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Dipole potential as a driving force for the membrane insertion of polyacrylic acid in slightly acidic milieu

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ABSTRACT

In this work, we report on the interaction of polyacrylic acid with phosphatidylcholine bilayers and monolayers in slightly acidic medium. We found that adsorption of polyacrylic acid on liposomes composed of egg lecithin at pH 4.2 results in the formation of small pores permeable for low molecular weight solutes. However, the pores were impermeable for trypsin indicating that no solubilization of liposomes occurred. The pores were permeable for both positively charged trypsin substrate N-benzoyl-L-arginine ethyl ester and negatively charged pH-indicator pyranine. Two lines of evidence were obtained confirming the involvement of the membrane dipole potential in the insertion of polyacrylic acid into lipid bilayer. (i) Addition of phloretin, a molecule which is known to decrease dipole potential of lipid bilayer, reduced the rate of a polyacrylic acid induced leakage of pyranine from liposomes. (ii) Direct measurements of air/lipid monolayer/water interface surface potential using Kelvin probe showed that adsorption of polyacrylic acid at pH 4.2 induced a decrease in both boundary and dipole potential by 37 and 62 mV for ester lipid dioleoylphosphatidylcholine (DOPC). Replacement of DOPC by ether lipid 1,2-di-O-oleyl-*sn*-glycero-3-phosphocholine (DiOOPC) which is known to form monolayers and bilayers with only minor dipole component of membrane potential showed that addition of PAA produced similar response in the boundary potential (by 50 mV) but negligible response in dipole potential of monolayer. These observations agree with our assumption that dipole potential is an important driving force for the insertion of polyacids into biological membranes.

1. Introduction

The ability of chain molecules to form multiple bonds with surfaces and low molecular weight substances determines unrelenting interest to synthetic polymers in various fields of science and engineering. Model bilayer membranes and the membranes of living cells are also substrates for polymer adsorption and targets for their action [1,2]. In particular, interaction of synthetic polymers with lipids and proteins in the membranes and resulting modulation of membrane properties attracts significant research attention as a tool for biomedical applications, e.g. enhancing therapeutic response of drug formulations [3]. In this case the polymer should facilitate penetration of physiologically active substances through biomembranes.

Most attention in this field was paid to the study of polycations and uncharged amphiphilic copolymers exhibiting high affinity to biological membranes due to electrostatic and hydrophobic interactions [4–6]. In contrast, fewer efforts were made to study the interaction of polyanions with lipid membranes, since the latter usually bear negative net charge and therefore are not expected to interact with anionic species under physiological conditions.

However, the remarkable series of works carried out by C. Tirrell et al. disclosed the ability of some weak polyacids to permeabilize uncharged or negatively charged lipid bilayers in pH-dependent manner. In particular, it was found that hydrophobic poly-(2-ethylacrylic acid) [7–11] and poly-(2-propylacrylic acid) [12] can solubilize phosphatidylcholine bilayers at pH <5.5 forming mixed polymer-lipid micelles. The same authors found that hydrophilic polyacrylic and polymethacrylic acids also can permeabilize lipid membranes but at pH <4.5 [13]. Covalent attachment of polyacrylic acid to phosphatidylcholine liposomes acquired them pH-sensitivity, such liposomes being permeabilized at acidic pH [11,14].

Interaction of hydrophilic polyacids with phosphatidylcholine vesicles at pH <5 is accompanied by aggregation of vesicles, as

Abbreviations: PAA, polyacrylic acid; BAEE, N-benzoyl-L-arginine ethyl ester; DODPC, 1,2-di-O-octadecyl-*sn*-glycero-3-phosphocholine; DPPC, dipalmitoylphosphatidylcholine; EYPC, egg yolk lecithin; RH-421, 4-{4-[4-(Dipentylamino)phenyl]-1,3-butadienyl}-1-(4-sulfobutyl)pyridinium; TMA-OH, tetramethylammonium hydroxide; Tris, Tris-hydroxymethyl aminomethane base

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demonstrated via by dynamic light scattering technique, while no solubilization of the liposomes occurred [15,16]. Electrostatic character of polymethacrylic acid adsorption on the supported lipid bilayer at pH 6 was demonstrated using FT-IR spectroscopy in the mode of attenuated total reflection [17]. Direct measurements of the membrane ζ-potential showed that phosphatidylcholine liposomes acquire slight positive charge at pH <5 which is decreased and becomes negative upon polyacid adsorption [16,18]. These results strongly suggest that electrostatic interaction is the main driving force for such interaction. The objective of the present work was to investigate the underlying factors guiding insertion of hydrophilic polyacrylic acid into lipid bilayers under slightly acidic conditions.

2. Materials and methods

2.1. Materials

Egg yolk phosphadidylcholine (EYPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), 2-di-O-(9Z-octadecenyl)-*sn*-glycero-3-phosphocholine (18:1 diether PC) and 1,2-di-O-octadecyl-*sn*-glycero-3-phosphocholine (di-18:0 ester PC) were purchased from Avanti Polar Lipids, USA, polyacrylic acid (PAA), M.w. 5000, Triton X-100, pyranine (trisodium 8-hydroxypyrene-1,3,6-trisulfonate), bovine trypsin, glycine, N-α-benzoyl-L-arginine ethyl ester, and 4-{4-[4-(Dipentylamino)phenyl]-1,3-butadienyl}-1-(4-sulfobutyl)pyridinium hydroxide (RH-421) were purchased from Sigma-Aldrich Corp., USA. Tetramethylammonium hydroxide (TMA-OH) was purchased from MP Biomedicals, USA. All buffer components were of highest grade.

2.2. Liposomes

Small unilamellar liposomes were prepared using as described elsewhere [19]. Briefly, 0.5 ml of chloroform solution containing 10 mg of EYPC was evaporated thoroughly under reduced pressure at 40 °C to obtain lipid film. The film was suspended in 1 ml of buffer solution for 90 min and then underwent to 3-fold freeze–thawing procedure. Then the suspension was sonicated under argon atmosphere using Cole-Parmer 4710 Ultrasonic generator (22 kHz, 30 W, 3 pulses, 150 s each). Hydrodynamic diameter of the obtained vesicles was evaluated by quasi-elastic light scattering technique using Malvern Autosizer 2c equipped with 632.4 nm Ar laser.

2.3. Trypsin- and pyranine-loaded liposomes

To obtain liposomes loaded with trypsin, lipid film was hydrated in 1 ml of 10 mg/ml solution of trypsin in 5 mM glycine-TMA-OH buffer, pH 9.2. The liposomes were sonicated at 4 °C (22 kHz, 30 W, 5 pulses, 60 s each) and then separated from free trypsin using gel-permeation chromatography on Sepharose CL-4B column (0.8 cm × 12 cm) equilibrated with 5 mM citrate-Tris buffer pH 4.2, supplemented with 0.4 M sucrose for osmotic pressure compensation. Similar procedure was used for the preparation of pyranine-loaded liposomes, lipid film being hydrated in 1 ml of 0.5 mM pyranine solution in 0.3 M glycine-TMA-OH buffer, pH 9.2.

2.4. Measurement of Trypsin activity

4 ml of 10 mM BAEE solution in 0.1 M KCl was placed in the cuvette of pH-stat ("Radiometer" Copenhagen) and kinetics of background hydrolysis of the substrate was measured, maintaining pH 8.0 due to automatic addition of 5 mM NaOH solution. Then 100–300 μl of the sample containing 10–500 pmoles of catalytically active enzyme was added and kinetics of BAEE hydrolysis was measured at the same pH.

2.5. Spectra of potential-sensitive fluorescent probe RH-421, 4-{4-[4-(dipentylamino)phenyl]-1,3-butadienyl}-1-(4-sulfobutyl)pyridinium hydroxide, in liposomes

0.2 μM solution of the probe was added to 0.4 mg/ml suspension of liposomes in 5 mM Tris-citrate buffer solution and incubated for 1 h at 37 °C. Then fluorescence excitation spectra were recorded at the fluorescence wavelength 620 nm and $I_{440}^{620}/I_{540}^{620}$ ratio was estimated for each sample.

2.6. Monolayers

Monolayers of dipalmitoylphosphatidylcholine (DPPC) were formed by spreading 10 μl of 2 mg/ml DPPC solution in freshly distilled chloroform on the surface of a subphase containing 5 mM citrate-Tris buffer pH 4.2 in double-distilled water with or without polyacrylic acid. The surface was cleaned by repeated "blank" runs prior to measurements. The solvent was allowed to evaporate for 10 min before compression. Surface pressure and surface potential isotherms (π-A and V-A isotherms) were measured simultaneously using a Teflon Langmuir trough (7.5 × 36 cm^2) "LB2-NT-MDT" (Zelenograd, Moscow) equipped with a Wilhelmy balance and a surface potential meter (Kelvin probe). The setup was mounted in a dust-free chamber. The monolayer surface potential was measured by vibrating (frequency 300 Hz) a 15-mm diameter polished brass plate with respect to the potential of the pure subphase surface (the latter was taken as zero). Ag/AgCl reference electrode was placed into the subphase. The accuracy and long-term stability in surface potential experiments were 5 mV and 10 mV/h, respectively. For surface pressure measurements the accuracy was mainly limited by occasional air flows and vibration of the water surface (~0.1 mN/m). Both isotherms (π-A and V-A) were measured by slow compressing a lipid monolayer at a rate of ~30 cm^2/min. All measurements were performed at room temperature 21 ± 1 °C.

3. Results

3.1. Effect of polyacrylic acid on the permeability of EYPC bilayers in slightly acidic medium

To evaluate the influence of polyacrylic acid on the permeability of EYPC membranes, we employed liposomes loaded with trypsin, a water-soluble enzyme localizing inside the waterpool of liposomes with negligible incorporation into the membrane. This enzyme is stable in the wide range of pH and is unable to penetrate through lipid bilayers. Furthermore, specific substrates of trypsin are positively charged and they are also commonly unable to penetrate through lipid bilayers. Therefore, trypsin-loaded liposomes exhibit only minor enzymatic activity (Fig. 1), obviously due to adsorption of minute quantities of the enzyme on the external surface of the vesicles. Distortion of liposomes in the presence of 0.05% of Triton X-100 results in the sharp increase in the catalytic activity (dashed line in Fig. 1).

To study the effect of polyacrylic acid on the permeability of lipid membranes under slightly acidic conditions, trypsin loaded liposomes were diluted with 10 mM citrate buffer pH 4.2 and mixed with various amounts of polyacrylic acid. After 15 min incubation, 0.1 ml aliquots were taken from these samples and added to 10 mM BAEE solution in 100 mM KCl, pH 8.0 to measure trypsin catalytic activity. The observed tryptic activity gradually increased in the samples with increasing amount of added polyacrylic acid (Fig. 1, curve 1) and upon addition of 2-fold molar excess of PAA in respect to EYPC, an entire payload of all trypsin contained in the sample was exposed to the substrate.

Trypsin exposure to the substrate may result from either membrane solubilization or formation of small pores permeable for

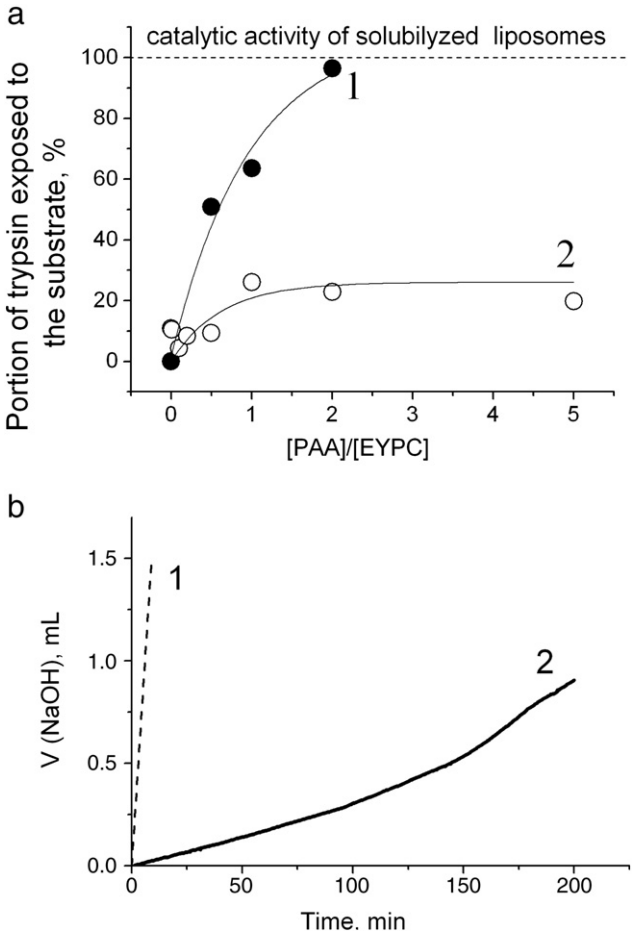

Fig. 1. (a) Dependence of the amount of trypsin exposed to the substrate on [PAA]/[EYPC] molar ratio. EYPC liposomes (12.6 mM) were loaded with trypsin solution (0.22 mM) in 0.3 M glycine-0.1 M TMA-OH, pH 9.2 (1) or the same solution containing 10 mM benzamidine, a competitive inhibitor of trypsin (2). Liposomes were suspended in 5 mM citrate-Tris, pH 4.2 and various amounts of polyacrylic acid were added (6.3–63 mM). The samples were incubated for 15 min and then 0.3 ml of each sample were mixed with 4 ml of 10 mM BAEE in 0.1 M KCl and the rate of BAEE hydrolysis was recorded. (b) Kinetics of BAEE hydrolysis by trypsin loaded into EYPC liposomes at pH 4.26 after liposomes solubilization by 0.05% Triton X-100 (1) and after 15 min incubation with 63 mM of PAA (5-fold excess to all EYPC containing in the sample) at pH 4.26 (2). The mixture was incubated 15 min and then 300 μl of the solution was added to 4 ml of BAEE at pH 8.0 and the rate of BAEE hydrolysis was recorded.

the substrate but not the enzyme. Membrane solubilization and formation of lipid-polymer complexes under slightly acidic conditions were proved for the hydrophobic derivatives of PAA — poly-(2-ethylacrylic acid) and poly-2-(propylacrylic acid) [7–10]. In order to discriminate between these mechanisms, liposomes were loaded with trypsin in the presence of 0.01 M of its competitive inhibitor, benzamidine. Inhibition constant of benzamidine, K_i, is 39 μM [20], therefore solubilization of liposomes the resulting ~10,000-fold dilution of the inhibitor in the external milieu should lead to complete dissociation of the complex. Indeed, addition of 0.05% of Triton X-100 led to a release of all the trypsin contained in the liposomes. In contrast to this, even in the presence of 5-fold excess of polyacrylic acid in respect to EYPC, activity of the enzyme remained about a quarter of its activity in the sample containing solubilized liposomes. These data strongly contradict the solubilization mechanism and point towards a formation of small pores permeable for the substrate and the inhibitor. In this case, the enzymatic hydrolysis of BAEE is kinetically limited by diffusion of BAEE into liposomes and benzamidine and the product of hydrolysis (N-benzoyl-L-arginine) to the external medium.

Formation of pores in liposome membranes may result from formation of transmembrane pH-gradient. To verify this, we prepared non-gradient liposomes loaded with trypsin reversibly inhibited by acidic pH (pH 4.2). If pH-gradient would be the reason for PAA-induced leakage of liposomes, no activity would be recorded in this case. When these liposomes were solubilized by 0.05% Triton X-100 and an aliquot of this sample was added to BAEE solution at pH 8.0, large activity was observed (Fig. 1b, curve 1). In contrast, if the same sample was mixed with 63 mM PAA, incubated for 15 min at pH 4.2 and then added to BAEE solution, much lower activity was recorded (Fig. 1b, curve 2), but in the course of time this activity gradually increased resulting in the upwards bending of the kinetic curve (Fig. 1b, curve 2). This observation indicates that in this case also trypsin was not withdrawn from liposomes but protons, buffer components and the substrate slowly diffused through small pores formed by PAA in liposome membrane. So, it may be concluded that polyacrylic acid alters membrane permeability independently, whether transmembrane pH-gradient is present or not. So, pH value of PAA solution is the critical factor that determines its ability to permeabilize lipid membrane.

Being a cationic molecule, BAEE can be transported through the membrane in an electrostatic complex with a polyanion, however, this mechanism would imply a passage of anionic molecules through the bilayer. To investigate this, we studied a PAA induced leakage of trianionic dye pyranine from EYPC vesicles. Pyranine exhibits strong fluorescence at pH >8, while acidification to pH <6 shifts its excitation maxima by 50 nm to the lower wavelengths [21]. Therefore the leakage of pyranine from liposomes loaded with alkaline buffer (pH 9.4) to the external milieu with pH 4.2 would result in the quenching of dye fluorescence.

As shown in Fig. 2, in the absence of PAA liposomes fluorescence changed negligibly (curve 1). Addition of PAA led to a sharp drop of pyranine fluorescence and further decrease according to exponential law (Fig. 2, curve 2). This kinetics can be fitted by biexponential equation

$$(I / I_0) = A_1 \ \exp(-k_1 t) + A_2 \ \exp(-k t_2), \quad (1)$$

where k_1 and k_2 — effective constants of pyranine leakage and A_1 and A_2 parameters show the relative contributions of the corresponding terms to the whole process.

The sharp decrease in fluorescence at a moment of PAA adding is obviously due to the aggregation of liposomes, since the magnitude of

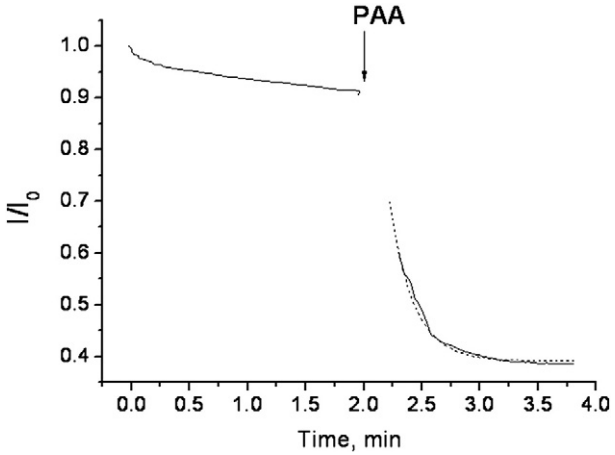

Fig. 2. Kinetics of fluorescence changes (λ_{ex} = 455 nm, λ_{em} = 509 nm) in the suspension of pyranine-loaded pH-gradient liposomes before (1) and after (2) addition of polyacrylic acid. EYPC liposomes (0.63 mM) loaded with 0.3 M glycine-0.1 M TMA-OH, pH 9.2 were suspended in 5 mM citrate-Tris, pH 4.2 and 0.63 mM of polyacrylic acid was added.

this leap is maximal at the concentrations of PAA corresponding to maximal aggregation. This aggregation was observed previously and is due to multi-point electrostatic interactions of PAA with EYPC bilayer possessing slight positive charge under acidic conditions [16]. The further decrease in pyranine fluorescence corresponds to the superposition of at least three processes: (i) growth and flotation of liposomal aggregates; (ii) spontaneous and (iii) PAA-mediated leakage of pyranine from liposomes:

$$\Delta I = I_{aggregation} + I_{leakage} = I_{aggregation} + I_{background} + I^{polymer-mediated}_{pores} \quad (2)$$

To confirm pyranine leakage directly, liposomes were incubated with PAA (C(PAA)/C(EYPC) = 1.2) for 15 min, which resulted in a decrease in pyranine fluorescence by 82% (ΔI in Eq. (2)). Subsequently, the liposomes and their aggregates were separated from the released pyranine by gel-permeation chromatography on Sepharose CL-4B column which revealed that ca. 70% of pyranine was eluted in the peak corresponding to the released pyranine ($I_{background} + I^{polymer-mediated}_{pores}$). Taking into account that the preparation of pyranine-loaded vesicles comprises their purification under the same conditions, it is concluded that free pyranine emerged in the sample due to a PAA-induced release and the contribution of aggregation processes in the observed changes of fluorescence equals 12% in this case. It means that PAA-mediated pores gate not only cationic BAEE molecules, but also trianionic molecules of pyranine.

Comparing mode of action of different polyacids on EYPC membrane permeability, we have previously shown that not all polyacids, but only those capable of multipoint interactions with the liposomal membrane can immerse into lipid bilayer and induce formation of pores [16]. In our subsequent experiments, we aimed to investigate the nature of intermolecular forces that determine immersion of polyacrylic acid into phosphatidylcholine bilayer at low pH.

It is commonly accepted that hydrophobic interactions favor immersing of solutes into lipid bilayers [2], and they mainly determine interaction of hydrophobic poly-(2-ethylacrylic acid) with lipid bilayers [7–10]. However, it seems doubtful that hydrophilic polyacrylic acid chains with no documented propensity to form secondary structures at this low pH can interact with lipid membrane due to this factor. Another plausible mechanism of PAA interaction with EYPC bilayers relies on hydrogen bonding with ester oxygen of phosphatidylcholine molecules. Such interaction may be responsible for an expansion of lipid bilayers and a resulting insertion of polyacid in the glycerol residues region of the bilayers. However, such interaction can hardly explain a formation of perforating defects in the membrane. The third possible driving force of the insertion of polyacrylic acid into lipid bilayer might be dipole potential of lipid bilayer.

The dipole potential is an electrical potential within phospholipid membranes, which arises from the alignment of dipolar residues of the lipids and water dipoles in the region between the aqueous phases and the hydrocarbon-like interior of the membrane. For a fully saturated phosphatidylcholine membrane, the value of dipole potential is estimated to be about 250–400 mV [22,23], positive in the membrane interior. Fig. 3 displays diagram of intramembrane fields in a bilayer composed of phospholipids.

Uncharged repeat units of weak polyacids and polymeric loops possess dipole moment. Entering into the water-lipid interface due to Coulomb interactions, they fall in the field of action of intramembrane potential which can draw polymeric loops into the membrane-water interface thus favoring expansion of lipid bilayer. Several arguments for the involvement of the membrane dipole potential in the interaction of polyacrylic acid with phosphatidylcholine bilayer in slightly acidic milieu will be presented below.

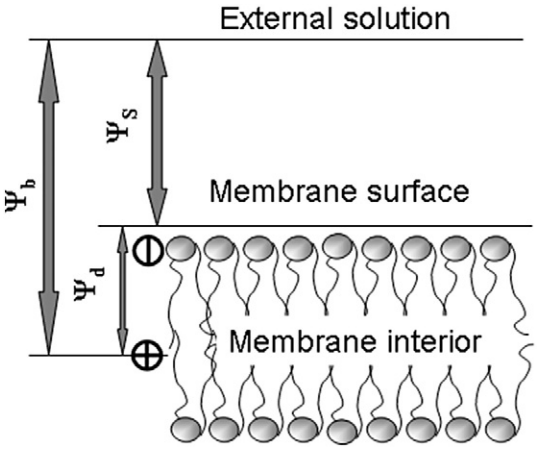

Fig. 3. Scheme of **e**lectrical potentials of lipid bilayer: Ψ_s — is the surface potential, that arises from charged residues at the membrane/solution interface, Ψ_d — is the dipole potential of lipid bilayer, it results from the alignment of dipolar residues of the lipids and associated water molecules within the membrane, Ψ_b — boundary potential, i.e. overall potential between the membrane interior and the bulk.

3.2. Use of dipole potential modifying substances

To study the influence of dipole potential on the ability of polyacrylic acid to permeabilize lipid bilayers, we evaluated the effect of dipole modifying substances (phloretin and 6-ketocholestanol) on the polyacrylic acid induced membrane permeabilization. In case protonated polyacrylic acid enters bilayer due to interaction between intramembrane field and dipole moment of repeat units or loops of the polymer, negative pole of the polymer dipole should be directed to the bilayer interior. It is therefore expected that the substances decreasing dipole potential should inhibit formation of pores, and those which increase dipole potential should cause opposite action.

Addition of phloretin, a molecules which is known to decrease dipole potential of lipid membrane [24,25] caused a deceleration of pyranine leakage from liposomes in the presence of polyacrylic acid in a concentration-dependent manner (Fig. 4a). Taking into account that phloretin is an uncharged molecule and at low concentration hardly can interact with polyacrylic acid, these data imply that reduction of intramembrane field in the presence of phloretin impedes insertion of the polyacid into lipid bilayer.

It would be reasonable to expect that dipole potential increasing sterol, 6-ketocholestanol, [26] would increase the rate of polyacrylic acid induced leakage of pyranine. However, addition of 10% of 6-ketocholestanol into lipid bilayer decreased the rate of PAA-induced membrane permeabilization nearly by the order of magnitude (Fig. 4b, curves 1 and 2). Similar result was obtained when polyacrylic acid was added to EYPC liposomes containing 10% (mol.) of cholesterol (Fig. 4b, curve 3). These facts may be explained by well-known increase in the bilayer area expansion modulus [28] and microviscosity [27], caused by cholesterol in phosphatidylcholine bilayers. The decrease in the membrane responsiveness of cholesterol-containing bilayers to poly-(2-ethylacrylic acid) has been previously reported in [29]. It is reasonable to suggest that sterols increase deformation free energy of lipid bilayers thus impeding polyacrylic acid penetration into water/lipid interface and this effect overbalances the increase in the membrane dipole potential induced by 6-ketocholestanol.

3.3. Influence of polyacrylic acid on the fluorescence properties of potential-sensitive dye RH-421

If polyacrylic acid indeed enters lipid bilayer due to a gradient of transmembrane field, its insertion should decrease dipole potential. To assess dipole potential of liposome membrane we used a potential-

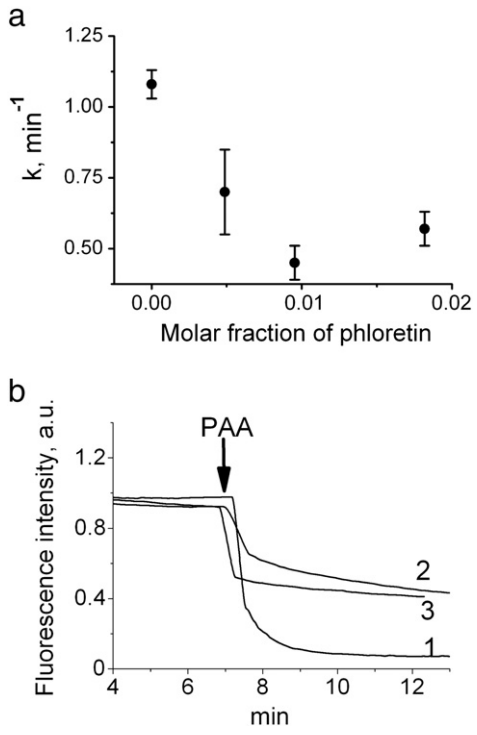

Fig. 4. Dependence of effective rate constant of polyacrylic acid induced pyranine leakage from pH-gradient liposomes on the molar fraction of phloretin added to EYPC liposomes. (a) Kinetic traces of polyacrylic acid induced pyranine leakage from EYPC pH-gradient liposomes (1), liposomes containing 10% (mol.) of 6-ketocholestanol (2) and 10% of cholesterol (3). (b) Equimolar amounts of polyacrylic acid were added to liposomes (0.63 mM) loaded with 0.5 mM pyranine solution in 0.3 M glycine-TMA-OH buffer pH 9.2, external solution contained 5 mM citrate-Tris buffer, pH 4.2 supplemented with 0.3 M sucrose, 30 °C.

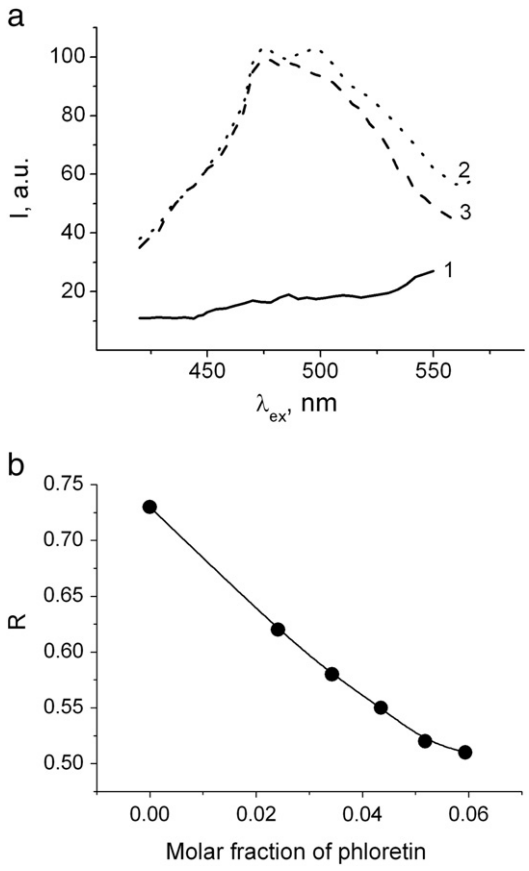

Fig. 5. Fluorescence spectra of RH-421 in citrate-Tris buffer solution at pH 4.2 (1), 7.0 (2) and in the presence of EYPC liposomes (0.5 mg/ml) at pH 4.2 (3) and 7.0 (4). (a) Effect of phloretin on the ratio of RH-421 fluorescence intensity produced by excitation at 440 nm to that produced by excitation at 540 nm, $R = I_{440}/I_{540}$. (b) Liposomes were prepared in 5 mM citrate-Tris buffer, 30 °C.

sensitive dye, RH-421. The ratio of the RH-421 fluorescence intensity produced by excitation at 440 nm to that produced by excitation at 540 nm, $R = I_{440}/I_{540}$, linearly increases with the growth of dipole potential of lipid bilayer [30].

RH-421 possesses tertiary amino group with a pK_a value of 4.7 in its aqueous solutions and 4.1 in DOPC bilayers [31], therefore its binding to liposomes at pH 4.2 is different from that observed at pH 7.0 (Fig. 5a). R value measured at pH 4.2 was 0.73 that is substantially lower than that measured at pH 7.0 (1.28). The latter value is close to that reported by Clarke ($R = 1.372$) in EYPC vesicles at 7.0 [32]. The changes in R value measured at pH 4.2 and 7.0 are obviously due to the changes in electronic structure and polar headgroup area of lipid molecules at lower pH. It cannot be excluded that changes in the boundary potential of lipid bilayer at pH 4.2 also contribute to the observed R value.

As shown in Fig. 5b, addition of phloretin at pH 4.2 gradually decreased R value, indicating that RH-421 can be used as dipole potential indicator under slightly acidic conditions.

Addition of polyacrylic acid to the solution of RH-421 at pH 4.2 did not alter its spectrum (data not shown) indicating that slightly charged polyacrylic acid does nor interact with RH-421 in slightly acidic medium. Interaction of polyacrylic acid with EYPC liposomes in the presence of RH-421 slightly decreased R value from 0.73 to 0.69 (Fig. 6, curve 1). However, this effect is very small compared to the conventional effectors of dipole potential, such as phloretin (see Fig. 5b). Interestingly, the value of polyacrylic acid induced drop of dipole potential considerably increased when 10% of 6-ketocholestanol was added to lipid bilayer. This dipole potential modifier enhanced the R value from 0.73 up to 0.89, while addition of polyacrylic acid in the presence of 6-ketocholestanol decreased R down to 0.75 (Fig. 6, curve 2). This result comes in a seeming contradiction with the above observation of 6-ketocolestanol-induced reduction of the rate of polyacrylic acid mediated pyranine leakage (see Fig. 4b). We believe that a plausible reason for this lies in the different response of the membrane permeability and its dipole potential to an increase in stiffness of lipid bilayer induced by addition of 6-ketocholestanol.

It is reasonable to suggest that if PAA macromolecule spans through lipid bilayer, its influence on dipole potential of both leaflets of the bilayer would be similar and opposite by sign. Hence, the resulting change of dipole potential traceable by RH-421 would be insignificant, since RH-421 is randomly distributed between bilayer leaflets [33]. Indeed, as shown in Fig. 6 (curve 1), addition of PAA to

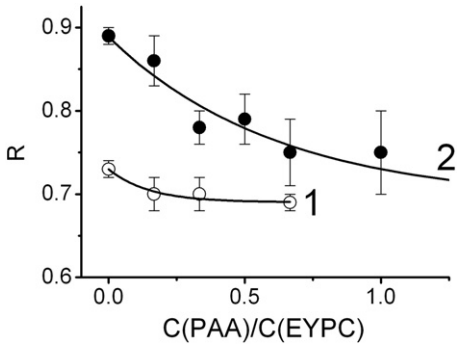

Fig. 6. Ratio of the RH-421 fluorescence intensity produced by excitation at 440 nm to that produced by excitation at 540 nm, $R = I_{440}/I_{540}$, as a function of molar excess of polyacrylic acid over lipid present in the sample of EYPC liposomes (1) and EYPC/6-ketocholestanol (9:1) liposomes (2), 5 mM citrate-Tris buffer, pH 4.2, 30 °C.

EYPC had no effect on RH-421 fluorescence. If lipid membrane contains 6-ketocholestanol or cholesterol, PAA incorporation into lipid bilayer is hampered, so PAA influence is restricted to the external leaflet of lipid bilayer. This may result in a decrease in dipole potential of the external monolayer, while internal one experiences much less influence of the polymer. Therefore, the overall changes in dipole potential are more pronounced (Fig. 6, curve 2). To further verify this reasoning, we studied effect of PAA on dipole potential of lipid monolayers formed on air/water interface.

3.4. Effect of polyacrylic acid on the properties of lipid monolayers

Potential drop on air/lipid monolayer/water interface was reported to be strongly dependent on the drop of dipole potential [23]. Surface potential of lipid monolayer ΔV measured by Kelvin electrode can be represented as a superposition of two components, one of which, ΔV_0, does not depend upon lipid concentration in the monolayer and in first approximation may be regarded as boundary potential of lipid monolayer, while the second is proportional to the density of monolayer and represents dipole potential:

$$\Delta V = \Delta V_0 + 12\pi\mu_\perp / A, \quad (3)$$

where $\mu_\perp$ — is the 'surface dipole moment' expressed in milliDebye (mD) units, and A — is the lipid molecular area in $Å^2$/molecule.

Compression of DOPC monolayer formed on slightly acidic water solution resulted in a monotonic growth of both, surface pressure and surface potential (Fig. 7a and b). Increase in the surface pressure up to 10 mN/m, which corresponds to an area per lipid polar headgroup about 75 $Å^2$, resulted in a growth of surface potential up to 200 mV. Somewhat lower values of surface potential (about 150 mV), [34] were reported previously for DOPC monolayers formed at pH 7.4. Addition of 2 mM of polyacrylic acid to the subphase resulted in a considerable growth of surface pressure by 4–5 mN/m at intermediate compression extents (Fig. 7a). This fact indicates that the polymer-lipid interactions lead to an expansion of lipid monolayer. Similar expansion was observed previously when a cationic polymer was added to the monolayer containing negatively charged O-pyromellitylgramicidin charged lipid monolayer [35]. Besides monolayer expansion, adsorption of polyacrylic acid also induced a strong reduction of its surface potential by nearly 100 mV in the whole range of surface pressures (Fig. 7b). Compression of the monolayer up to the pressures exceeding 35 mN/m resulted in the squeezing out of the polymer from lipid monolayer. This conclusion is made from the concordance of π-A isoterms obtained in the absence and in the presence of the polymer (Fig. 7a).

To further confirm the involvement of dipole potential in the interaction of polyacrylic acid with lipid membranes, we compared the effects induced by this polymer in monolayers formed by ester and ether lipids. It is known that dipole potential of bilayers or monolayers built from ether lipids is much lower than of those from naturally occurring ester phospholipids. If dipole component of surface potential is involved in the polyacid-lipid interaction, such interaction should be weaker with the monolayer formed by ether phospholipids. As shown in Fig. 7b, addition of polyacrylic acid to monolayer formed by 1,2-di-O-(9Z-octadecenyl)-*sn*-glycero-3-phosphocholine (18:1 diether PC) caused the increase in surface pressure by only 1–2 mN/m, i.e. to two-fold lesser extent than it was observed with DOPC monolayer. In accordance with the previously reported data, surface potential of 18:1 diether PC monolayer was less than that of DOPC by about 110 mV. Addition of polyacrylic acid into the subphase of such monolayer at pH 4.2 resulted in a reduction of this value by approximately 57 mV that is also substantially lower than in the case of ester lipid DOPC (Fig. 7c). These data imply that polyacrylic acid interacts with ether lipid monolayer weaker than with that formed by ester lipid DOPC.

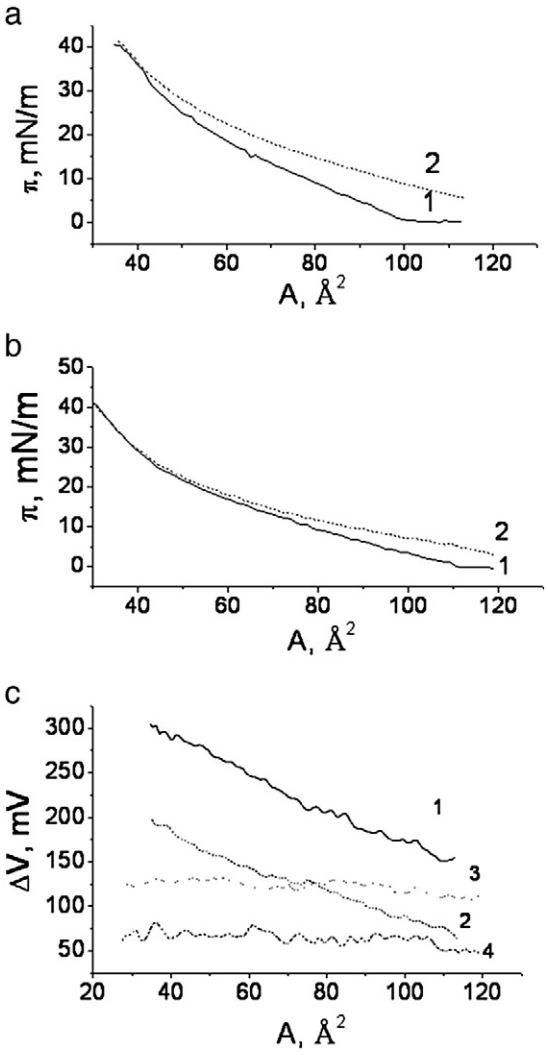

Fig. 7. Dependence of surface pressure (a, b) of DOPC (a) and 18:1 diether PC (b) monolayer on the area per lipid molecule in the absence (1) and in the presence of 2 mM of polyacrylic acid (2). Panel (c) represents dependence of surface potential of DOPC (1, 2) and 18:1 diether PC (3, 4) monolayers on the area per lipid molecule in the absence (1, 3) and in the presence (2, 4) of 2 mM of PAA. The subphase solution contained 5 mM citrate-Tris buffer, pH 4.5.

Similar regularities were observed upon interaction of polyacrylic acid with monolayers built from saturated ester lipid DPPC and its ether analog di-18:0 ether PC. In this case polyacrylic acid caused an expansion of lipid monolayer, the effect for DPPC being nearly twice higher than that for di-18:0 ether PC. Increase in the surface pressure up to 30 mN/m resulted in the squeezing polyacrylic acid out from the monolayer. Surface potential of DPPC monolayer decreased by about 37 mV upon polyacrylic acid adsorption, while in the case of 18:0 ether PC this decrease was less pronounced (29 mV) pointing to a reduced affinity of polyacid to ether PC monolayer. This fact agrees with the above presented regularities of polyacid adsorption on unsaturated lipid monolayers (Table 1).

To evaluate influence of polyacrylic acid on the dipole potential of monolayers, following Eq. (3), we plotted surface potential ΔV versus 1/A for both lipids. Fig. 8 represents the data referred to intermediate compression extents of the monolayers, the data points corresponding to gas phase and approaching collapse of monolayer being excluded. It is seen that all graphs are linear. Parameters of Eq. (3) for monolayers of both lipids are presented in Table 1. It is seen, that DPPC monolayer exhibited ΔV_0 (65 mV) and $\mu_\perp$ (551 mD) values close to those obtained previously for POPC at pH 6.8 by Brockman (124 mV and 473 mD, respectively) [23]. In contrast to this, monolayers of

Table 1
Limiting areas per molecule (A_0), collapse pressures (π_c), maximum surface potentials (ΔV_0), and effective dipole moments ($\mu_\perp$) for pure monolayers of studied phospholipids at pH 4.2 and after addition of 2 mM of polyacrylic acid into subphase.

	A_0	ΔV_0, mV	$\mu_\perp$, mD	Ψd[a], mV
DOPC	69 ± 4	92 ± 5	244 ± 9	133 ± 0.1
18:1 diether PC	64 ± 3	104 ± 5	33 ± 9	19 ± 0.1
DOPC + PAA	80 ± 5	55 ± 4	138 ± 6	65 ± 0.5
18:1 diether PC + PAA	65 ± 4	54 ± 8	20 ± 12	12 ± 0.2
DPPC	58 ± 4	56 ± 4	570 ± 8	370 ± 0.6
18:0 diether PC	56 ± 5	176 ± 7	205 ± 10	138 ± 0.2
DPPC + PAA	70 ± 6	44 ± 5	307 ± 9	165 ± 0.1
18:0 diether PC + PAA	63 ± 4	147 ± 4	128 ± 6	77 ± 0.5

[a] Value of dipole potential was calculated from $\mu_\perp$ values with the molecular area of lipid, A_0.

ether lipid were characterized by higher value of ΔV_0 (158 mV) and much lower value of $\mu_\perp$ (232 mD), that agrees well with the fact that ester groups strongly contribute to the overall dipole moment of lipid molecules and dipole potential of lipid monolayer and bilayer.

Addition of polyacrylic acid to the monolayers under study was accompanied by a decrease in both ΔV_0 and $\mu_\perp$ values demonstrating that polyacid adsorption influences not only boundary potential of the monolayer, but also decreases its dipole potential. Importantly, the drop of boundary potential (ΔV_0) of unsaturated ether lipid monolayer was nearly negligible indicating lower adsorption of polyacrylic acid. This observation is in accord with the above mentioned lower expansion of ether lipid monolayer induced by addition of polyacrylic acid (Fig. 8a and c). The decrease of $\mu_\perp$ value induced by polyacrylic acid in ester lipid monolayer was also nearly two-fold to that induced in the monolayer composed of ether lipid. The corresponding decrease of dipole potential of lipid monolayer composed of ester lipid achieved 108 mV, whereas the effect of polyacid on the dipole potential of ether lipid monolayer was much lower (53 mV).

Taken together, the data obtained in the experiments with lipid monolayers clearly show that insertion of polyacid into lipid structures decreases their intrinsic dipole potential. It is reasonable to assume that intramembrane electric field is an important driving force for the insertion of polyacids into lipid membranes.

4. Discussion

Interest to the interaction of weak polyelectrolytes with phosphatidylcholine membranes in slightly acidic medium resides in their application as pH-sensitive motifs in the design of pharmaceutically active polymers [3,4]. About two decades ago it has been shown that interaction of hydrophobic polyacids with phosphatidylcholine bilayers in slightly acidic medium results in complete solubilization of liposomes and formation of polymer-lipid particles stabilized by electrostatic, hydrophobic and hydrogen bonds [7–9]. Previously, we have shown that in contrast to this, polyacrylic acid is unable to form such particles. In the present paper, we demonstrate that its adsorption on lipid membrane in slightly acidic medium results in the insertion of the polymer into lipid bilayer and formation of hydrophilic pores permeable for small ions, but impermeable for large molecules, protein or DNA. It means that polyacrylic acid containing polymer constructs can be used in pH-targeted delivery of small molecules. This principle has already been implemented by constructing polyacrylic acid grafted liposomes which were capable of releasing of their content in slightly acidic medium [11,14].

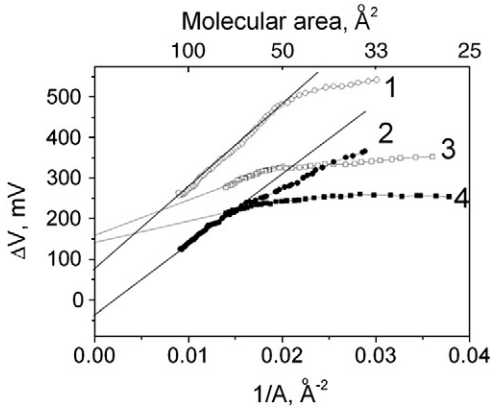

Fig. 8. Dependencies of the surface potential of DPPC (1, 2) and 18:1 diether PC (3, 4) monolayers on the reciprocal molecular area in the absence (1, 3) and in the presence (2, 4) of 2 mM of polyacrylic acid in the subphase. The data are taken from Fig. 6. Lines show extrapolation of the surface potential to the infinite molecular area and thus give ΔV_0 as intercept.

Bilayer insertion of a hydrophilic polyacid seems to be thermodynamically unfavorable due to the losses in the free energy of hydration water occurring upon transfer of hydrophilic carboxylic groups from water into hydrophobic environment. Indeed, as it is shown in Fig. 1a considerable permeabilization of phosphatidylcholine bilayer occurs upon addition of the polymer in concentrations comparable to that of lipid. Previously we have shown that adsorption of polyacrylic acid on lipid bilayers is very effective, only negligible concentrations of unbound polyacrylic acid being detected in the medium after separation of liposomes via centrifugal ultrafiltration [15]. Taken together, these facts confirm thermodynamic disadvantage of polyacid insertion into lipid bilayer. The squeezing of polyacrylic acid out from lipid monolayers upon excessive compression shown in the present paper (Fig. 8a and c) also supports this statement.

Large value of the membrane dipole potential is commonly recognized as an important factor determining orientation and functioning of membrane proteins [22]. Since polyacrylic acid is a weak polyelectrolyte (pK_a of the first ionizable repeat unit being about 4.2) its ionization degree at pH 4.2 does not exceed 5%. Therefore, once liposome surface being covered with polyacrylic acid, the polymer can form electroneutral loops or tails on liposome surface. Each repeat unit of polyacrylic acid has dipole moment with excess of negative charge on the carboxylic group (Fig. 9a). If the macromolecule is attached to the membrane surface via electrostatic forces, its electroneutral loops experience strong influence of the intramembrane field. It seems quite natural that this intramembrane field presents a factor which compensates the losses in conformational free energy and free energy of the hydration water and stimulates flexible polymer macromolecule to adopt conformation whose dipole moment is directed oppositely to the intramembrane field. Finally this field 'drags' the polymer loops into the membrane interior, causing expansion of lipid bilayer and formation of hydrophilic pores. Expansion of lipid packing was shown in the present work using monolayer technique (Fig. 7a and b) and the ability of polyacrylic acid to form hydrophilic pores is proved by its ability to cause activation of trypsin entrapped into liposomes (Fig. 1) and leakage of pyranine from liposomes (Fig. 2).

We obtained at least two lines of evidence that dipole potential is really involved in the insertion of polyacid into lipid bilayer. Firstly, phloretin, which is known to decrease dipole potential, was found to considerably reduce the rate of polyacrylic acid-induced pyranine leakage. Secondly, direct measurements of lipid monolayer surface potential showed that adsorption of polyacrylic acid induces the decrease in both boundary and dipole potential by about 60 and 108 mV for ester lipid DPPC. Addition of polyacrylic acid to the monolayer of 18:1 ether PC produced negligible response in dipole potential of monolayer (about 7 mV) and more significant but also relatively low response in its boundary potential (about 50 mV) (Table 1). This observation agrees with our assumption that carbonyl groups of lipid molecules are essential for the interaction of lipid with polyacids.

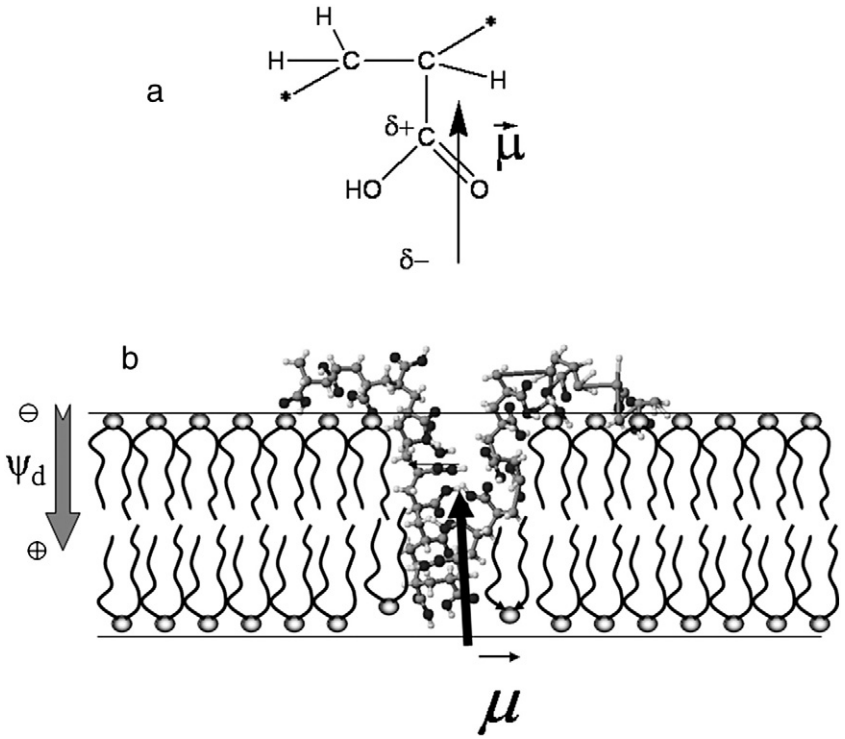

Fig. 9. Structure of a repeat unit of polyacrylic acid and the direction of its dipole moment (a) and tentative threaded configurations of polyacrylic acid in the complex with lipid bilayer (b). Gray arrow shows the directions of intramembrane field (dipole potential Ψ_d), and black arrow shows the direction of dipole moment of the loop of the polymer chain entering lipid bilayer.

Can dipole potential favor insertion of other polyacids into lipid bilayer? Our previous study on the ability of different polyacids to adsorb on and permeabilize egg lecithin membranes allows assuming that the polymers adopting helical conformation in slightly acidic medium such as polymethacrylic, polyglutamic acids and DNA, are nearly unable to form pores in lipid bilayer [16]. It can be hypothesized that in this case dipole moment of repeat units is canceled out and insertion of such polymers is only marginally influenced by the membrane dipole potential. In the latter two cases, large ionization degree of the polymers also interferes with their insertion into lipid bilayer. However, insertion of helical peptides possessing dipole moment directed lengthwise the helix should strongly depend upon dipole potential of the membrane as shown in [35] by example of gramicidin A. This situation should take place also in case of GALA-containing peptides and other cell penetrating peptides [36].

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