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Effect of Polyacrylic Acid on Phase State of Lipids and Diffusion in Lipid-Water System

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Abstract. Lipid vesicles interacting with polyanions are promising for controlled drug delivery. However, different aspects of the interaction of these polymers with lipids are far from complete understanding. In this work we studied the influence of polyacrylic acid (PAA) with small concentrations (1–4 mol%) on the change of the phase state, lateral diffusion of these lipids in lamellar phase and transmembrane water diffusion in macroscopically oriented bilayers of lipid-water systems formed by dimiristoylphosphatidylcholine (DMPC) and dioleoylphosphatidylcholine. Measurements were performed by ^{31}P nuclear magnetic resonance (NMR) spectroscopy and the ^{1}H NMR technique with a pulsed field gradient. It was found that the presence of PAA does not change the lamellar structure of the system. However, a part of bilayers changes their originally flat geometry and forms vesicles with a higher surface curvature. Macroscopic orientation of bilayers disappeared. For DMPC the presence of PAA leads to a shift of the gel-to-liquid crystalline phase-transition temperature to higher temperatures. An increase of PAA concentration leads to a monotonous decrease in the lateral diffusion coefficient of lipids that is caused, probably, by the ordering of lipids in bilayers. The transbilayer diffusion coefficient of water increases in the presence of PAA, but it depends slightly on the PAA concentration. An increase of pH leads to a change of the lipid lateral and transbilayer diffusion coefficients to the values typical for a pure bilayer.

1 Introduction

Interest in the application of water-soluble polymers in biotechnology and medicine is growing rapidly [1]. It was shown for various model systems that different classes of polymers interact with lipid membranes in a different way and, as a rule, essentially change the structure and properties of membranes [2–6], including the membrane fusion, selective membrane permeabilization, and formation of lateral domains. So, a vesicle with covalently attached, adherent or entrapped polymer can be used as a novel responsive delivery system, particularly to deliver deoxyribonucleic acid and drugs. For the practical application of the polymers for drug delivery it is important to know, firstly, the details of changes produced by the molecular interaction of the polymer with lipids, and the ef-

fects of this interaction, particularly, on the phase state, local and translational mobility and permeability. Among a diversity of lipid-polymer compositions, lipid vesicles prepared with polyanions are very promising for controlled drug delivery [1]. The usage of such polymers is very attractive due to their relatively low prices and variety of properties. However, different aspects of the interaction of polyanions with lipids are far from complete understanding.

The usage of weak polyacrylic acids (PAAs) can be rather interesting because of their secondary structure sensitive to pH. It was shown that hydrophobic PAAs (poly-α-ethylacrylic and poly-α-propylacrylic) completely disrupt membranes of liposomes, leading to the formation of hydrophilic pores and even mixed micelles with lipid molecules at pH 6.5 [7, 8]. At essentially low pH (<5.0), hydrophilic PAA also can form hydrophilic pores in membranes of liposomes [2]. Many aspects of the interaction of PAA with bilayers are not understood, particularly, the effect of polyanions on the phase state of bilayers and mobility of lipid and water molecules was not sufficiently studied till now.

In this work, we applied ^{31}P nuclear magnetic resonance (NMR) spectroscopy and ^{1}H NMR with pulsed magnetic field gradient (PFG) for studying the system state (phase state and distortion of bilayers geometry from the plane one), the lateral diffusion of lipids and transbilayer diffusion of water in the system formed by synthetic phosphatidylcholines homogeneous in composition in the presence of PAA.

2 Experimental

2.1 Materials and Sample Preparation

The lipid compounds used were 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), both from Avanti Polar Lipids (Birmingham, AL). Molecular structures of the lipids are presented in Fig. 1. These structures differ in the length of hydrocarbon chains and the presence of a double bond in both chains of DOPC.

PAA was obtained from Sigma (St. Louis, MO). The molecular weight was 5000 and the profile length was 17 nm.

For ^{31}P NMR, we used water suspension of vesicles of lipids. The mixture of lipid and PAA was dissolved in ethanol, dried in N_2 gas flow and hydrated by addition of 95 wt% of water with careful vortexing. To make the sample more homogeneous it was frozen and thawed 5 times. One day before measurements the sample was kept in the dark at room temperature.

For preparation of oriented samples the mixture of lipid and PAA was prepared by dissolving in a mixture of methanol and propanol (1:4 by volume) at a concentration of 15 mg/ml. 25 μl of the solution was put onto each of 60 glass plates (5 by 14 mm). The solvent was evaporated at atmospheric pressure and then under vacuum at 30 °C for 6 h. Glass plates were stacked and placed in a humid atmosphere in order to form hydrated bilayers and additional water (either MQ or D_2O, pH 6.5) was added through filter paper. The sample was

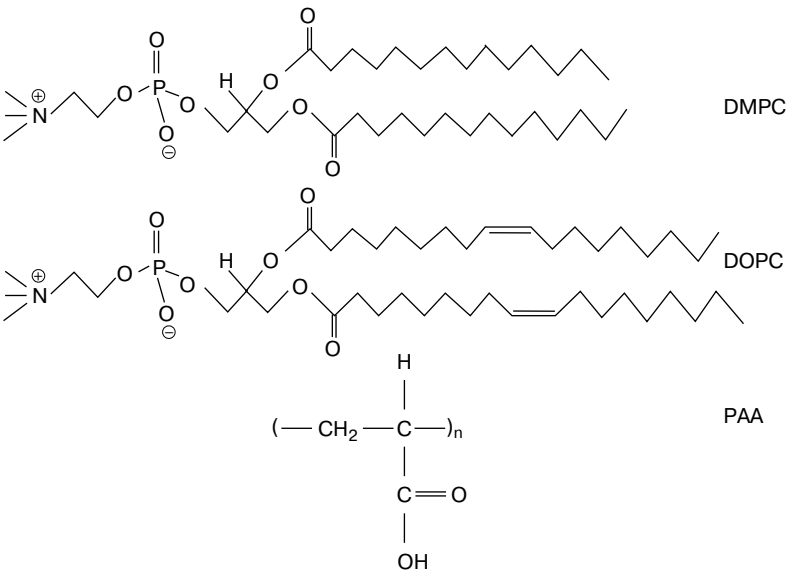

Fig. 1. Structures of molecules of lipids and PAA.

sealed before measurements. Crossed polaroids were used to check the orientation of the hydrated bilayers. More details about the sample preparation can be found in ref. 9.

2.2 NMR: Techniques and Measurements

^{31}P NMR spectra were obtained on a Unity-300 spectrometer of Varian Associates Inc. at a frequency of 121 MHz. Free induction decays after 90° radiofrequency pulses were used and the repetition delay was 1–2 s. The spectra width was 100 kHz.

^{1}H PFG NMR diffusion measurements were performed at 60 MHz on a home-built NMR diffusometer with a maximum magnetic field gradient amplitude of 30 T/m. By rotating the stack of glass plates the bilayer normal can be oriented at the so-called magic angle of 54.7° with respect to the main magnetic field. This causes the dipolar interactions to vanish with a significant reduction of the line widths [10]. A stimulated echo pulse sequence was used. Diffusion decays $A(k)$ were used for analyses, where $A(k)$ were either echo amplitudes or integral values of the corresponding spectra, $k = \gamma^2\delta^2g^2t_d$, where γ is the gyromagnetic ratio of proton, δ is the duration and g is the amplitude of the field gradient pulses, $t_d = (\Delta - \delta/3)$ is the diffusion time, Δ is the time interval between the first and the second gradient pulses in the pulse sequence. The value varied under measurements was either g or δ, while the other parameters were kept constant. Under these conditions in a one-component bulk liquid, $A(k)$ is

$$A(k) = A(0)\exp(-\gamma^2\delta^2 g^2 t_d D), \quad (1)$$

where $A(0)$ is the value of $A(k)$ without a pulsed gradient, D is the measured self-diffusion coefficient. The diffusion time was varied from 7 to 250 ms. The main magnetic field and PFG had the same direction in all experiments. Lateral diffusion coefficient D_L was calculated by multiplying the measured self-diffusion coefficient D_s by a factor of 1.5 [9]. The relative error in the self-diffusion coefficient determination was less than 10%.

To measure the transbilayer water diffusion, the normal to bilayers was oriented along the PFG. In this case no signal from lipids was present in the proton NMR spectra and only signal from water protons was observed [10]. The measured water diffusion coefficient through bilayers depends on the diffusion coefficient of bulk water and the permeability of bilayers [11, 12].

The temperature of measurements was in the interval of 25–60 °C. The precision of maintenance and control of temperature was better than 1 °C.

3 Experimental Results

3.1 ^{31}P NMR Spectroscopy

^{31}P NMR spectra for DMPC and DOPC at different PAA concentrations are presented in Fig. 2a and b, respectively. At PAA concentrations varying from 0 to 0.5 mol%, the spectrum shape is asymmetric (Fig. 2a) with the main peak being in low field and the shoulder in high field; the chemical shift anisotropy is about 50 ppm. This shape of the spectra corresponds to the lamellar phase of the phospholipid–water system [13]. The shape of the spectrum changes in the PAA concentration range of 0.5–1 mol%. The analysis revealed a new additional symmetric peak appearing in the central part of the plot. This new spectrum is typical for the isotropic rotation of molecules in small-size lipid aggregates (vesicles or micelles). It is known that systems of phospholipids with PAA do not form micelles [8], so most probably in the presence of PAA a part of DMPC bilayers separates from initially plain bilayers and forms small vesicles typical for pure lipid.

Spectra for the DOPC-PAA system in the concentration interval of 0–7.5 mol% are shown in Fig. 2b. In this case the spectrum for the lamellar phase DOPC is not changed in the presence of PAA.

Thus, ^{31}P spectra demonstrate that in the concentration interval shown in Fig. 2 the presence of PAA does not change the lamellar phase of phosphatidylcholines but in DMPC-PAA a part of the system forms small vesicles.

3.2 Gel–Liquid Crystal Phase Transition

Temperature T_m of the gel–liquid crystal phase transition for DOPC bilayers is about −20 °C, and for DMPC it is about 24 °C [14]. The gel phase of DOPC

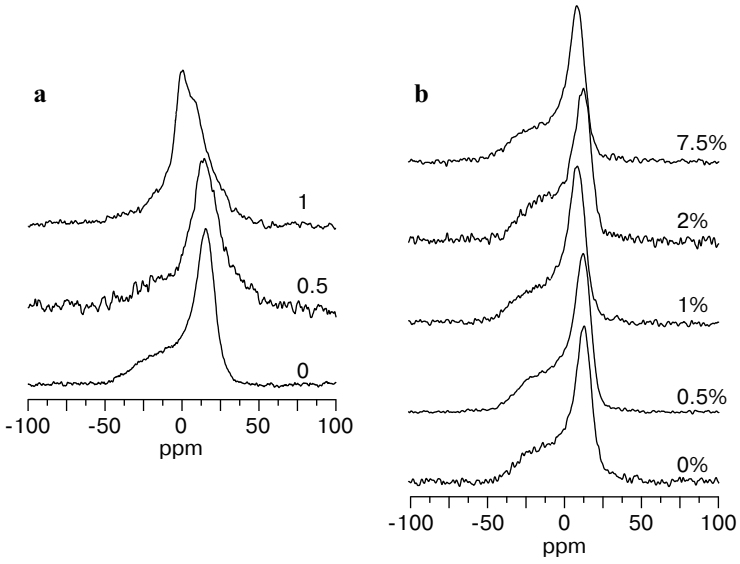

Fig. 2. ^{31}P NMR spectra for DMPC (**a**) and DOPC bilayers (**b**) with indicated PAA concentrations in mol%. $T = 30$ °C.

is out of the temperature range of our study and does not influence the possibility to measure the lateral diffusion of lipids. However, for DMPC bilayers oriented at the magic angle, the echo signal from lipids was not observed below 24 °C because of the low rotational mobility of lipids in the gel phase [15], but the signal appeared after heating to 25 °C due to the phase transition of the bilayer into the liquid crystalline state. In the presence of PAA at concentrations of 0.27 and 0.8 mol%, no echo signals were observed in DMPC bilayers. Therefore, measurements were performed at temperature of 30 °C and higher. Further increase of the PAA concentration to 2 mol% leads to the disappearance of the echo signal at 30 °C; however, at 35 °C the signal was observed again and the shape of the diffusion decay (DD) became nonexponential. The cause of this nonexponential shape will be discussed below by the example of the DOPC-PAA system. The appearance of the echo signal at a higher temperature means that the presence of PAA in the system leads to a shift of the phase-transition temperature (gel–liquid crystal) to a higher temperature range. For DMPC-PAA bilayers, this shift is proportional to the PAA concentration in the studied range of PAA concentrations, 0.27–2 mol%.

3.3 Lateral Diffusion of Lipids

It is known that in the PFG NMR experiment the echo decay is determined by molecular translational displacements, so in homogeneous system without any mechanical obstacles it can be described by Eq. (1). Previous research [15] and

our current measurements showed that in pure bilayers of both lipids, DMPC and DOPC, oriented at magic angle DDs are exponential (Eq. (1)). For bilayers of these lipids with PAA, DDs were also exponential except the cases specified below. This means that lateral diffusion in each sample in the studied diffusion range can be described by the unique lateral diffusion coefficient of lipids, D_L. The temperature dependence of D_L for pure DMPC at 30–60 °C is close to that of the Arrhenius type (Fig. 3) and deviates from linear when T is close to the phase-transition temperature ($T_m \sim 24$ °C). This deflection can be explained by the presence of gel-phase domains in the liquid-crystalline bilayers of DMPC (it was also confirmed by differential scanning calorimetry curves). Indeed, gel-phase domains play the role of obstructions for the lipid molecule diffusion in the liquid crystalline phase [16]. The temperature dependence of D_L for DOPC in the studied temperature interval is close to that of the Arrhenius type because $T_m = -20$ °C for DOPC is far from the temperature interval of the study.

DDs for DOPC-PAA bilayers are shown in Fig. 4. DDs for bilayers with 1 and 2 mol% of PAA remain exponential, similarly to pure DOPC. However the slope of DDs, which is determined by the lateral diffusion coefficient of DOPC, decreases when the fraction of PAA increases. This fact demonstrates the decrease of the lateral diffusion coefficient of DOPC with increasing PAA concentration.

In the system with 4 mol% of PAA the dependence of the signal echo amplitude on the angle in the vicinity of the magic angle was weaker and DD was biexponential (solid line in Fig. 4). In the initial part of the decay we can see a signal from water which is characterized by the diffusion coefficient $3.5 \cdot 10^{-11}$ m^2/s that is less than the interbilayer water diffusion coefficient of about $2 \cdot 10^{-10}$ m^2/s [12]. After Fourier transformation of the echo for the sample oriented at magic angle and angles different from the magic angle we obtained a broadened spectrum with lines of water, choline and acyl protons of DOPC. This is

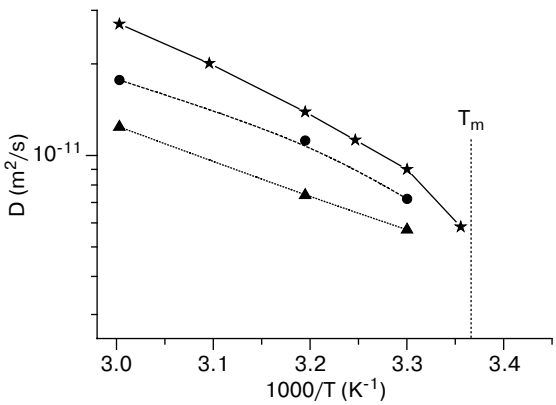

Fig. 3. Temperature dependences of the lateral diffusion coefficient in DMPC bilayers containing 0 (stars), 0.27 (circles) and 0.8 mol% of PAA (triangles). T_m is the temperature of the gel-to-liquid crystal phase transition for pure DMPC bilayers.

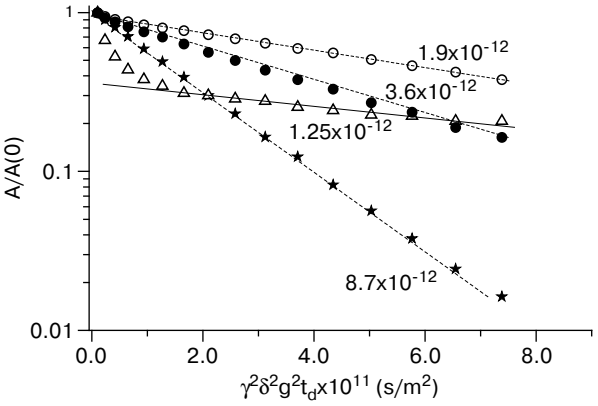

Fig. 4. DDs of stimulated spin echo for the lateral diffusion in DOPC bilayers with the normal oriented at the magic angle to the main magnetic field. PAA concentrations are 0 (stars), 1 (solid circles), 2 (triangles) and 4 mol% (open circles). $T = 25$ °C. DDs for lipids are approximated by lines with indicated lateral diffusion coefficients in square meters per second.

in agreement with ^{31}P NMR data (Fig. 2b) according to which lipids form a lamellar phase at this concentration of PAA in the system and the geometry of a part of bilayers can be deflected from the originally plain one because the system forms vesicles alongside the flat bilayers. Diffusion of water inside vesicles is additionally restricted and water molecules provide a new diffusion component with the apparent diffusion coefficient of $3.5 \cdot 10^{-11}$ m^2/s. For the DMPC-PAA system, we observed the same behavior as for DOPC-PAA, but the nonexponentiality of DD which we related to the deflection from flat geometry for a part of bilayers was observed at 2 mol% of PAA.

The temperature dependences of D_L in samples with PAA were also of the Arrhenius type as in the samples without PAA. The typical picture is shown in Fig. 3 for the DMPC-PAA system.

3.4 Transbilayer Water Diffusion

The ^{1}H NMR spectrum for flat bilayers oriented at angles different from the magic angle, particularly along the normal to the constant magnetic field and PFG direction, contains a signal only from water [11]. Figure 5 shows curves of DDs in the DOPC-PAA system that are typical for all measurements of this type. DDs for water transverse to pure DOPC (solid stars in Fig. 5) looks similar to other one-component bilayers [11]: it is the sum of two parts corresponding to the diffusion of water through bilayers (dashed line, $D_\perp \sim 1 \cdot 10^{-12}$ m^2/s) and diffusion through defects of the bilayer cracks (initial parts of decays with diffusion coefficients in the range of about $10^{-10}-10^{-11}$ m^2/s, shown by arrows). For comparison, DD for water along bilayers (open stars in Fig. 5, $D_\parallel \sim 1.4\times 10^{-11}$ m^2/s) which was obtained for the normal to bilayers oriented normal to

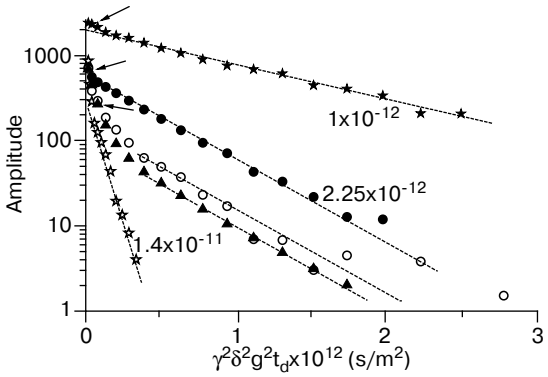

Fig. 5. DDs of stimulated spin echo for lateral diffusion in DOPC bilayers with the normal oriented at 0° (solid stars) and 90° (open stars) to the main magnetic field. DDs for DOPC bilayers with 1 (solid circles), 2 (open circles) and 4 mol% of PAA (triangles). Diffusion time is 500 ms. $T = 25$ °C. Dashed lines show slopes corresponding to the transbilayer water diffusion. Arrows show parts of the decays related to the bilayer's defects.

the PFG is also shown. As we can see in Fig. 5 (triangles and open and solid circles), the presence of PAA changes DDs. The measured value of the transbilayer diffusion coefficient increases from $1 \cdot 10^{-12}$ to about $2.25 \cdot 10^{-12}$ m^2/s; it is almost independent of the PAA concentration. When PAA concentration increases, the fraction of the component of DD corresponding to the transbilayer diffusion decreases, whereas the fraction of defects of bilayers increases.

3.5 pH Influences on PAA–Bilayer Interaction

It is evident from our experimental results (Figs. 3–5) that PAA interacts with lipids reducing their lateral diffusion coefficient and increasing the transbilayer diffusion of water. It is known that PAA interacts with bilayers at pH values below 5.5 [2]. Features of our flat-oriented samples do not allow the pH measurements. Therefore, we had measured pH in water dispersions of DOPC-PAA and obtained a value of about 6.4.

The interaction of PAA with a membrane weakened as the increase of pH exceeded 7.0 [2]. To check the effect qualitatively we added a drop of 0.1 M NaOH solution on the back of the DOPC with 1 mol% of PAA bilayer stack. Afterwards the sample was sealed and kept for two days at room temperature for equilibration. For comparison, the same procedure was performed with a sample of pure DOPC bilayers. In the case of pure DOPC bilayers the introduction of NaOH did not influence the lateral lipid diffusion and transbilayer water diffusion. The results for the DOPC-PAA system are shown in Fig. 6. The addition of NaOH increases the lateral diffusion coefficient of lipids from $3.6 \cdot 10^{-12}$ to $5.1 \cdot 10^{-12}$ m^2/s in good agreement with the lateral diffusion coefficient of pure DOPC bilayers (Fig. 6a). The transbilayer water diffusion coefficient de-

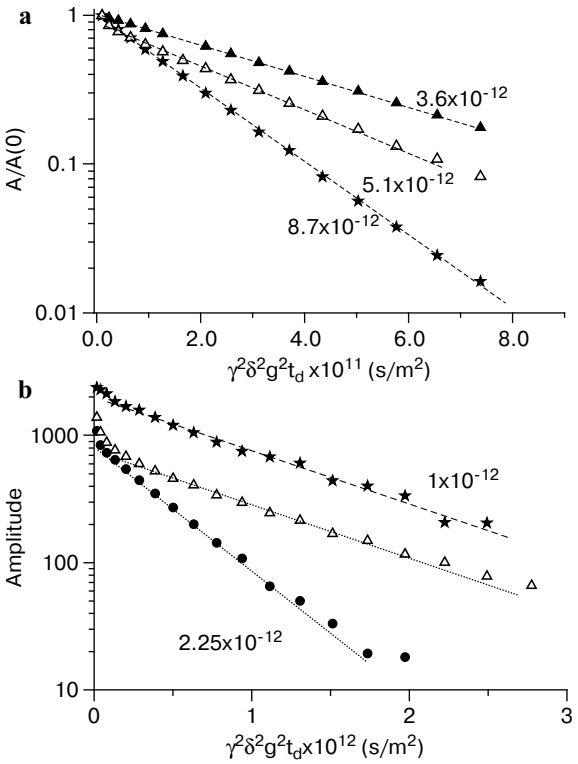

Fig. 6. a DDs of stimulated spin echo for the lateral diffusion in DOPC bilayers with the normal oriented at magic angle to the main magnetic field. Stars, pure DOPC bilayers; solid triangles, DOPC with 1 mol% of PAA; open triangles, DOPC with 1 mol% of PAA after addition of NaOH. $T = 25$ °C. **b** DDs of stimulated spin echo for the lateral diffusion in DOPC bilayers with the normal oriented at 0° to the main magnetic field. Stars, pure bilayers; circles, DOPC with 1 mol% of PAA; triangles, DOPC with 1 mol% of PAA after addition of NaOH. Diffusion time is 500 ms. $T = 25$ °C. Dashed line shows the slope corresponding to the transbilayer diffusion of water through the pure DOPC bilayer. Dotted lines show the slope corresponding to the transbilayer water diffusion through bilayers of DOPC with 1 mol% of PAA.

creases from $2.5 \cdot 10^{-12}$ m^2/s to the value typical for bilayers without PAA, $1 \cdot 10^{-12}$ m^2/s (Fig. 6b).

4 Discussion

It is seen from ^{31}P NMR spectra that at neutral pH, lamellar phases of DOPC and DMPC do not change in the presence of PAA. The presence of PAA in DMPC bilayers leads to an increase of the temperature of the phase transition from gel to liquid crystal and to a decrease of the lateral diffusion coefficient of lipids. Moreover, at concentrations of about 2 mol% of PAA, a part of the

system forms vesicles. It should be noted that twisting of phosphatidylcholine bilayers in the presence of PAA was observed earlier for large unilamellar vesicles [17]. All mentioned experimental facts confirm the interaction of PAA with the membrane.

Another group of facts, the decrease of the membrane permeability and the stronger effect of PAA on the geometry of DMPC bilayers (saturated chains) in comparison with DOPC (monounsaturated chains), shows that PAA strongly interacts with the saturated chain bilayer. It is known that the weak acidity PAA can bound with lipid bilayers of zwitterionic phosphatidylcholine and this interaction is complex in its nature [2]. The mechanism of the interaction (deepening) of PAA with bilayers is not known. In ref. 7, there is a description of embedding of different polyanion, polyethylacrylic acid into the DOPC bilayers. It was shown that the effect of polyacid on the permeability of charged and zwitterionic bilayers is similar, testifying the minor role of electrostatic interactions with polar heads of the lipid molecules. It was also shown that the permeability of DOPC bilayers grows linearly with the polymer concentration and exponentially with the decrease of pH. Thus, the formation of a part of the bilayers with relatively high permeability (channel) in the presence of the PAA is not a cooperative process.

Effects which we observed under the PAA–bilayer interaction partially coincide with effects observed earlier in the presence of PAA and other polyanions with phosphatidylcholine bilayers, particularly the increase of temperature of gel–liquid crystal transition [8] and the increase of bilayer permeability [1, 7, 8]. Our studies allowed us to reveal some features of the system behavior which are related to the so-called deepening of PAA molecule into membranes. The exponential shape of transbilayer water DDs (Fig. 5, curves 2–4) shows that bilayers are homogeneously permeable and PAA molecules do not form extensive areas of multibilayers with permeability different from surroundings. The resulting bilayer permeability is an average of the permeability of areas with channels and passive permeability of pure bilayer parts.

The next effect is the only weak dependence of transbilayer water diffusion on the PAA concentration (Fig. 5). As the concentration of PAA increases the fraction of bilayer defects also increases (Fig. 5). These defects are located in the areas of contacts of multilamellar vesicles [11]. Thus in the presence of PAA, the system breaks into smaller multilamellar vesicles, so more water becomes involved in the space between them. The weak dependence of the transbilayer water diffusion coefficient on the PAA concentration may be a result of the simultaneous manifestation of two effects. The increase of permeability of the membrane due to the growth of the number of channels formed by PAA is compensated by the decrease of the surrounding permeability due to the ordering of lipid carbohydrate tails in the presence of deepened PAA molecules.

A number of effects of PAA on bilayers of phosphatidylcholines was found in the current study for the first time. So the interaction of PAA with membrane leads to the decrease of lipids lateral diffusion (Figs. 3 and 4). It was known that the interaction of polyions with bilayers can lead to the lateral phase decomposition in liquid crystalline phase and the formation of lateral domains of

lipid molecules more strongly interacting with polyion [18]. In our case (polyanion PAA), monoexponential DDs for the lateral diffusion of lipids mean that either lateral domains are not formed or these domains are very small (less than 1 mm) and lipid molecules easily exchange between domains and surroundings.

In the case when foreign molecule (for example, cholesterol) is embedded into bilayers, the lateral diffusion coefficient typically decreases [15]. The effect is accompanied by a decrease of the membrane passive water permeability [11] because both these effects are caused by the free molecular volume in bilayers. However in our case, the lateral diffusion of lipids and bilayer permeability are controlled evidently by two different mechanisms. Water permeability is increased because of the presence of channels which are formed due to the deepening of PAA molecules, whereas hydrocarbon chains of lipids are ordered because of the interaction with rigid PAA molecules that leads to the decreased D_L for lipids.

5 Conclusions

Our study has demonstrated the possibility of a complementary study by ^{31}P NMR and NMR diffusometry of the pH-controlled interaction of polyions with lipid bilayers by the example of PAA and two phosphatidylcholines. Small concentrations of PAA do not influence the system lamellar phase organization, however, the phase transition temperature from gel to liquid crystal increases and a part of bilayers changes its geometry from plane to curved. The lateral diffusion of lipid decreases and the bilayer permeability increases. The interaction of PAA with lipids is controlled by pH that can be observed through two effects: the lipid lateral diffusion and water diffusion through the bilayers.

The results of this study can be useful to understand the mechanisms and effects accompanying the embedding of polyanions into biomembrane. It can be used in studying the interaction mechanisms of charged molecules with biomembranes and for the development of systems for controlled drug delivery.

Acknowledgments

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