a pseudo-first-order process and is characterized by an effective rate constant k_0 that was readily measured from the rate of quenching of DOX fluorescence inside the liposomes (Figure 5A, curve 1). A similar measurement of DOX permeation kinetics in the presence of a copolymer gave a constant k_P (curves 2−4), the ratio k_P/k_0 being a measure of the copolymer effect on the bilayer permeability toward DOX at a given copolymer concentration.

The increase in the copolymer concentration led to a linear growth of the k_P/k_0 values (Figure 5B). The slopes of these lines, denoted as β_{DOX} (Figure 5B), are concentration-independent parameters of the copolymer effect on the rate of DOX permeation. The proportionality between k_P/k_0 values and the copolymer concentration C_0 was observed for all copolymers up to CMC. It has been reported previously that solubilization of low molecular weight compounds in Pluronic micelles may decrease the rate of their accumulation in the cells (*19*). To avoid these effects, the concentrations of the copolymers were varied in the intervals below or close to CMC in all experiments. It was found that, at higher concentrations (about 10 × CMC), leakage of liposomes and dissipation of the pH gradient were observed.

In the experiments described above, we have used unilamellar liposomes composed only of EL, while natural cell membranes contain a large variety of other phospholipids and sphingolipids. To understand if our model is relevant to the effects induced by the amphiphiles in a more complicated system, we studied the effects of the copolymers on liposomes composed of two-component lipid mixtures. One of the components was always EL, while the other was either bovine brain SM as a marker of protein-rich rafts or egg CL as a marker of mitochondria. The content of SM and CL was varied in the range 0−50% (mole). The effect of Pluronic L61 on the permeability of mixed liposomes containing up to 50% (mole) of the additive was the same (β_{DOX} was 0.04 ± 0.01 μM^{-1}) as in the case of liposomes composed of pure EL. It means that the addition of SM and CL into the membrane does not influence the ability of Pluronic L61 to accelerate DOX permeation.

The relationship between the structure and the ability of a copolymer to accelerate DOX permeation into liposomes is seen from Table 2 (column 3). All Pluronics studied in the present work were able to accelerate DOX permeation in accordance with our previously reported results (*38*, *39*, *42*). At the same time, the effect strongly depended on the molecular structure of the copolymer. The effects of copolymers of the I, II, and III series containing the lowest amounts of EO and differing in the length of their PPO block indicate that increasing the length of the hydrophobic block augmented the copolymer effect on the rate of DOX permeation. At the same time, a comparison of Pluronics inside each series, differing in the length of their hydrophilic EO blocks, showed that an increase in the content of EO repeat units in the copolymer decreased its ability to facilitate DOX permeation. Thus, the relationship between the copolymer structure and its ability to facilitate DOX permeation (Table 2, column 3) was quite similar to the correlation observed for flip-flop.

The hydrophilic homopolymers PEO and PG (Table 2, series V) did not accelerate DOX permeation at all, while block copolymers of PPO and PG did, especially in the case of $PO_{30}EO_6G_2$ (Figure 5B, curve a, $\beta_{DOX} = 0.06$ μM^{-1}). However, their influence weakened steeply with an increase in the DP of their PG blocks (Table 2, series VI).

Hexadecyl-PG copolymers (Table 1, series VII) were also able to accelerate DOX permeation, albeit at a relatively high concentration 0.4−1 mM, and were ineffective at a concentration less than 0.4 mM. However, the addition of these copolymers at a concentration above 1 mM (close or above CMC) resulted in an unusual kinetics of DOX permeation. Once initiated, the exponential decay of DOX fluorescence turned into growth, indicating DOX efflux from the vesicles. Presumably, this was due to liposome disruption and/or formation of large holes in lipid membranes leading to dissipation of transmembrane pH gradient. Thus, hexadecyl-PG copolymers increased the membrane permeability toward DOX in a narrow concentration range.

In contrast to hexadecyl-modified polyglycerols, PPO-PG copolymers affected the properties of the lipid bilayer at rather low concentrations (4−25 μM), and no substantial leakage of the internal buffer was observed. Thus, the effect of $PO_{30}EO_6G_n$ copolymers on the permeation of the uncharged form of DOX was not due to formation of pores in

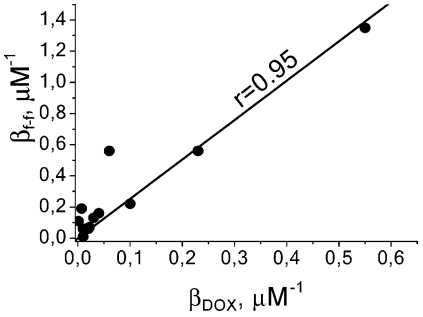


FIGURE 6: Correlation between the copolymer influence on the rate of lipid flip-flop (β_{f-f}) and DOX transport (β_{DOX}). Each point corresponds to the effects caused by one copolymer on both processes; r is the coefficient of correlation.

the bilayer; obviously, it was caused by facilitation of DOX diffusion through the bilayer.

Analysis of the copolymers' influence on the rates of two different processes in the bilayer, flip-flop and DOX transport (Table 2, columns 3 and 4, respectively), showed that the copolymers exhibiting flippase activity could also increase membrane permeability toward DOX. A comparison of β_{DOX} and β_{f-f} values revealed high mutual correlation ($R = 0.95$, $N = 13$, Figure 6). This fact indicates that both effects are manifestations of the same phenomenon, a disturbance of the lipid bilayer caused by amphiphilic copolymers.

Both β_{f-f} and β_{DOX} values are determined by a superposition of the copolymer abilities (1) to bind to the membrane and (2) to disturb its packing. Obviously, these properties may be independent of each other; i.e., copolymers can have high ability to adsorb on lipid bilayers, but once bound they can have no effect on the rate of flip-flop or DOX permeation. At the same time, another copolymer with a relatively low affinity to lipid membranes, still being adsorbed, may induce high disturbance of the bilayer. It seems relevant to compare the copolymers' flippase activity and its ability to bind to lipid membranes. Since the binding of a substance to a lipid membrane correlates with its partition coefficient between aqueous phase and an organic solvent (*53*, *57*), we analyzed the bulk hydrophobicity of all copolymers studied and compared it with the copolymer ability to accelerate dynamic processes in the membrane.

Evaluation of Copolymer Hydrophobicity. To evaluate the hydrophobicity of the copolymers, we measured their partitioning in a biphasic water−hexane system and calculated the respective log $P_{water-hexane}$ values. The estimation of the partition coefficients of surfactants meets an obvious difficulty due to the possibility of formation of micelles and reversed micelles both in water and in the organic phase, respectively (*53*, *57*−*59*). However, it has been shown that the distribution of a surfactant between water and oil remains constant below CMC (*59*). In this case, solubilization processes do not contribute substantially to the measured values of the partition coefficients. Therefore, we measured the copolymer partitioning at concentrations 1.5−2 times lower than the CMC.

The values of log P that we obtained are presented in Table 2, column 4. It was found that an increase in the length of the hydrophobic PPO block in Pluronics L61, L81, and L101 increases copolymer hydrophobicity (Table 2, column 4). The log P values increased linearly with the number of PO repeat units (Figure 7A). The increment for one PO unit calculated

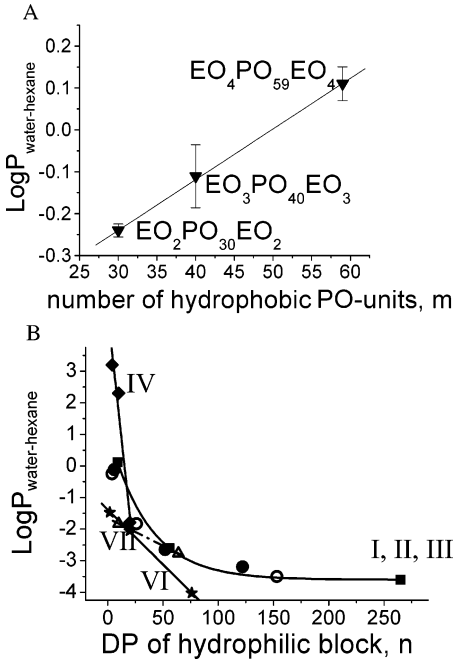


FIGURE 7: Relationship between the number of hydrophobic (A) and hydrophilic (B) repeat units in the copolymer and their bulk hydrophobicity. Ordinate: hydrophobicity evaluated as the logarithm of their partition coefficients in a water−hexane biphasic system. Symbols: series I (○), II (●), III (■), IV (◆),VI (★), and VII (△).

from Figure 7A was found to be very low (0.012 ± 0.010). It means that only polymers containing tens of PO repeat units are hydrophobic, while amphiphilic copolymers in our study exhibit low hydrophobicity.

An increase in the number of hydrophilic ethylene oxide or glycerol repeating units in the copolymer decreased their partition coefficient in the water−hexane mixture (Figure 7B). Importantly, the variation in DP of the PPO block from 30 for Pluronic L61 to 59 for Pluronic L101 increased the hydrophobicity by only 0.35 log P unit. At the same time, an increase in the length of the PEO block by 22 EO repeat units (from 4 for Pluronic L61 to 26 for Pluronic L64) resulted in a more pronounced decrease of the copolymer hydrophobicity by 1.59 log P units (Table 2, column 4). Thus, variation in the DP of the PPO block caused less pronounced changes in the copolymer hydrophobicity than that due to the PEO block. Pluronics with close content of EO repeat units [10% (w/w) in L61, L81, and L101; about 50% in L64, P85, and P105; and about 80% in F68, F87, and F108 Pluronics] have similar hydrophobicities. It means that the bulk hydrophobicity of Pluronics is determined mainly by the EO/PO ratio in the copolymer rather than by the DP of its PPO block.

An increase in the EO content in alkyl-PEO surfactants (series IV) resulted in a nearly linear decrease in their hydrophobicity (Figure 7B, line IV) in accordance with the previously reported data (*58*). The log $P_{water-hexane}$ value for Brij 30 ($DP_{PEO} = 4$) measured in the present work (3.20 ± 0.21) was nearly equal to that obtained by Greenwald et al. (*59*) for partitioning of this surfactant to isooctane (log $P_{water-isooctane} = 3.19$). This fact confirms our estimation.

It is essential that the steepness of the log P curve sharply decreases with the increase in the number of hydrophilic EO repeat units (Figure 7B). The log P values of highly

hydrophilic Pluronics containing more than 100 EO repeat units leveled off (Figure 7B). Obviously, the bulk hydrophobicity of these copolymers quickly reached a limit close to that of long EO homopolymers.

The last column in Table 2 represents water−lipid bilayer partition coefficients available in the literature for some of the copolymers. It is noteworthy that these values are about 3 orders of magnitude higher than those measured in the water−hexane system. This fact indicates that the incorporation of amphiphilic copolymers into membranes occurs much easier than into a bulk hydrocarbon phase. Obviously, copolymer incorporation into the membrane requires less dehydration as compared to its transfer into the bulk oil phase.

However, the comparison of log $P_{water-lipid}$ values published previously and log $P_{water-hexane}$ data measured in the present paper fit a straight line log $P_{water-lipid} = 3.03 + 0.97$ log $P_{water-hexane}$ with a slope close to unity and intercept about 3. This proportionality means that the hexane−water partition coefficients followed the copolymer affinity for the lipid bilayer. Therefore, the coefficients log $P_{water-hexane}$ can be used as a hydrophobicity scale of the copolymers' ability to partition into a lipid bilayer.

DISCUSSION

In the present work, we have examined the relationship between the structure of amphiphilic copolymers and their ability to change the properties of a lipid bilayer in liposomes. To this end, we have tested 19 amphiphiles differing in length and chemical structure of their hydrophobic and hydrophilic blocks. Three series of PEO-PPO block copolymers (Pluronics) were compared with detergents of the alkyl-PEO (Brij) series. To assess the role of the hydrophilic block, we have tested novel copolymers containing hyperbranched polyglycerols as their hydrophilic block. All copolymers were examined for their ability to influence two membrane properties: lipid flip-flop and permeability toward the antitumor drug DOX.

The model systems used for the investigation of these processes were proposed previously and thoroughly studied. The technique of flip-flop measurement based on quenching of NBD-labeled lipid fluorescence by dithionite was originally proposed for living cells (*60*) and further applied for studies of much slower translocation of lipids in artificial membranes (*50*).

To study the kinetics of DOX interaction with cell membranes, two types of liposomes were proposed: (1) liposomes filled with DNA that bind DOX with an effective constant about 2 μM (*61*−*63*) and (2) pH-gradient liposomes (*52*). The characteristic rates of DOX permeation in both systems were found to be similar. This fact indicates that the rate of the whole process is limited by drug diffusion through the lipid bilayer and does not depend on the way of formation of the transmembrane gradient of DOX chemical potential (*61*, *62*). Although our present data may or may not be directly relevant to the mechanisms of chemosensitization of MDR cells, there are some common features of the copolymer interactions with artificial and cell membranes. Thus, the rate of DOX accumulation both in cells and in liposomes is determined by permeation of the uncharged form of the drug (*62*). The important role of the hydrophobic block of Pluronics was disclosed by Batrakova et al. in studies of cultured cells (*20*, *21*), and the same conclusion was obtained in the present study on liposomes (Figure 4). A good agreement was observed previously between the influence of Pluronics on the microviscosity of normal and tumor blood cells (*10*), multidrug-resistant cells (*17*−*19*), and liposome membranes (*38*). Thus, the results obtained on liposomes are in accordance with those obtained on living cells. It means that an artificial liposome system adequately mimics initial stages of polymer interaction with cell membranes.

The ability of each copolymer to accelerate flip-flop and DOX permeation was determined at various copolymer concentrations below CMC. It was found that both effects were proportional to the copolymer concentration, in agreement with the previously reported data (*39*, *42*). The slope of this dependence (Figure 3) is a concentration-independent parameter, which reflects the ability of a polymer to influence the corresponding process. The characteristic rates of flip-flop and DOX permeation differ by several orders of magnitude (*50*, *61*). Nevertheless, the copolymer effects on these processes showed a close mutual correlation (Figure 6). The capacity to enhance both processes has been previously described for Pluronic L61 (*39*). Now we can see that this property is common for a broad spectrum of amphiphiles of various chemical structures. This finding strongly indicates that the effects of amphiphiles on both processes are manifestations of a common cause, which we denote as the ability of a copolymer to induce disturbances in the liquid-crystalline packing of lipid membrane.

The main results of the present work were obtained using one-component EL liposomes. However, we found that the copolymers exhibited the same effects on mixed liposomes containing up to 50% (mol) of CL, a mitochondrial marker, or SM, the marker of protein-enriched lipid rafts. This fact supports the possibility of the copolymers' interaction with lipid rafts, membrane "dwellings" of P-gp, and with mitochondrial membranes, the putative targets of Pluronic effect in living cells (*14*, *18*−*20*).

The key question of our study concerns the features of the chemical structure of the copolymer that ensure its ability to adsorb onto a lipid bilayer and affect its properties. We found that the length of the copolymer hydrophobic block is of prime importance for its disturbing ability (Figure 4). It could be suggested that the bilayer disturbance caused by the copolymers should depend on their ability to bind to the membrane. Previously, H. Heerklotz and J. Seelig have shown that surfactant partitioning into a lipid membrane correlates with its CMC (*43*). The importance of CMC values of Pluronics for their chemosensitizing effects was also demonstrated by Batrakova et al. (*18*). Nevertheless, we found that the correlation between log β_{f-f} and log CMC was poor ($R = -0.37$), indicating that the copolymer−membrane interactions could hardly be mimicked by the formation of copolymer micelles. Besides, this result points to existence of some other parameter that contributes to the copolymer disturbing ability.

To reveal the factors essential for the copolymer flippase activity, we turned to the theoretical predictions made by Lipowsky (*37*) and Balgavy and Devinsky (*65*), who showed that inclusions into lipid bilayers induced deformations in the membrane. It was reasonable to suggest that such

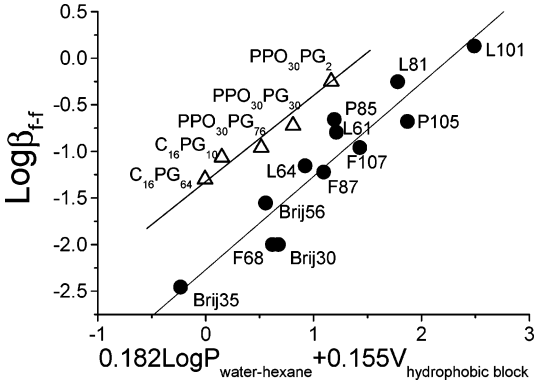


FIGURE 8: Correlation between the effects induced by copolymers of series I−IV (●) and VI and VII (△) on the rate of NBD-PE flip-flop (log β_{f-f}) and the linear combination of their bulk hydrophobicity (log $P_{water-hexane}$) and van der Waals volume of their hydrophobic block ($V_{hydrophobic\ block}$).

deformations should be proportional to the volume of the inserted compound. Keeping this in mind, we calculated the van der Waals volumes of the hydrophobic block for all copolymers under study using Hyperchem 7.0 software (Hypercube Inc.). These volumes varied from 0.8 nm^3 for hydrocarbon radicals to 3.8 nm^3 for PPO chains with $m = 50$. Thus, we built a two-parameter correlation between log β_{f-f} and the linear combination of the copolymer bulk hydrophobicity (log P) determined in the water−hexane system (Table 2, column 4) and the volume of its hydrophobic block. We found that the latter two parameters, log $P_{water-hexane}$ and $V_{hydrophobic\ block}$, were linearly independent, and since the mutual correlation coefficient for the investigated copolymers was less than 0.1, they could be used for correlation analysis. The best fit of experimental data was achieved with a linear combination of log $P_{water-hexane}$ and $V_{hydrophobic\ block}$ multiplied by the corresponding coefficients 0.182 log $P_{water-hexane}$ + 0.155$V_{hydrophobic\ block}$. These coefficients were obtained using multiple regression analysis of the data with the NCSS 2000 program (copyright by Jerry Hintze). The results are presented in Figure 8. They distinctly break up into two nearly parallel separate lines, one of which refers to PEO-based copolymers, i.e., Pluronics and Brij surfactants ($R = 0.95$), and the other refers to polyglycerols ($R = 0.85$).

The relative contributions of the copolymer hydrophobicity and the volume of their hydrophobic block may be evaluated from the comparison of the coefficients by which they are multiplied in the correlation equation (*4*). The coefficient by which the volume is multiplied in the correlation equation (0.155, *T*-value 8.18) is close to that for log *P* (0.182, *T*-value 3.95), indicating a significant contribution of the size of the hydrophobic block into the disturbing ability of a copolymer. It should be emphasized that the calculated van der Waals volumes of the hydrophobic blocks represent the minimal space occupied by the polymer. This parameter does not take into account the conformation of the polymer in a lipid bilayer or its radius of gyration. The volume occupied by a real polymer coil in the membrane is determined by a number of factors including the persistence length of the polymer and its thermodynamic compatibility with the lipid surrounding. The accuracy of the estimation decreases for polymers with long hydrophobic blocks. However, even using the minimal estimates of $V_{hydrophobic\ block}$, we found that its contribution in the copolymer disturbing ability was comparable to that of log *P*.

The fact that Brij copolymers and Pluronics can be described by a common correlation indicates that the effects of these amphiphiles have common nature and they differ only quantitatively by the volume of their hydrophobic blocks. It may be concluded that the disturbances induced by these copolymers are mainly caused by insertion of their hydrophobic blocks into the membrane and are increased as the size of these blocks grows.

The intercept of the line corresponding to polyglycerols is higher than that for PEO-based surfactants by 0.7, indicating that polyglycerol corona makes additional (about 5-fold) contribution to the disturbance in the headgroup region of the membrane in comparison to the PEO hydrophilic block.

This additional disturbing ability of these copolymers not described by the volume of their hydrophobic block may be due to several reasons. One of the possible explanations for the differences in the flippase activity of Pluronics and PPO-PG copolymers may be based on the differences in the block architecture of these copolymers. Pluronics are three-block copolymers, and two modes of their incorporation into bilayer are discussed in the literature (*40*, *41*, *66*, *67*). First, the hydrophobic PPO block may adopt a coil conformation with both PEO blocks directed to the external side of the membrane (U-conformation). In the case where Pluronics are added to liposomes during their preparation, the polymer may incorporate into the membrane by spanning its hydrophobic block through the hydrophobic region, while PEO blocks direct to opposite sides of the membrane (spanned conformation). Kostarelos et al. hypothesized that the latter mode of incorporation seems to be less probable in the case of addition of the copolymer to preformed vesicles, as it requires dragging of the hydrophilic PEO blocks through the hydrophobic region of the membrane (*66*). At the same time, it is known that block architecture has a strong influence on the ability of PEO/PPO block copolymers to form micelles and incorporate into lipid bilayers. It is known that two-block copolymers are characterized by lower CMC values (*68*). It may be supposed that the larger disturbing ability of polyglycerol-based surfactants in comparison with Pluronics may be determined by their two-block architecture, which determines their enhanced ability to incorporate into a bilayer.

Another reason of additional disturbing ability of polyglycerol-based copolymers may arise from their bulky hydrophilic corona. Homopolymers of polyglycerol do not exhibit any ability to interact with bilayers (Table 2, series V). However, attachment of a hydrophobic anchor, hexadecyl or PPO block, provides them with the ability to incorporate into the fatty acid region of the bilayer. The hydroxyl groups of polyglycerols may form hydrogen bonds with the headgroups of lipids, resulting in the incorporation of the polymer into the polar region of the membrane. Large volume and stiffness of the hyperbranched hydrophilic block of these polymers would provoke disturbances in this region and thus cause additional disturbances in the membrane. If so, dendrimers and other hyperbranched polymers should also be effective in the disturbance of membrane structure. Indeed, polyamidoamine dendrimers were recently shown to facilitate DNA permeation into living cells more effectively than linear polycations (*69*).

The quantitative structure−activity relationship analysis of the copolymer effects on lipid bilayer described in the present paper permits formulation of requirements for the amphiphilic copolymer structure that ensure its ability to disturb lipid bilayers. First, the copolymer should have a large hydrophobic block. Obviously, its molecular weight should be comparable to or exceed that of lipid molecules. The hydrophobicity of this block should not be very high because this would impart the copolymer high tendency to micellization that was shown to be unfavorable for the copolymer chemosensitizing activity (*20*). In contrast to alkyl-containing surfactants, Pluronics ideally meet this requirement. The log *P* value for each PO repeat unit is only 0.012 ± 0.01, while for each methylene group it is about 0.55. Block copolymers with hydrocarbon chains whose size is comparable to that of polypropylene oxide in Pluronics would be water insoluble and, therefore, could not be used in biological systems. It may be hypothesized that copolymers consisting of other ether monomers with polar groups in their hydrophobic block would also meet this requirement.

The results presented rely essentially on homologous sets of amphiphiles with polyether-based hydrophilic heads and two types of hydrophobes. Certainly, this cannot encompass the complexity of lipid−surfactant mixtures, neither can it address the effects on biological membranes containing a significant amount of proteins. The commonality found between these molecules, however, is important and especially useful for developments in the areas specifically using these polyether-based compounds.

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