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Influence of the Headgroup on the Interaction of Poly(ethylene oxide)-Poly(propylene oxide) Block Copolymers with Lipid Bilayers

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Abstract

The lipid headgroup plays an important role in the association of polymers with lipid bilayer membranes. Herein, we report how a glycerol headgroup versus a choline headgroup affects the interaction of poly(ethylene oxide)-*b*-poly(propylene oxide) (PEO-PPO) block copolymers with lipid bilayer vesicles. Unilamellar vesicles composed of phosphatidylcholine and

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpcb.0c00553.

Polymer characterization; summary of polymer binding to 5 mM POPC liposomes in D_2O, to POPC liposomes in 150 mM NaCl D_2O solution, and to POPG liposomes in 150 mM NaCl D_2O solution; NMR decay curves of 0.2 mg/mL F127 in D_2O and in 150 mM NaCl D_2O solution; summary of polymer binding of 0.2 mg/mL F127 to 5 mM POPC/POPG liposomes at various POPG molar percentages in the lipid bilayer in 150 mM NaCl D_2O solution; echo decay curves of polymers in the presence of 5 mM POPC and POPG liposomes in 150 mM NaCl D_2O solution; echo decay curves of 0.2 mg/mL F127 in the presence of 5 mM POPC/POPG liposomes with various POPG molar percentage in 150 mM NaCl D_2O solution; and results and discussion of Raman spectroscopy (PDF)

The authors declare no competing financial interest.

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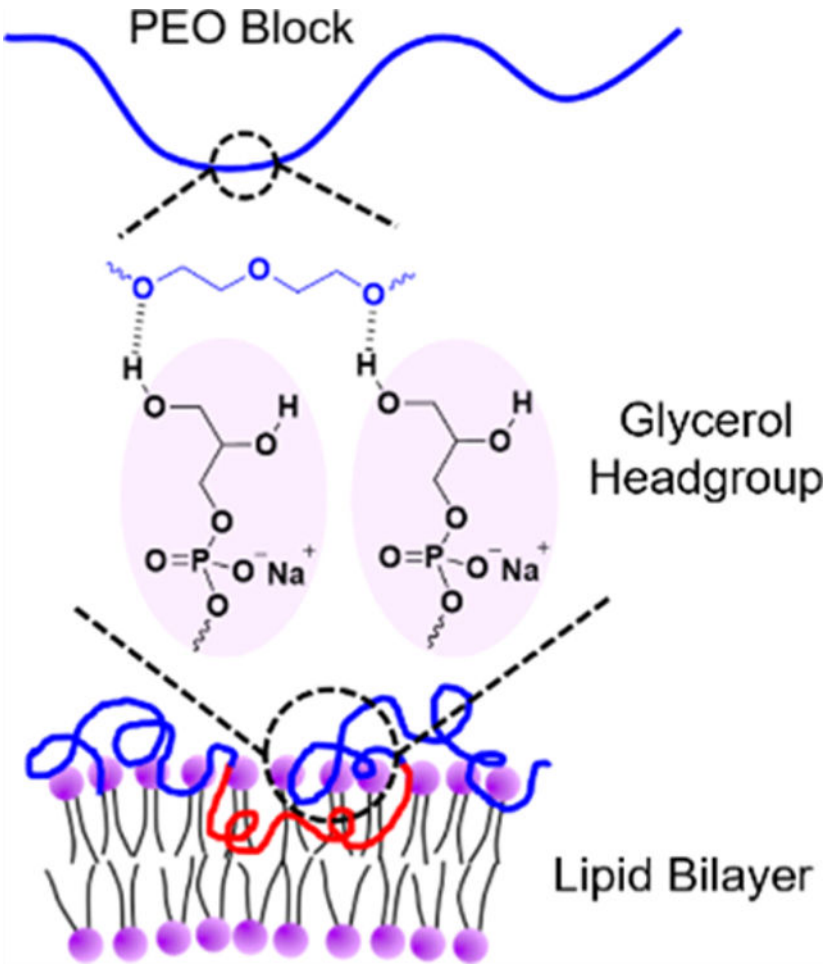
phosphatidylglycerol at various molar ratios were used as model membranes. The interactions between the block copolymers and lipid bilayers were quantified by pulsed-field gradient nuclear magnetic resonance (PFG-NMR) based on the distinctly different mobilities of free and bound polymers. All the investigated polymer species showed significantly higher binding with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) sodium salt (POPG) liposomes than with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) liposomes, indicating stronger association with the glycerol headgroup compared to the choline headgroup. This effect did not become significant until the composition of mixed POPC/POPG liposomes contained more than 20 mol %POPG. A plausible explanation for the enhanced polymer binding with POPG invokes the role of hydrogen bonding between the glycerol headgroup and the ether moieties of the polymers.

Graphical abstract



INTRODUCTION

The major components of plasma membranes are phospholipids which form the bilayer scaffold separating the internal content of living cells from the exterior matrix.[1,2]

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Phospholipids are amphiphilic molecules generally comprising two hydrocarbon chains and a polar headgroup consisting of a phosphate group and a functional group (e.g., choline, ethanolamine, serine, glycerol, and inositol).[2] Headgroup chemistry, including electrostatic characteristics, can strongly affect the phospholipid membrane dipole potential,[3] head-group conformations,[4] phase transitions,[5–7] raft formation,[8,9] and interactions with drug molecules, polymers, peptides, and proteins.[10–17] Triblock copolymers composed of poly(ethylene oxide) (PEO; A) and poly(propylene oxide) (PPO; B) in an A-B-A architecture are commercially known as Pluronics. They have shown great potential in cell membrane stabilization and permeabilization, depending on the hydrophilic-hydrophobic balance of the polymer.[18–26] Exploring the influence of the lipid headgroup composition on the interactions of PPO-PEO block copolymers with lipid bilayers will enable a more comprehensive understanding of the mechanism of polymer-membrane association from a membrane perspective and help improve the design of model membrane systems.

Herein, we report an investigation of the effect of lipid headgroup composition on polymer-membrane association, by quantifying the block copolymer binding to liposomes composed of phospholipids with two different types of headgroups. Narrowly distributed unilamellar liposomes were selected as model membranes, the preparation of which gives precise control over the lipid bilayer composition and size. Polymers involved in this study include commercial Pluronics and laboratory-synthesized diblock analogs, and their chemical structures are shown in Scheme 1a,b, respectively. Polymer binding to liposomes was determined by pulsed-field gradient nuclear magnetic resonance (PFG-NMR) based on the distinctly different diffusivities of free and bound polymers.[27,28] Lipid headgroup composition was manipulated by mixing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, Scheme 1c) with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) sodium salt (POPG; Scheme 1d) at various molar ratios to compare the effect of glycerol headgroups with choline headgroups. Pure POPC liposomes were used as controls because phosphatidylcholine is one of the major components found in eukaryotic cell membranes and is widely used as the basic constituent in model membranes.[29–32] POPG, on the other hand, is a relatively minor component in eukaryotic cell membranes and is mostly found in bacterial cell membranes.[33] Nevertheless, given that POPG has the same phase transition temperature and almost the same chemical structure except for the headgroup as POPC, it can be considered as a simple model to study how the presence of net charge and hydroxyl groups affect membrane interactions with the copolymers. It has been reported that the net charge and hydroxyl groups carried by the headgroup of POPG play important roles in headgroup-headgroup interactions, which could change the orientation and conformation of the headgroups, as well as the area per lipid.[34–37] The results obtained from this study demonstrate the critical role that lipid headgroup composition plays on polymer-membrane association and provides guidance to future studies using more complicated membrane compositions than a two-component membrane system.

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EXPERIMENTAL SECTION

Materials.

Pluronics F68, P84, P103, P105, F108, and F127 were provided by BASF. Ethylene oxide (EO, ≥99.5%), propylene oxide (PO, ≥99%), potassium, naphthalene, potassium *tert*-butoxide, *n*-butyllithium, 18-crown-6 ether, sodium chloride (NaCl, ≥99.0%), silver trifluoroacetate (AgTFA), and α-cyano-4-hydroxycinnamic acid (≥98%), were purchased from Sigma-Aldrich and used as received. POPC and POPG in chloroform were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. Deuterium oxide (D_2O, 99.9 atom % D) and chloroform-*d* ($CDCl_3$, 99.8 atom % D + 0.05% V/V TMS) were purchased from Cambridge Isotope Laboratories, Inc. Ultrapure water with a resistivity of 18.2 MΩ·cm was obtained from a Millipore Direct Q-3 water system (EMD Millipore, Billerica, MA).

Polymer Synthesis and Characterization.

Diblock polymers *t*PPO_{14}-PEO_{46} and *t*PPO_{29}-PEO_{68} were synthesized by anionic polymerization as described previously,[27] where the subscripts represent the average number of repeat units in the PPO and PEO blocks and "*t*" signifies a *tert*-butyl end group on the PPO block, which originates from the *tert*-butyl potassium initiator used for the PPO synthesis. The number-average molecular weight (M_n) and dispersity ($Đ$) of commercial triblock Pluronics and laboratory-synthesized diblocks were characterized by matrix-assisted laser desorption ionization (MALDI) mass spectroscopy (AB SCIEX TOF/TOF 5800). The weight fraction of PEO (w_{PEO}) was determined from the molar ratios of PPO to PEO measured by ^1H NMR spectroscopy. Details of the characterization procedures and the corresponding results were reported in our previous studies;[27,28] data relevant to the current study are summarized in Table 1.

Liposome Preparation.

Liposomes were prepared using an extrusion method described elsewhere.[27–29,38–40] Briefly, the bicomponent liposomes were prepared by mixing POPC with POPG in chloroform at a specific ratio, followed by evaporating the organic solvent under nitrogen. The resulting dry lipid thin films of various compositions were then hydrated with D_2O or 150 mM NaCl salt solution in D_2O at 37 °C for an hour. Vortex was applied every 5 min to facilitate the formation of large multilamellar liposomes. The final unilamellar liposome solution was obtained by repeated extrusion through a polycarbonate membrane with 100 nm diameter pores using an Avanti Mini-Extruder assembly. The hydrodynamic radius R_h and size dispersity of the liposomes were characterized by dynamic light scattering (DLS), as described previously.[28]

PFG-NMR Measurements.

Polymer-lipid bilayer association was evaluated by quantifying the polymer binding to the liposomes via PFG-NMR as described in our previous studies.[27,28] The experiments were conducted on a Bruker AVANCE III 500 MHz NMR spectrometer with a 5 mm broadband fluorine observe probe. A stimulated pulse sequence named "ledbpgp2s" (longitudinal eddy current delay experiment using bipolar gradients acquired in two dimensions) was applied,

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and a series of 1D ^{1}H spectra were recorded corresponding to increasing gradient strength G (from 2 to 95% of the maximum strength). The echo-attenuated intensity I observed from each 1D ^{1}H spectrum can be correlated to G based on the following equation

$$\ln\left(\frac{I}{I_0}\right) = -\gamma^2\delta^2G^2D\left(\Delta - \frac{\delta}{3}\right) \quad (1)$$

where I_0 is the intensity at zero gradient strength, γ is the gyromagnetic ratio of ^{1}H (42.6 MHz/T), δ is the length of the gradient pulse (δ = 5 ms), and Δ is the diffusion time (Δ = 300, 500, and 700 ms for polymer-liposome mixtures and Δ = 700 ms for pure polymer solutions). The diffusion coefficient D can be extracted from the slope of a linear fit to eq 1. Polymer diffusivity was obtained from the attenuated intensity of the PEO peak because of its strong signal at zero gradient strength. The decay curves of POPG liposomes were also obtained, using the ^{1}H signal of POPG alkyl chains because the ^{1}H signal of the headgroup can be barely observed.

In polymer-liposome mixtures, the binding of a fraction of the polymers to the liposomes gives rise to two distinct diffusivities of polymers, that is, free and bound polymers (D_{free} and D_{bound}, respectively). The diffusivity of the bound polymers is about an order of magnitude slower than that of the free polymers, assuming that the bound polymers have the same diffusivity as the liposomes and that the liposome diffusivity changes little after polymer binding. These two distinct polymer diffusivities result in a bi-exponential signal decay profile, which can be expressed by

$$\begin{aligned}\frac{I}{I_0} = {} & f_{bound}\exp\left(-\gamma^2\delta^2G^2D_{bound}\left(\Delta - \frac{\delta}{3}\right)\right) \\ & + (1 - f_{bound})\exp\left(-\gamma^2\delta^2G^2D_{free}\left(\Delta - \frac{\delta}{3}\right)\right)\end{aligned} \quad (2)$$

where f_{bound} is the molar percentage of the bound polymers. The fraction of bound polymers can be obtained from the biexponential fit to the signal decay profile of polymers in the presence of liposomes (see Figure 1 for an example). Note that f_{bound} is the only fitting parameter because D_{free} and D_{bound} can be directly extracted from the initial and final slopes of the decay profile, respectively. Additionally, as also reported in our previous studies, molecular exchange of block copolymer between the bound and free states is negligible based on the close overlap of the three decay profiles at different diffusion time points, as illustrated in Figure 1. All samples were measured at 27 °C with a polymer concentration of 0.2 or 1 mg/mL and a liposome concentration of 5 mM.

RESULTS AND DISCUSSION

Liposome Size Characterization.

The size and dispersity of the liposomes composed of POPC and POPG are summarized in Table 2. An NaCl solution at physiologic concentration (150 mM) was used as the solvent instead of pure D_2O in order to screen electrostatic interactions between anionic POPG lipids, as discussed in more detail below. The size of neat POPC liposomes remained the same after switching the solvent from D_2O to the salt solution. The liposome size decreases

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slightly as the molar percentage of POPG increases in the POPC/POPG lipid bilayer. However, the dispersity of the liposomes was not affected as the values of the reduced second cumulant μ_2/Γ^2 determined by DLS in all cases remained around 0.1. Note also that the liposome diffusivity remains 1 order of magnitude lower than that of the free polymers despite the minor reduction in the liposome size.

Effect of the Lipid Headgroup.

The headgroup effect was investigated by comparing block copolymer binding to POPC liposomes containing a choline group and POPG liposomes with a glycerol group. Salt solutions containing 150 mM NaCl in D_2O were used instead of pure D_2O to stabilize the liposomes by screening the electrostatic interactions between the negatively charged headgroups of POPG. Additional remarks about this effect can be found at the end of this subsection. Polymer binding to POPC liposomes in D_2O, to POPC liposomes in 150 mM NaCl, and to POPG liposomes in 150 mM NaCl is displayed in Figure 2a; the same data plotted on a logarithmic scale are shown in Figure 2b. Values of the binding percentage and related parameters used in fitting the data are summarized in Table S2. In order to assess whether adding salt makes a difference, the polymer binding to POPC liposomes in D_2O (gray bars) is compared to that in 150 mM NaCl (red bars). Some polymers show slightly more binding with POPC lipid bilayers in salt solution than in D_2O. The presence of salt can lead to a slight decrease in the CMC,[41–44] which may result in a stronger drive for the hydrophobic block of the polymers to form a micellar core or insert into the hydrophobic bilayer interior, rather than interacting with water molecules.[27] It is to be noted that the physiologic salt concentration used is not high enough to induce micellization, so the polymers still remain as free chains, despite the polymer concentration moving slightly closer to the CMC.[42–44] This point is supported by the linearity of the PFG-NMR profiles of free polymers in salt solution, as shown in Figure S1, which illustrates the echo decay curves of F127 in D_2O and in 150 mM NaCl. The two linear fits give virtually identical diffusivities, indicating that the presence of 150 mM NaCl does not induce polymer micellization. Therefore, changing the solvent from D_2O to 150 mM NaCl slightly increases the polymer binding to POPC lipid bilayers, but the effect is not significant, especially considering the error bars associated with the measurement of polymer binding. Here, we note that P103, P105, and F127 were measured at a lower concentration (i.e., 0.2 mg/mL instead of 1 mg/mL) because of lower CMCs compared to those of other polymers that have been evaluated.[45,46] The polymer concentration was kept below the CMC because the presence of micelles would result in a third value of polymer diffusivity (i.e., D_{micelle}), in addition to D_{free} and D_{bound}, and thereby complicate the data analysis.[47]

The effect of a choline versus a glycerol headgroup was probed by quantifying the polymer binding to POPC liposomes (red bars) and to POPG liposomes (blue bars) in 150 mM NaCl, as shown in Figure 2. NMR decay curves of P105 are shown in Figure 1a,b as representative examples of block copolymer binding to POPC and POPG liposomes in 150 mM NaCl, respectively. The polymer signal intensity in the second linear region is much higher in the presence of POPG liposomes versus POPC liposomes, indicating that P105 has more association with the POPG bilayer membrane. Similarly, all the other polymer species that were evaluated, except F68, show significantly higher binding with POPG than POPC, as the

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blue bars are much taller than the red bars in Figure 2. In the case of F68, both POPG and POPC lipid bilayers show very low binding and there is no statistically significant difference between the two lipids. This weak association between F68 and lipid bilayers can be attributed to the relatively short hydrophobic PPO blocks and large content of hydrophilic PEO blocks (ca. 80 wt %).[27] The NMR decay curves of all the other polymer species in addition to P105 are shown in Figure S2.

Next, we investigated how polymer binding varied with increasing POPG content in the lipid bilayer. F127 was selected as a representative polymer to illustrate binding as a function of the molar percentage of POPG in POPC/POPG bicomponent liposomes in 150 mM NaCl (Figure 3). Binding of F127 at each POPG molar percentage is summarized in Table S3, and the corresponding echo decay curves are shown in Figure S3. Figure 3 shows that the binding of F127 increases as the POPG content increases, with the headgroup effect becoming significant when the bicomponent lipid bilayer contains more than 20 mol % POPG. A possible reason that the glycerol group of POPG enhances polymer binding is hydrogen bonding. Earlier work in the literature demonstrated that POPG lipid bilayers can form strong intermolecular hydrogen bonds within the hydrophilic headgroup layer because of the presence of the hydroxyl groups.[34–36] It was also found that POPG can form hydrogen bonds with specific sites on some proteins, resulting in tight binding between the lipid headgroup and the protein.[48] Additionally, it was suggested that ether oxygen in PEO favors hydrogen bonding with the surrounding hydroxyl groups.[49–53] In this context, we suggest that the headgroup of POPG could form hydrogen bonds with PPO-PEO block polymers, where the hydroxyl groups function as hydrogen bonding donors, while the ether oxygen of the PEO or PPO blocks are potential hydrogen bonding acceptors. A schematic of this proposed mechanism is shown in Figure 4. The fact that the headgroup effect is not significant until the bilayer contains more than 20 mol % POPG could be because multiple hydrogen bonds need to be formed between one polymer molecule and two or more glycerol groups in order to produce enhanced polymer binding. We speculate that there is a threshold POPG content in the lipid bilayer, below which the lipid bilayer is unable to form enough hydrogen bonds with the polymers because of the lack of glycerol groups, and thereby shows little increase of polymer binding.

In an attempt to verify the proposed binding mechanism, Raman spectroscopy was employed to probe hydrogen bonding between the polymers and the POPG headgroups. However, because of the limitations on the signal resolution, we were unable to obtain definitive evidence of hydrogen bonding. These experiments and associated discussion are present in the Supporting Information.

Additionally, PFG-NMR was employed to show that using 150 mM NaCl instead of pure D_2O can stabilize the liposomes by screening the electrostatic interactions between the negatively charged headgroups of POPG. In pure D_2O (Figure 5a), the PFG-NMR decay profile for the POPG liposomes after mixing with polymers (red circles) decays much more rapidly than that of the neat liposomes (black squares), notwithstanding the noisy signal obtained with the neat liposomes. Faster diffusion and the nonlinear decay profile of the liposomes in the presence of polymers suggest that the liposomes are disrupted into smaller vesicles or aggregates with high dispersity. In the absence of salt, the repulsive interactions

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between the POPG headgroups become significant, which we speculate makes the liposomes unstable and vulnerable to disturbances induced by polymer association. We attribute the highly disperse diffusivity of the copolymers in the presence of liposomes, as evidenced by the nonlinearity of the PFG-NMR decay (blue triangles), to polymer association with the disrupted polydisperse liposomes. In contrast, as shown in Figure 5b, the bi-exponential character of the polymer decay curves in the presence of liposomes is recovered with a 150 mM NaCl solution. The plateau region (i.e., final slope of the polymer biexponential decay) indirectly indicates that the integrity of the liposome structure is retained. Additional indirect evidence of liposome integrity is that liposome diffusion, after mixing with polymers (red circles), as shown in Figure 5b, no longer resembles that in Figure 5a which is a much faster decay, although a quantitative diffusion coefficient could not be clearly resolved because of a noisy signal. We attribute the noisy signal of the liposomes to the broad NMR peak because of the poor solubility of the hydrocarbon chains of lipid in water, which significantly reduces the signal-to-noise ratio.

CONCLUSIONS

We have investigated the effect of the lipid headgroup on the interactions of PPO-PEO block copolymers with lipid bilayers. Polymer binding to POPC liposomes and to POPG liposomes dispersed in 150 mM NaCl solution was studied in order to ascertain the roles of glycerol versus choline headgroups. PFG-NMR was employed to determine polymer binding based on the quantitative measurement of the relative amounts of the free and bound polymer. All the selected polymer species showed higher binding to POPG liposomes than to POPC liposomes. Binding of F127 as a function of the mole fraction of POPG in POPC/POPG bicomponent liposomes revealed that the enhanced binding effect becomes substantial when the lipid bilayer contains more than 20 mol % POPG. Enhanced polymer association with POPG is tentatively attributed to hydrogen bonding between the glycerol groups in POPG and the ether oxygen atoms in the polymers. These findings suggest that other lipids containing headgroups relevant to mammalian cells, such as phosphatidylserine, may play a role in the physiological action of PPO-PEO block copolymers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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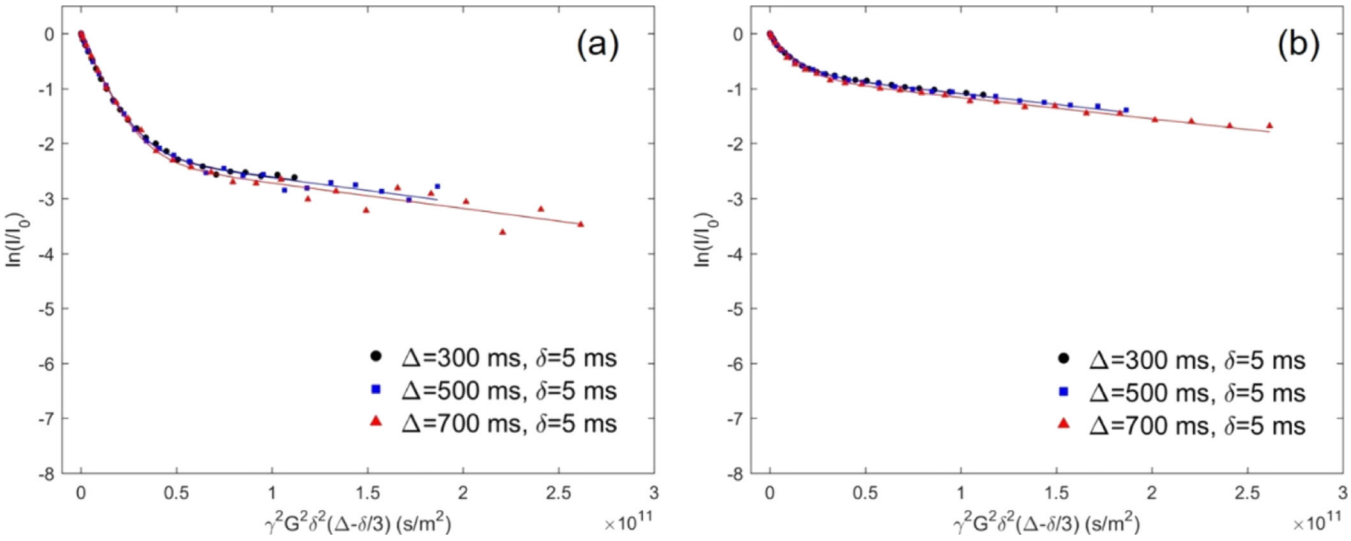

Figure 1.

Experimental and fitted echo decay curves of the protons from PEO for 0.2 mg/mL P105 in the presence of 5 mM liposome at 27 °C with Δ = 300, 500, 700 ms and with fixed δ = 5 ms. The value of f_{bound} in eq 2 was fit to the data. (a) POPC liposome and (b) POPG liposome solution in 150 mM NaCl.

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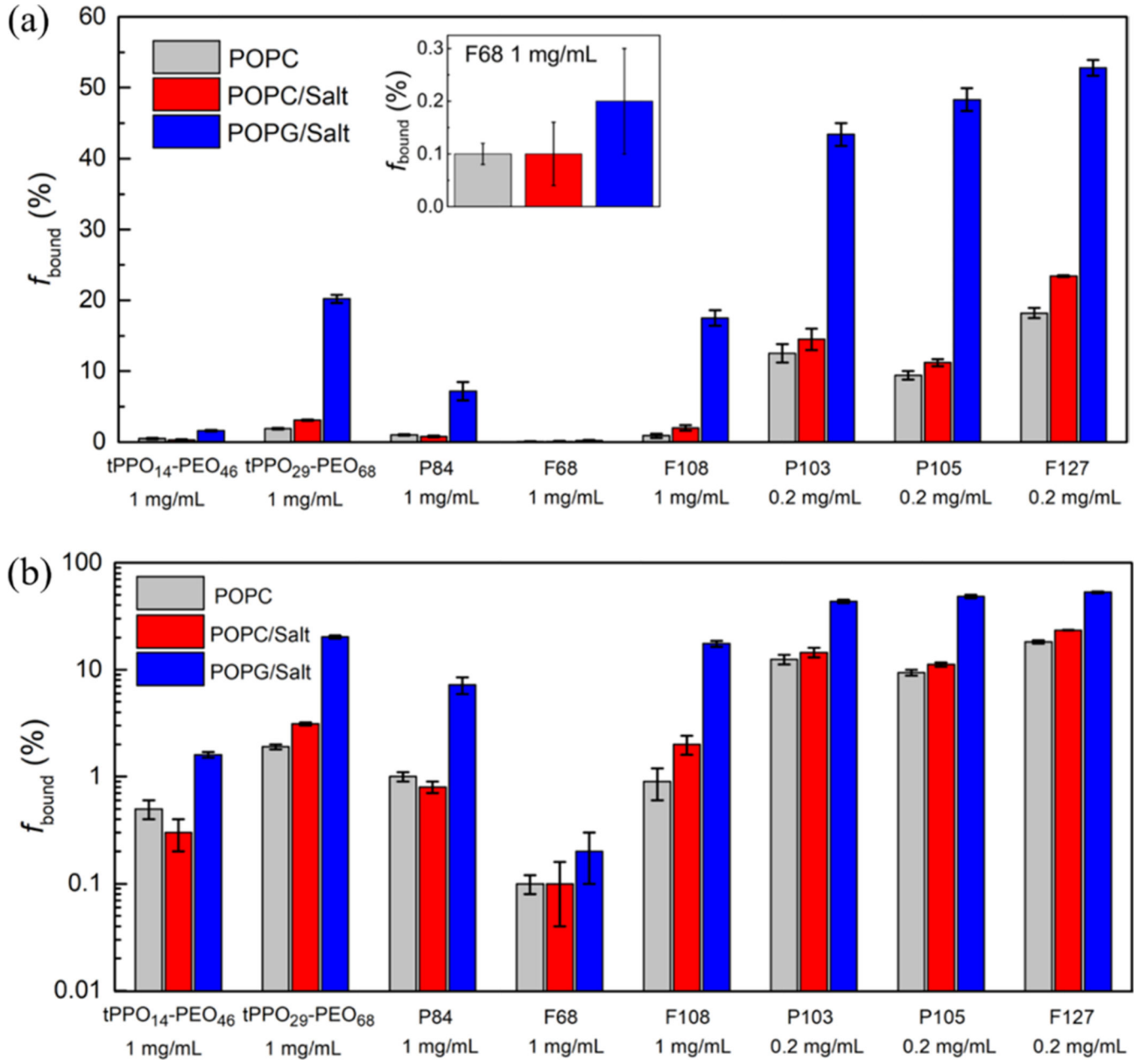

Figure 2.

(a) Polymer binding with POPC liposomes in D_2O (gray), with POPC liposomes in 150 mM NaCl D_2O solution (red), and with POPG liposomes in 150 mM NaCl D_2O solution (blue). The inset shows a zoom-in view of the binding of F68. (b) Data replotted on a logarithmic scale.

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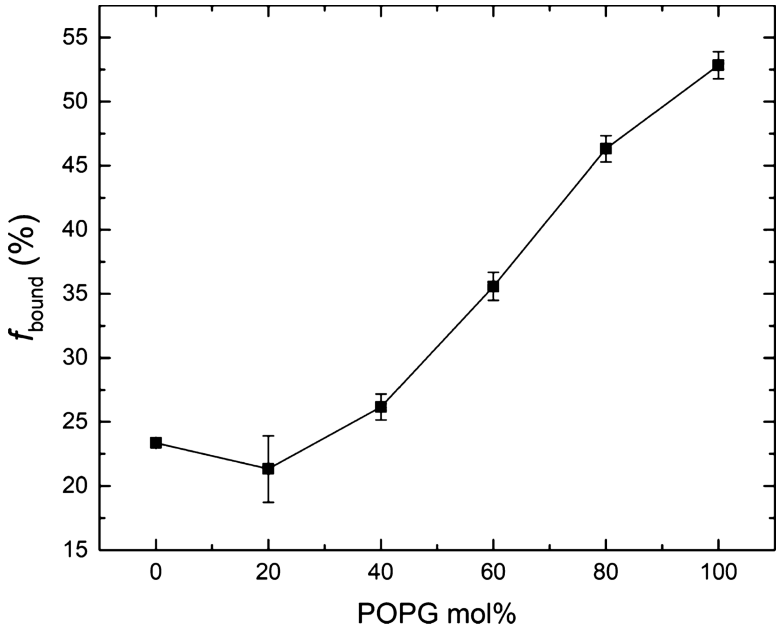

Figure 3.

Polymer binding percentage of 0.2 mg/mL F127 as a function of POPG molar percentage in the POPC/POPG lipid bilayer in 150 mM NaCl.

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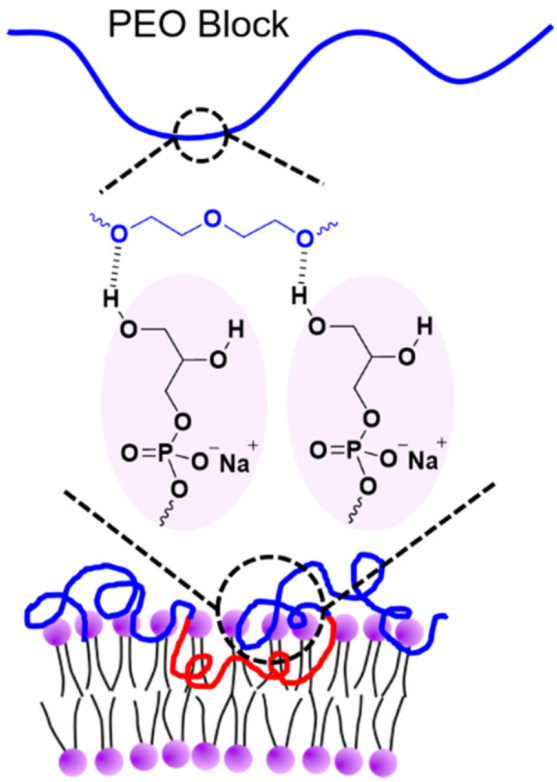

Figure 4.
Schematic of possible hydrogen bonding between a PEO block and glycerol headgroups of POPG in the lipid bilayers.

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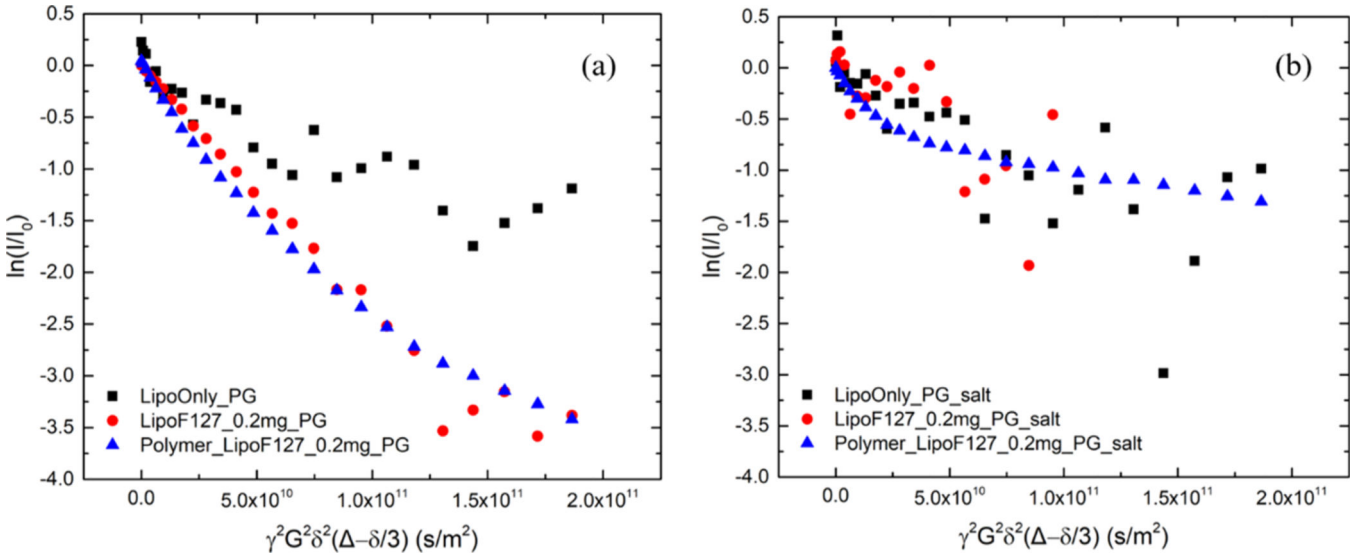

Figure 5.

Echo decay curves of 5 mM neat POPG liposomes (black squares), 5 mM POPG liposomes in the presence of 0.2 mg/mL F127 (red circles), and 0.2 mg/mL F127 in the presence of 5 mM POPG liposome (blue triangles) at 27 °C in (a) D_2O and (b) 150 mM NaCl D_2O solution. The decay curves of POPG liposomes were obtained using the ^1H signal of POPG alkyl chains because the ^1H signal of the headgroup is barely observable. The noisy signal of liposomes is attributed to the broad signal peak because of poor solubility of alkyl chains in water, which significantly decreases the signal-to-noise ratio.

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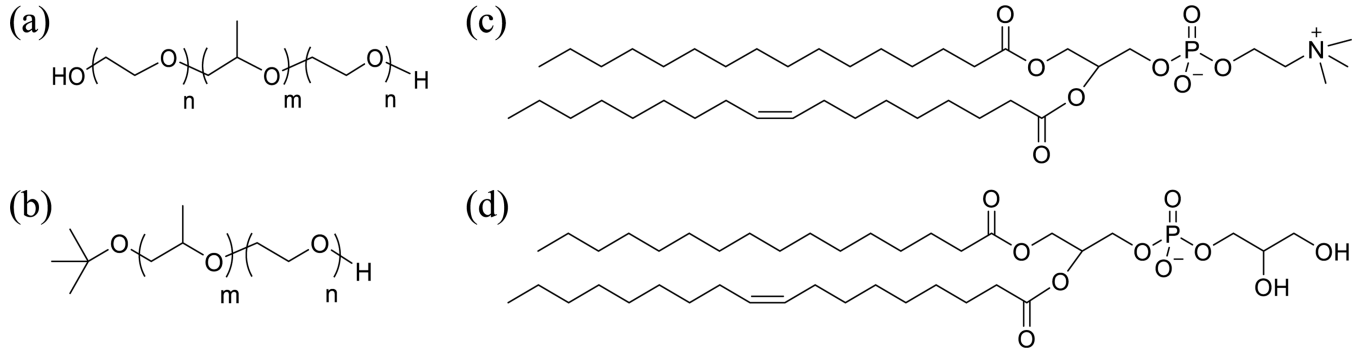

Scheme 1.

Chemical Structures of (a) Pluronic, (b) a Diblock Polymer with a *tert*-Butyl Endgroup on the PPO Block, (c) Phosphatidylcholine, and (d) Phosphatidylglycerol

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Table 1.

Polymer Characterization

	M_n[a] (g/mol)	$Đ$[a]	w_{PEO}[b]	N_{PO}[c]	N_{EO}[c]
F68	8200	1.01	0.81	27	75
F108	15,900	1.01	0.82	49	148
F127	13,200	1.01	0.72	64	108
P103	5200	1.02	0.33	60	19
P105	6500	1.02	0.50	56	37
P84	4200	1.03	0.42	42	20
tPPO_{14}-PEO_{46}	2900	1.03	0.70	14	46
tPPO_{29}-PEO_{68}	4700	1.04	0.63	29	68

[a] Determined by MALDI mass spectroscopy.

[b] Determined from molar ratios by ^{1}H NMR spectroscopy.

[c] Calculated from number-average molecular weight (M_n) and weight fraction of PEO (w_{PEO}). For triblock Pluronics, N_{EO} represents the number of repeat units of EO in one PEO block. Triblock N_{PO} and N_{EO} values differ slightly from those provided by the supplier, as reported in refs 27 and 28.

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Table 2.

Characterization of the Liposome Size by DLS

POPG molar percentage (%)	R_h [a] (nm)	μ_2/Γ^2 at 90°
0 [b]	70	0.04
0	70	0.08
20	66	0.08
40	66	0.11
60	61	0.07
80	62	0.08
100	62	0.10

[a] R_h measured by DLS has approximately 5% uncertainty.

[b] Measured in D_2O. All the other samples were measured in 150 mM NaCl solution in D_2O.