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Interaction of a Poly(acrylic acid) Oligomer with Dimyristoylphosphatidylcholine Bilayers

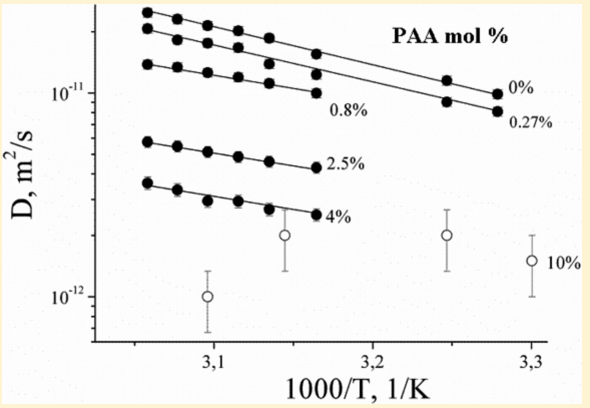
Andrey Filippov,*[,†,‡] Bulat Munavirov,[†] Tobias Sparrman,[§] Valentina Ishmuhametova,[†] Maya Rudakova,[†] Prashant Shriram,[§] and Staffan Tavelin[§]

[†]Kazan (Volga Region) Federal University, 18 Kremlevskaya St., Kazan 420008, Republic of Tatarstan, Russian Federation

[‡]Luleå University of Technology, Luleå SE-91187, Sweden

[§]Umeå University, SE-901 87 Umeå, Sweden

ABSTRACT: We studied the influence of 5 kDa poly(acrylic acid) (PAA) on the phase state, thermal properties, and lateral diffusion in bilayered systems of dimyristoylphosphatidylcholine (DMPC) using ^{31}P NMR spectroscopy, differential scanning calorimetry (DSC), ^{1}H NMR with a pulsed field gradient, and ^{1}H nuclear Overhauser enhancement spectroscopy (NOESY). The presence of PAA does not change the lamellar structure of the system. ^{1}H MAS NOESY cross-peaks observed for the interaction between lipid headgroups and polyion protons demonstrated only surface PAA−biomembrane interaction. Small concentrations of PAA (up to ∼4 mol %) lead to the appearance of a new lateral phase with a higher main transition temperature, a lower cooperativity, and a lower enthalpy of transition. Higher concentrations lead to the disappearance of measurable thermal effects. The lateral diffusion coefficient of DMPC and the apparent activation energy of diffusion gradually decreased at PAA concentrations up to around 4 mol %. The observed effects were explained by the formation of at least two types of PAA−DMPC lateral complexes as has been described earlier (Fujiwara, M.; Grubbs, R. H.; Baldeschwieler, J. D. *J. Colloid Interface Sci.*, **1997**, *185*, 210). The first one is characterized by a stoichiometry of around 28 lipids per polymer, which corresponds to the adsorption of the entire PAA molecule onto the membrane. Lipid molecules of the complex are exchanged with the "pure" lipid bilayer, with the lifetime of the complex being less than 0.1 s. The second type of DMPC−PAA complex is characterized by a stoichiometry of 6 to 7 lipids per polymer and contains PAA molecules that are only partially adsorbed onto the membrane. A decrease in the DMPC diffusion coefficient and activation energy for diffusion in the presence of PAA was explained by the formation of a new cooperative unit for diffusion, which contains the PAA molecule and several molecules of lipids.



INTRODUCTION

The main purpose of targeted drug delivery is to lower side effects (lowering the dose, coating the drug, etc.) and increase the chances of delivering the drug close to the site of infection. Thin-walled lipid-based systems are of special interest because lipids are known to be biodegradable,[1,2] tissue compatible,[2] may form different structures in an aqueous environment (which is typical of living organisms),[3−5] and can incorporate either hydrophobic or hydrophilic drug molecules because of their amphiphilic nature. One remarkable trend in developing lipid-based drug carriers is modifying lipid structures with various polymers.[6−10] Polyacrylic acid (PAA) is among perspective polymers for this application. Recently, it has been shown that hydrophobic polyacids [(poly(methacrylic acid), poly(α-ethacrylic acid), and poly(α-propylacrylic acid)] can completely disrupt liposome membranes, leading to the formation of hydrophilic pores and even mixed micelles with lipid molecules at pH 6.5.[9,11] It has been stated that the main driving force in hydrophobic polyions' interactions with membranes is the hydrophobic interaction.[11] However, it is evident that the effect of hydrophilic PAA on bilayers cannot be explained in the same way.

In recent studies, vesicles of phosphatidylcholine (PC) modified by PAA were investigated by fluorescence spectroscopy (FS), differential scanning calorimetry (DSC), and surface pressure measurement techniques in an effort to understand the mechanism of pH-dependent vesicle destabilization induced by the polymer.[12] PAA destabilizes PC vesicles at pH values lower than 4.1 and at temperatures above the main phase-transition temperature (T_m), inducing vesicle aggregation. A DSC study using multilamellar vesicles of dipalmitoylphosphatidylcholine in the presence of PAA demonstrated a gradual disappearance of the main transition peak after the pretransition peak with the accompanying appearance of a peak at a higher temperature. The DSC peak was split into two components: a

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peak corresponding to the pure vesicles and a broad peak at a higher T_m with a higher enthalpy (ΔH). A decrease in the phase-transition cooperativity was manifested in the broadening of the peak width. According to FS data, the effect of PAA on vesicles was minimal around neutral pH, where PAA is highly negatively charged, probably because of electrostatic repulsion between PAA molecules. At low pH, the anionic character of the polymer decreases and its hydrophobic character increase because of protonation of the carboxyl groups. The reduction of polymer mobility has been observed in the presence of PC vesicles at pH values where the carboxyl groups of the polymer are protonated (pK_a 4−4.5). The reduced mobility of the polymer is most likely due to the complexation of PAA with PC vesicles through hydrogen bonding. The strength of the PAA complexation is dependent upon polymer concentration. The complexation is weaker at high polymer concentrations. At low concentrations, the complexation was strong, most likely because a long segment of the polymer can be bound to the vesicles. At high polymer concentrations, polymer strands compete for binding to the vesicle surface and a shorter segment of polymer binds to the vesicle. Seki and Tirrell suggested that hydrogen bonding between the carboxylic group of the polymer and the phosphate group of the phospholipid may be the driving force for polymer adsorption on the vesicles.[13] According to their suggestions, the phosphodiester group serves as an H-bond acceptor and poly(carboxylic acids) form strong interpolymer complexes with the H-bond acceptor.

In more recent articles,[7,8] it has been shown that the PAA−lipid bilayer interaction is more complex than previously thought. Introducing PAA into oriented bilayers contributes to the lateral mobility of lipids and the appearance of a new "phase", characterized by decreased values of lateral diffusion coefficients.[8] The PAA−biomembrane interaction is strongly dependent on the polymer molecular mass, polymer concentration, and pH of the environment.[7,8] High-molecular-mass PAA (∼100 kDa) may completely disrupt the bilayer, leading to the formation of micellelike structures, and low-molecular-mass polymer fractions (∼1 kDa) induce vesicle aggregation, keeping the bilayer intact.

In this work, a combination of physical methods, such as ^{1}H pulsed field gradient NMR, DSC, ^{1}H NOESY NMR, and ^{31}P spectroscopy, were used to investigate the particular properties of the interaction of low-molecular-mass PAA with dimyristoylphosphatidylcholine lipid bilayers.

MATERIALS AND METHODS

1,2-Dimyristoyl-*sn*-glycero-3- phosphocholine (dimyristoylphosphatidylcholine) (DMPC) was purchased from Avanti Polar Lipids (Birmingham, AL). Poly(acrylic acid) with a degree of polymerization of $n \approx 69$ and a molecular weight of 5 kDa was purchased from Sigma-Aldrich (St. Louis, MO).

DMPC vesicles were prepared by a standard "thin film hydration method". A required amount of DMPC was dissolved in a sufficient quantity of methanol. A thin film of DMPC was obtained after evaporating methanol under a stream of N_2, followed by overnight drying in a vacuum evaporator. The film that formed was dispersed in an adequate amount of a water−PAA solution to form the required molar solution (2 mM) and vortex mixed. The vesicles were subjected to five freeze−thaw cycles in liquid N_2 and a 40 °C water bath for homogenization. This procedure typically results in the formation of homogeneous unilamellar vesicles.[14] The formation of vesicles with a size of 25 ± 2 nm in "pure" DMPC was proven by a dynamic light scattering method. The presence of PAA leads to the aggregation of DMPC vesicles in accordance with previous finding[15] that did not allow size measurements of individual vesicles. We also applied an alternative method of preparing lipid vesicles. A lipid−water suspension was sonicated for 10 min using a Bransonic B12 (Branson Ultrasonics Co., Danbury, CT) at a frequency of 50 kHz, an output of 80 W, and a temperature of 293 K. The sample-preparation procedure did not affect the obtained results. All of the prepared samples were measured within 24 h.

For lipid lateral diffusion measurements, we prepared samples of oriented lipid bilayers with PAA concentrations in the range of 0−10 mol %. Lipid vesicles with PAA were prepared as described above. They were applied to glass plates (0.1 mm thickness and 14 mm × 4.7 mm area) by covering one side of the plates with an amount to make the lipid concentration 15 mg/mL. The plates were dried at room temperature and stored under vacuum overnight. Afterwards, 20−45 plates were stacked on the top of each other and placed in a glass tube. The sample tube was placed in a humid atmosphere at a temperature of 40 °C (higher than the gel−liquid transition temperature of DMPC, T_m = 24 °C[16]) for several days. During this time, hydrated and oriented bilayers were formed. After the desired water content was obtained, as judged by the weight of the sample, the tube was sealed and left for several hours for final equilibration. The quality of the bilayer orientation and the possible presence of nonlamellar phases were checked by ^{31}P NMR spectroscopy.

^{31}P NMR spectra, recorded at 162 MHz, were acquired with a Hahn echo pulse sequence with a repetition time of 2 s, a 3.2 μs 90° pulse, and an echo delay time of 50 μs.

Pulsed Field Gradient NMR. Diffusion measurements were performed on a Chemagnetics Infinity NMR spectrometer operating at a ^{1}H frequency of 100 MHz. The spectrometer was equipped with a specifically designed goniometer probe that enables macroscopically aligned bilayers to be oriented with the bilayer normal at the magic angle (54.7°) with respect to the main magnetic field. The rotation of the lipid molecules around their axis leads to partial averaging of the dipolar proton interactions, thus the ^{1}H NMR spectrum of lipid molecules can be obtained without any additional averaging.[17]

For all measurements, the stimulated spin-echo pulse sequence was used. Diffusion decays $A(k)$ were obtained, where $A(k)$ is the spectrum integral $k = \gamma^2\delta^2g^2t_d$, γ is the ^{1}H gyromagnetic ratio, δ is the duration, g is the amplitude of the gradient pulse, $t_d = (\Delta - \delta/3)$ is the diffusion time, and Δ is the time between identical gradient pulses. In the measurements, g was 1.5 T/m and t_d was 111 ms, whereas δ was varied in the range of 1−8 ms. $A(k)$ in the case of a one-component bulk liquid (e.g., pure water) depends on the experimental parameters as

$$A(k) = A(0)\exp(-kD) \quad (1)$$

where D is the diffusion coefficient. In all experiments, the main stationary magnetic field and the pulsed field gradient were aligned in the same direction. The lateral diffusion coefficient of lipids (D_L) was obtained after multiplying the apparent diffusion coefficient (measured at the magic angle) by 1.5.[17]

Differential Scanning Calorimetry. Measurements were performed on a MicroCal VP-DSC microcalorimeter (Täby, Sweden). DSC thermograms were obtained in heating and cooling scans at a pressure of 20 psi and at temperatures from 5 to 60 °C with a scan rate of 20 °C/h. Four temperature scans were recorded, where the first scan was discarded to maintain a common thermal history of all samples. The appearance of the following scans was always identical. Data handling (baseline subtraction and peak integration) procedures were performed with MicroCal Origin software.

^{1}H NOESY MAS NMR. The nuclear Overhauser enhancement spectroscopy NMR experiment with magic angle spinning was conducted on a Chemagnetics Infinity NMR (Varian, Fort Collins, CO) widebore spectrometer at a resonance frequency of 400 MHz and a MAS spinning frequency of 8 kHz with a 4 mm zirconium double gas bearings rotor. Mixing times were 50, 200, and 300 ms. Spinning frequencies were around 9 kHz, and the 90° pulse was 8.0 μs.

Initially, the system was prepared with the PAA concentration of as high as 20 mol %. Afterwards, the system spontaneously separated into

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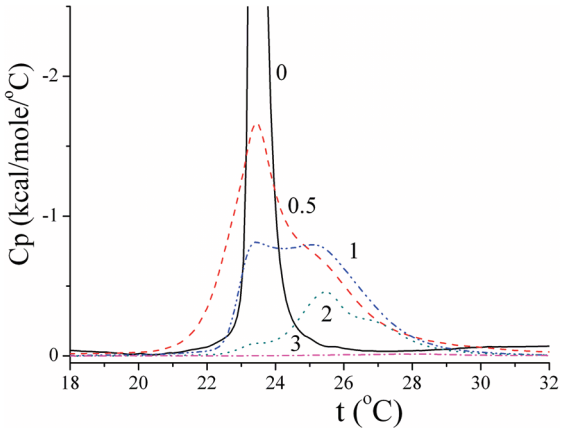

Figure 1. DSC thermograms for DMPC/PAA systems at the indicated concentrations of PAA.

two phases: a dense, white phase of DMPC/PAA (in the form of white flakes) and a transparent water solution of PAA. The dense phase of DMPC/PAA aggregates was separated from the water solution and placed in an NMR ampule and a zirconium NMR rotor for measurements. In preparing this type of sample, we tried to determine if (i) PAA interacts with DMPC membranes according to a binding stoichiometry, where the distribution of PAA between the water solution and DMPC aggregates is determined by this stoichiometry, which could be estimated from NMR measurements and (ii) the NOESY experiment with magnetization transfer between protons of interacting chemical groups of PAA and DMPC could be informative in identifying these interacting chemical groups. For this purpose, the ^{1}H NMR spectra of PAA and DMPC were previously obtained; they are shown in Figure 5 with the signal assignment.

RESULTS

^{31}P NMR spectra for the system with different PAA concentrations were asymmetric and consistent with a lamellar phase.[18] However, at concentration higher than 1 mol % PAA, a new symmetric peak appeared at around 0 ppm as described previously.[8] We related this peak to small vesicles because the system with phospholipids and a small-molecular-mass PAA does not form micelles.[7] Thus, the lateral phase organization of DMPC that agrees with previously reported results was maintained.[8,9]

The results of the DSC measurements are shown in Figure 1, and the thermodynamic parameters of the DMPC gel-to-liquid phase transition are presented in Table 1. The thermogram for the DMPC vesicles shows a pretransition temperature of 12.4 °C (not shown) followed by a main transition temperature of 23.5 °C. The measured transition temperature and enthalpy of the transition are close to those reported earlier.[16] An increase in PAA concentration leads to the disappearance of the pretransition peak, shifting T_m of DMPC to higher temperatures and to gradually decreasing enthalpy. DMPC in pure bilayers shows a symmetric peak, but in the presence of PAA, the peak becomes more complex and can be assumed to be the sum of at least two lines. One line has the same width and position as that of pure DMPC (with the center at around 23.5 °C), and its fraction decreases with increasing PAA content. The second peak is broader and is positioned at a higher temperature, around 25.5 °C. For the sample with 2 mol % PAA, a third peak appeared with a higher T_m and a lower cooperativity. Finally, at 3 mol % PAA and greater, no thermal effects were observed. This type of

Table 1. Temperatures and Enthalpies of Gel-to-Liquid Phase Transitions of DMPC in the Presence of Poly(acrylic acid)

PAA concentration (mol %)	pH	T_m (°C)	ΔH (kcal/mol)
0	6.4	23.5	6.03
1	5.9	25.0	5.1
2	5.2	25.5	3.34
3	4.8	26.0	1.7
4	4.6	26.6	

behavior demonstrates that in the presence of up to ~3 mol % PAA part of the bilayer remained in the same state as bilayers without the polyacid, whereas other parts of the bilayer formed a new (different) phase or phases with increased T_m and lower cooperativity. This new phase cannot be related to the formation in the presence of PAA vesicles with smaller sizes, which were obtained from ^{31}P NMR data, because a decrease in vesicle size typically leads to a decrease in T_m that has been reported, particularly for DMPC.[16]

In the experiments described above, the pH was changed in the range of 6.4−4.6 (Table 1) accordingly to a varied concentration of PAA. In another experiment, we changed the pH of the system to be far from the normal physiological conditions by adding NaOH or HCl. It was observed that an increase in pH to 8.0 leads to the complete disappearance of the PAA effect whereas a decrease in pH down to ~2.5 does not influence the DSC data, in accordance with the data obtained by pulsed field gradient NMR.

In the pulsed field gradient ^{1}H NMR experiment on pure DMPC multibilayers oriented at the magic angle, an echo signal from the lipids was not observed below 24 °C. This was conditioned by dipolar interactions of the lipid protons, resulting in rather small NMR transverse relaxation times (T_2) of these protons in the gel phase and by broadening the proton NMR line. After the system was heated to above 24 °C, the rotational mobility of the lipid molecules in the liquid-crystalline phase averaged the dipolar interaction of the protons; as a result, the echo signal appeared. However, in the presence of PAA, no echo signals were observed in DMPC bilayers at 25 °C, even at a PAA concentration of as low as 0.27 mol %; it appeared only at 30 °C. For this shift in temperature, the echo appearance agrees with the temperature dependence of the DMPC phase transition in the presence of PAA, as described above (Figure 1), nevertheless a difference of macroscopic structure of multibilayers studied by pfg NMR from unilamellar vesicles analyzed by DSC. At any PAA concentration, we observe a strong angular dependence of the NMR signal with a maximum signal for bilayers oriented at the magic angle. This fact demonstrates that not any part of the lipids can form micelles. From the point of view of NMR, the micelle is a particle with isotropically rotating lipid molecules. This is because of the rotation of the particle as a whole and the diffusion of molecules around the center of the micelle. This motion could average the dipole−dipole interaction of protons, resulting in the disappearance of the strong angular dependence of the NMR signal.

^{1}H NMR spectra obtained after the Fourier transformation of the stimulated spin echo in pfg NMR experiments at 30 and 40 °C are shown in Figure 2a,b. An increase in PAA concentration in the system leads to a change in the spectra. Typically, characteristic spectra of solid-supported oriented multibilayers of phosphatidylcholines at temperatures above T_m have the most

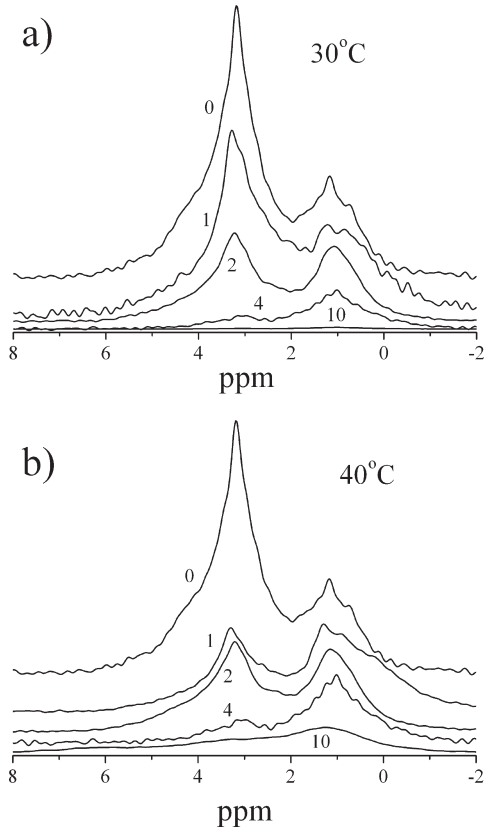

Figure 2. ^{1}H NMR spectra of DMPC/PAA at the indicated concentrations of PAA obtained after the Fourier transformation of the signal of stimulated spin echo at τ = 11 ms, τ_1 = 100 ms, and δ = 1.5 ms. Temperatures were (a) 30 and (b) 40 °C. Lines are shifted vertically for convenience.

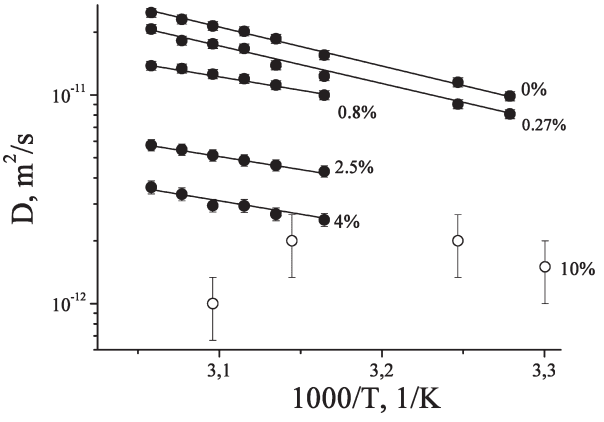

Figure 3. Dependence on temperature of the lateral diffusion coefficient, D_L, of lipid molecules for oriented DMPC multibilayers with the indicated concentrations of PAA. Solid lines show the best fits according to Arrhenius' law.

prominent peaks at ~1.3 ppm (−CH_2− chains) and ~3.4 ppm (−CH_3 cholines).[19] Under these conditions, we can see that the larger decrease in the signal intensity of the peaks corresponds to the lipid headgroups, followed by a decrease in the chain's peak as the PAA content increases. The signal from lipids can be observed even at rather high concentrations of PAA (as high as 4−10 mol %), and the increase in temperature allows one to observe the spectra with

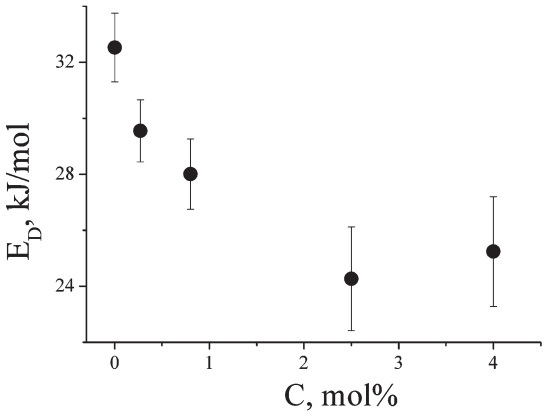

Figure 4. Activation energies of DMPC lateral diffusion for oriented multibilayers with different PAA concentrations.

progressively higher concentrations as seen in Figure 2 for samples with 4 and 10 mol % PAA.

Diffusion decays in pure DMPC bilayers and in bilayers with PAA have a monoexponential form, which can be described by eq 1 and can be characterized by a single diffusion coefficient that changes with the concentration of PAA and the variation of temperature. Diffusion coefficient values obtained at different PAA concentrations and temperatures are shown in Figure 3; D_L values for the pure DMPC bilayer are equal to those reported previously.[19] An increase in PAA concentration in the system leads to a progressive decrease in D_L.

The temperature dependence of D_L on pure DMPC at 30−60 °C is close to that of Arrhenius-type plots (Figure 3), which can be described by the equation

$$D_L = D_0 \exp\left(\frac{E_D}{RT}\right) \quad (2)$$

where D_0 is the pre-exponential factor that is independent of temperature, E_D is the apparent activation energy for diffusion, RT is the thermal energy, R is the universal gas constant, and T is the temperature. A similar dependence of D_L on temperature was observed in DMPC/PAA samples at PAA concentrations of up to 4 mol %. For a sample with 10 mol % PAA, the temperature trend for D_L was not obvious because of small NMR signal values (Figure 2) and small D_L values (Figure 3). Activation energies calculated from the slopes of these temperature dependences for samples with PAA concentrations of 0−4 mol % are shown in Figure 4. In this Figure, it is seen that the increase in PAA in the system from 0 to more than 2 mol % leads to a reduction in activation energy from the E_D value that is proper for the DMPC bilayer, 31 kJ/mol,[19,20] to a value of E_D that is decreased by a factor of 0.75.

Two-dimensional NOESY NMR spectra generally have similar forms for mixing times of 50, 200, and 300 ms, but the most apparent cross-peaks were observed at 300 ms. The spectrum obtained at 300 ms is shown in Figure 6; the plot shows intermolecular cross-peaks corresponding to dipole−dipole interactions of protons for the following groups:

(1) −CH_2 group of DMPC (C2) and −CH_2 group of PAA (a);
(2) choline −CH_3 group of DMPC (γ) and −CH group of PAA (b).

No cross-peaks are observed between the −CH_2 of the lipid's chains and any PAA protons.

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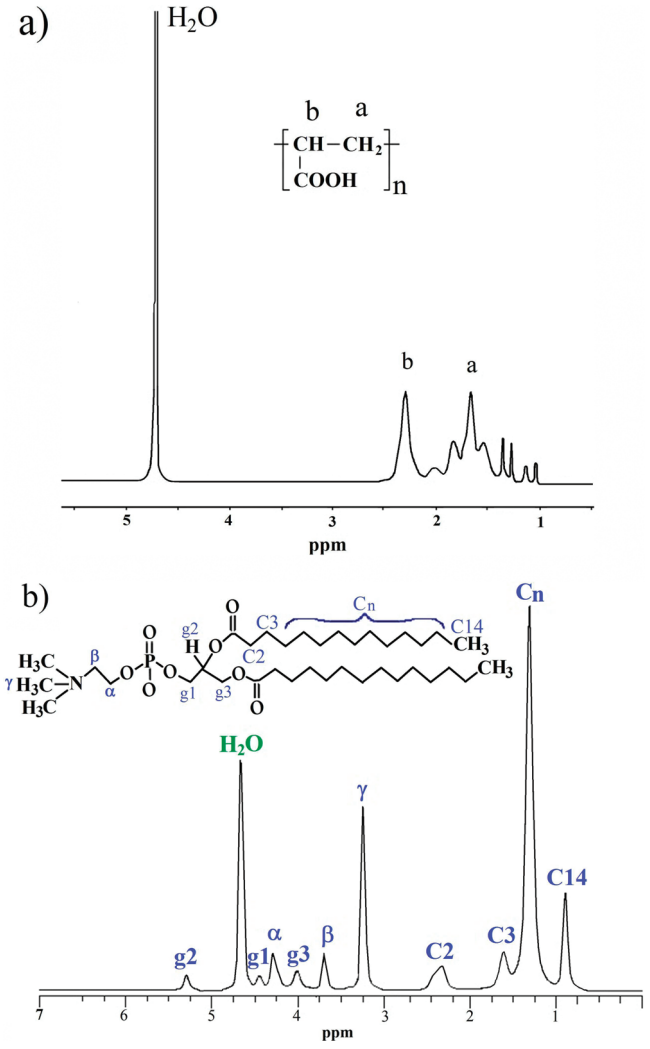

Figure 5. ^{1}H MAS NMR spectra of (a) PAA obtained for a 10 mol % PAA solution in D_2O and (b) DMPC obtained for multilamellar vesicles of DMPC in D_2O. The measurement temperature was 35 °C. Spectra were aligned according to the literature for PAA[2] and for DMPC.[30]

NMR longitudinal relaxation times (T_1) of choline, $-CH_2$, and PAA protons were in the range of 0.54−0.61 s, so the amplitudes of the corresponding peaks under the experimental conditions are proportional to the real proton fractions in these groups and the ratio of interacting PAA and DMPC molecules can be estimated from the amplitudes. The estimation showed that the number of lipid molecules bound to one PAA molecule is approximately 6.27 (i.e., 6 to 7 lipids per PAA molecule).

DISCUSSION

First, we will discuss the effects of pH on a gel-to-liquid phase transition and the lateral diffusion coefficients in DMPC bilayers because the increase in the PAA concentration in the studied system leads to the change in pH of the water phase surrounding the lipid membrane (Table 1).

Earlier DSC measurements revealed that a wide pH range extended above 3.0 in which the mesophase properties of phosphatydylcholines do not change significantly.[16,21] Authors noted that the PC molecule has in its structure a physiologically relevant titrable group, phosphate oxygen. Therefore, electrostatic considerations assume that the mutual repulsion of similarly charged polar groups would result in a lateral expansion in the membrane plane. They will destabilize the ordered low-temperature gel phase and decrease the phase-transition temperature.[16,22] This is contradictory to what was really occurring in the membranes of PCs. A possible explanation involves favorable hydrogen bond formation that develops upon acid titration and that more than compensates for the charge repulsion.

The above-described compensatory mechanism can be conducted in a manner similar to the effective lateral diffusion of PCs. An additional confirmation of no influence of pH on the D_L has been obtained by analyzing effects of pH on mechanical properties of the lipid membranes. This is because D_L and properties such as the bending rigidity and elastic area compressibility are controlled through the intermolecular interactions, lipid chain ordering, and free area per lipid molecule. Zhou and Raphael[23] systematically characterized the effect of pH on the mechanical properties of stearoyloleoyl−PC membranes. They showed that both the elastic compressibility and bending rigidity are insensitive to pH, except for a decrease at pH 2.0. Finally, our direct measurements of D_L in pure DMPC bilayers by means of pfg NMR in the range of pH 2.5−8.0 showed no change in the D_L under these conditions. At the same time, it is known that not only H^+ but also Na^+ and Ca^{2+} ions do not significantly influence the lateral mobility in PC bilayers.[24] Thus, an increase in PAA concentration in the system that leads to the change in DCS thermograms and the lateral diffusion coefficient of lipid is obviously conditioned by the adsorption of PAA on the bilayers.

PAA Interaction with the DMPC Bilayer. It is now generally understood that there are several ways in which macromolecules may interact with lipid membranes: (i) adsorption on the bilayer hydrophilic surface, (ii) insertion into the hydrophobic core of the bilayer, and (iii) complete disruption of the bilayer with the formation of mixed polymer−lipid micelles or others aggregates.[25] Most of our results directly show that PAA interacts mainly with the surface of the DMPC bilayer and only with the lipid polar headgroups. First, NMR NOESY demonstrates cross-peaks between glycerol's $-CH_2$ of DMPC and the $-CH_2$ group of PAA and between the choline $-CH_3$ groups of DMPC and the −CH group of PAA (Figure 6). No cross-peaks between the DMPC chain's $-CH_2$ protons and any protons of PAA were observed at an even longer mixing time, 300 ms. Under this condition, magnetization could be transferred at a distance of less than or around 5 Å. Therefore, there are no PAA protons in close proximity to the DMPC chains.

The change in the ^{1}H NMR spectra of the lipid in the presence of PAA (Figure 2) also demonstrates the PAA−lipid headgroup interaction. At increasing PAA concentration, the intensity of the peak corresponding to the choline's $-CH_3$ protons decreases first, demonstrating the obstruction of their local mobility and leading to an increase in the T_2 relaxation rate. The intensity of the peak, corresponding to the chain's $-CH_2$ components, decreased at much higher PAA concentrations.

Some conclusions about the interactions of polyions with PCs have been reported previously. Feng et al.[25] studied poly(methylacrylic acid) association with DMPC bilayers by ellipsometry and AFM techniques and found that the polymer associated first with the outer headgroup region of the lipid bilayer. Seki and Tirrell[13] studied the DPPC−PAA system by DSC and ^{1}H NMR spectroscopy. These authors concluded that hydrogen bonds between the carboxyl group of the polymer and the phosphate group of phospholipids is the driving force in the adsorption of macromolecules on the vesicles. Hydrophobic

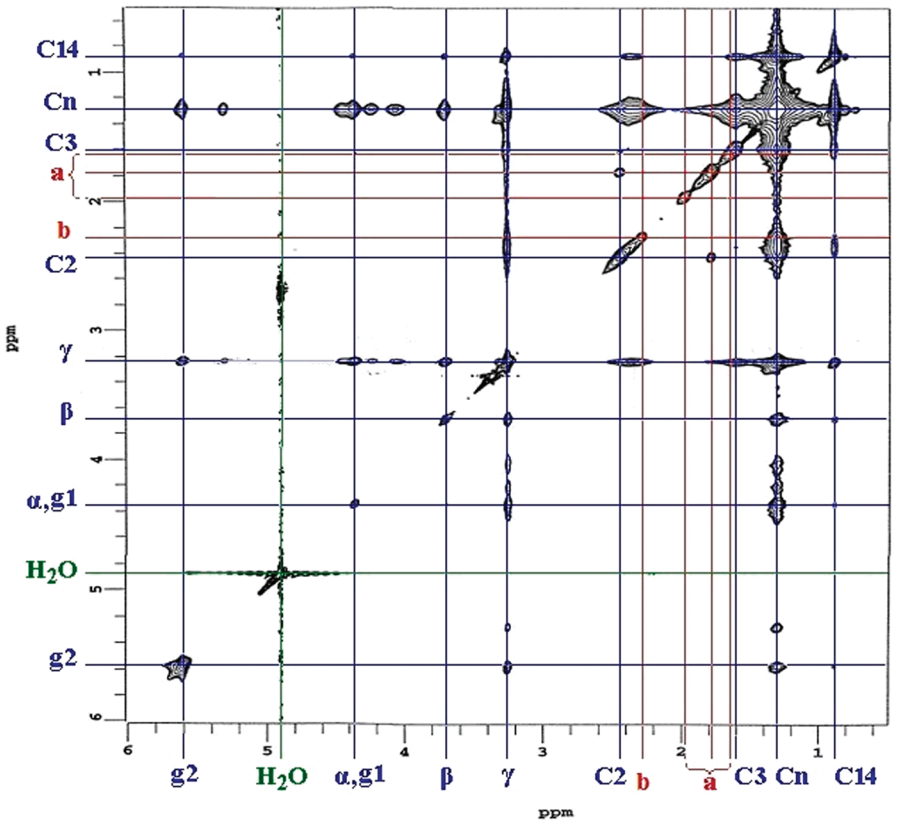

Figure 6. NOESY spectrum for DMPC multibilayered liposomes initially prepared with 20 mol % PAA. The mixing time was 300 ms. Peaks corresponding to PAA are marked with red lines, DMPC peaks are marked with blue lines, and water is marked with green lines. Letters are used to mark corresponding chemical groups of PAA, DMPC, and H_2O molecules. T = 40 °C.

contacts are not involved in the interaction because PAA does not interact with hydrophobic substances even at lower pH values. This hypothesis explains the observed dependence on pH as a result of the hydrogen bonding of un-ionized carboxyl groups and phosphodiester functional groups on the surface of the bilayer. It is possible that the losses of translational and configurational entropy accompanying this process are overcome by forming a critical number of hydrogen bonds in the polymeric chain. The only difference between our results and those of Seki and Tirrell is that they obtained a critical value of pH 4.6 for PAA−DPPC complexation whereas in our DMPC−PAA system the interaction occurs at a higher pH and the decrease in pH below the natural values due to the presence of PAA (Table 1) did not influence either the DSC or the pfg NMR results.

An alternative approach proposed that a polyion molecule undergoes a conformational change after protonation to bury effectively charged groups; subsequently, the molecule can be inserted into the core of a lipid bilayer.[26] This is not the case with PAA, which does not contain any hydrophobic groups. Only slightly increasing the pH changes the macromolecular conformation of PAA molecules with moderate molecular masses. Experimental data by dynamic light scattering showed macromolecule (M = 12 kDa) hydrodynamic radii of 6.6 nm at pH 9 and 5.8 nm at pH 4 [27] resulting from the chain hydration effect. Therefore, the effects of increased PC membrane permeability in the presence of PAA[8,26] do not occur by the same mechanism as the formation of hydrophobic channels by a polyion molecule.

PAA Influence on the Structure and Phase State of DMPC Bilayers. The presence of PAA does not change the lamellar phase organization of DMPC, but it leads to the appearance of new lateral phases that differ in thermodynamic properties, such as T_m and ΔH, from that of pure DMPC. In order of increasing PAA concentration, the first new phase appeared in the concentration range of up to around 4 mol % PAA. This phase has a higher distinct main phase transition temperature (around 26 °C), a lower cooperativity of the phase transition, and a lower phase-transition enthalpy. It should be noted that a similar new phase with a separate T_m and a lower cooperativity was observed in the DPPC−PAA system.[12] What causes the change in DMPC thermodynamic characteristics at PAA concentrations in the range of 0−4 mol % PAA? It may potentially be explained by the adsorption of PAA molecules. The liquid−gel phase transition of a lipid bilayer is entirely related to the crystallization of lipid chains. Therefore, increases in T_m and decreases in ΔH could occur as a result of changes in external conditions, leading to easier packing of lipid chains. The reason that these chains may form a solid phase at higher temperature is the decrease in the free energy of formation of the nucleus of the solid phase and the diminishing entropy of lipid polar groups as a consequence of their interaction with bulkier adsorbed macromolecules. It was proposed by Fujiwara et al.[12] that at low PAA concentrations a long segment of the polymer can be bound to the vesicles. Taking the area per molecule of DMPC[27] to be 60 Å^2 and the area covered by one molecule of PAA to be 10 × 170 Å^2,[28] the concentration of complete coverage of the membrane can be estimated to be 3.4 mol % PAA. This value is close to the upper boundary of the concentration interval of a new phase with a higher T_m and a low transition enthalpy. Thus, in the concentration range of 0 to ∼4 mol % PAA, the entire length of the PAA molecules can

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absorb onto the membrane surface, associating in a separate phase with certain thermodynamic characteristics, whereas the remainder of the membrane surface remains free of PAA. In this range of PAA concentration, macromolecules influence the local mobility of the lipid headgroup, as demonstrated by the ^{1}H NMR spectra in Figure 2.

In this new phase, PAA may affect the lipid headgroup conformation through the modification of headgroup hydrogen bonding and subsequent acyl chain packing. The disappearance of the pretransition peak also supports the idea that the lipid headgroup conformation is altered by PAA because the pretransition is associated with the freedom of the long axis of rotation of the PC molecules. Increased T_m is caused by increased van der Waals interactions between the lipid acyl chains as soon as there is a decreased local mobility of headgroups due to PAA adsorption.

At PAA concentrations higher than 4 mol %, all headgroups appeared to be bound to PAA molecules whereas PAA molecules only partially interact with the membrane. Other segments of the PAA molecule are free and protrude into the water phase above the bilayer.[12] For example, at 3.4 mol % PAA, all segments bind, whereas at concentrations corresponding to the saturation of the bilayer by PAA molecules estimated from ^{1}H NMR lines intensities (1:6 PAA/DMPC, in other words, 14 mol % PAA in the complex) only around 25% of the PAA segments are interacting with the membrane. In this concentration range, as seen in the ^{1}H NMR spectra (Figure 2), the adsorption of PAA influences not only the local motion of lipid headgroups but also the mobility of the lipid hydrocarbon chains. This is a probable mechanism preventing the regular arrangement of lipid chains that is required to form a solid lipid phase (gel phase) at lower temperatures.

PAA Influence on the Lateral Diffusion of DMPC. Lateral diffusion in bilayers is characterized by a single diffusion coefficient, which decreases with increasing PAA concentration as seen in Figure 3. At the same time, the DSC data demonstrate the presence of two phases for the system with PAA concentrations of up to 4 mol %. One of the phases has the same thermodynamic characteristics as the pure lipid bilayer, which means that the second phase formed in the presence of PAA is characterized by a lower diffusion coefficient. The presence of two phases differing with diffusion coefficients should result in two-component diffusion decay. However, in our case all of the diffusion decays were monoexponential, demonstrating the averaging of the translational molecular characteristic of the phases. This typically occurred because of the molecular exchange between these phases at a time less than the time of the NMR diffusion experiment (ca. 0.1 s). Averaged diffusion also characterizes the system at higher PAA concentration.

A decrease in apparent activation energy for the lateral lipid diffusion, E_D (Figure 6), can be caused by at least two factors. The first one is the change in the fraction of molecules observed by pfg NMR at temperatures in the studied temperature range as a result of a broader gel-to-liquid phase transition in the presence of PAA. It is also possible that as a result of PAA adsorption the cooperative unit for diffusion changes; it may contain PAA and several lipid molecules, so the diffusion process is characterized by lower D_L and E_D.

DMPC−PAA Complexation. PC−PAA complexation has been discussed in earlier papers.[12,13] Our study confirms that the DMPC−PAA system can form at least two types of complexes. These complexes form on the surface of the membrane, mainly because of hydrogen bonding between un-ionized carboxyl groups with the phosphodiester groups on the bilayer surface.[13] One type of complex forms at PAA concentrations lower than ∼4 mol %, when the entire length of the PAA molecule adsorbs on the membrane. The stoichiometry of this complex is around 28 lipid molecules to 1 molecule of PAA, and the thermodynamic parameters of the lateral phase formed by this complex are described above. The residence time of lipid molecules in the complex is less than 0.1 s, as seen from pfg NMR data. At higher PAA concentrations, the system forms complexes with a smaller stoichiometric ratio (around 6 to 7 molecules of lipid per polyion) because of the partial adsorption of the PAA molecules onto the membrane.

Our experimental results could be related to the most practically interesting effects of PAA adsorption on phospholipid vesicles, bilayer permeabilization. In the case of PAA derivatives, the effect of increased permeability is often explained by the formation of pores,[9,11] and pore formation is due to the insertion of the polymer molecule into the hydrophobic part of the bilayer. However, this mechanism is less possible for the PAA molecule, which does not contain hydrophobic groups. It is usually expected that if a polymer interacts only with the membrane surface then it cannot significantly change the membrane permeability.[29] However, permeabilization of the PC bilayer covered by PAA was observed not only for water[8] and the relatively small fluorescent probe pyranine but also for a substrate of the proteolytic enzyme trypsin.[26] Therefore, because there is no evidence of PAA insertion into the bilayer core, we can assume that the increased bilayer permeability is caused entirely by the increased defects of lipid bilayer followed by the PAA interaction with the lipid headgroups.

AUTHOR INFORMATION

Corresponding Author

*E-mail: andrey.filippov@ksu.ru. Tel: +7 843 5315189.

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