

# Binding of trivalent metal ions ( $\text{Al}^{3+}$ , $\text{In}^{3+}$ , $\text{La}^{3+}$ ) with phosphatidylcholine liposomal membranes investigated by microelectrophoresis

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**Abstract.** Interactions between trivalent metal ions ( $\text{Al}^{3+}$ ,  $\text{In}^{3+}$ ,  $\text{La}^{3+}$ ) and phosphatidylcholine (PC) liposomes are studied by microelectrophoresis. The dependence of the PC membrane surface charge density and zeta potential on  $p\text{H}$  ( $p\text{H}$  range from 2 to 10) of the aqueous metal chloride solutions is determined. The obtained results indicate the adsorption of  $\text{Al}^{3+}$ ,  $\text{In}^{3+}$  and  $\text{La}^{3+}$  ions on phosphatidylcholine model membranes, leading to changes in the electrical properties of the membranes. The theoretical considerations on equilibria occurring between phosphatidylcholine liposomal membrane and trivalent metal ions are presented. A mathematical model describing the interactions in a quantitative way is proposed.

## Introduction

Lipid membranes represent the most important biological interface and bind a wide variety of substances: proteins, surfactants, cations, anions. The ionic composition of the surrounding medium can influence on a number of membrane properties (*e.g.*, dipole potential, membrane surface charge) and membrane phenomena (*e.g.*, transport across the membrane, phase transition). The literature on interactions between membranes and anions as well as between membranes and cations is considerable [1–12]. Cations interact with biological and model membranes through electrostatic binding to phospholipids headgroups. The interactions are very strong with charged lipids, *e.g.* phosphatidylserine, but moderate with zwitterionic lipids, *e.g.* phosphatidylcholine. The strength of the cation-lipid interaction increases with the valence of the adsorbing cation [13, 14].

Trivalent cations have high affinity for lipids phosphate groups leading to changes in the physicochemical properties of membranes. A special place among trivalent ions takes aluminium, because of its prevalence in daily life, observed interference with several biological processes and toxic effects on the living organism. Al is related to a number of disease states, particularly those relating to oxidative stress [15]. The ion plays a role in the etiology of sporadic Alzheimer's disease and other neurodegenerative disorders [16].  $\text{In}^{3+}$  is closely related to aluminium ion in its physical and chemical properties,

$\text{La}^{3+}$  although not so closely related to aluminium ion has similar chemical properties with the ion [17].  $\text{In}^{3+}$  is applied in anticancer therapy and nuclear medicine [18, 19] while  $\text{La}^{3+}$  in the electronic industry.  $\text{La}^{3+}$ , as well as other lanthanide ions, possesses unique chemical properties which make them valuable probes of the interaction of  $\text{Ca}^{2+}$  with biological systems. The intoxication with these metals results in diseases of liver, kidney, lungs and the central nervous system in living organism, both animals and human. The data reported in the literature show the influence of trivalent ions on properties of lipid membranes. It was demonstrated that  $\text{La}^{3+}$  promotes fusion and permeabilization of phosphatidylserine vesicles [20]. Furthermore,  $\text{La}^{3+}$  can modulate the gating properties of a voltage-gated sodium channel, it also has significant impact on the structure and stability of the phospholipid membranes [21].  $\text{Al}^{3+}$  and related cations induce membrane rigidification, which may cause changes in the phase state of the bilayer or in membrane hydration that could lead to higher rates of lipid oxidation [22]. It was demonstrated that Al promotes phosphatidylcholine-phosphatidylserine liposome fusion, aggregation and lipid packing [23]. Also, the stimulatory effect of Al on  $\text{Fe}^{2+}$  initiated lipid peroxidation in erythrocytes, liposomes and microsomes was shown [24–26].

This work continues the systematic study of interactions of cation binding to model lipid membranes, undertaken by Figaszewski and co-workers [27–34]. The interactions of aluminium, indium and lanthanum ions with phosphatidylcholine membranes were investigated through experimental studies and theoretical

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considerations. Phosphatidylcholine is the most abundant lipid in mammalian cell membrane. This neutral, zwitterionic lipid possesses one negatively charged (phosphate) and one positively charged group (trimethylammonium). Therefore phosphatidylcholine is a good prototype lipid to study the effects of ion binding to biological membranes. Liposomes were used as model membranes. The microelectrophoresis method was used to determine the membrane surface charge density, which is not only a function of the membrane composition, but also depends on environmental factors such as *pH* and electrolyte concentration. An attempt was also made to describe in a quantitative way equilibria between zwitterionic (phosphatidylcholine) membrane and solution ions coupled with the determination of association constants characterizing the equilibria. Theoretical considerations are presented (see appendix).

## Experimental

### Materials

L- $\alpha$ -phosphatidylcholine (PC) from egg yolk, type XVI-E,  $\geq 99\%$  (TLC), lyophilized powder were purchased from Sigma-Aldrich and used without further purification. HPLC grade ( $\geq 99\%$ ) chloroform, was purchased also from Sigma-Aldrich. Inorganic chemicals were of analytical grade quality (NaCl  $\geq 99,7\%$ , AlCl<sub>3</sub>  $\geq 99\%$ , InCl<sub>3</sub>  $\geq 98\%$ , LaCl<sub>3</sub>  $\geq 99,99\%$ ) from POCH (Gliwice, Poland). All the salts were prepared freshly before use. All solutions and cleaning procedures were performed with water purified by means of a Milli-Q plus water purification system (Millipore, USA) with a resistivity of 18.2 M $\Omega$  cm.

### Preparation of liposomes

Liposomes were prepared by a sonication technique. The stock solution of phosphatidylcholine in chloroform (10 mg ml<sup>-1</sup>) was prepared. Then the solvent was evaporated under a gentle stream of argon to form a thin dry film. The resulting lipid film was hydrated with appropriate aqueous medium. Liposomes were formed by sonicating the suspension using an ultrasound generator UD 20 (Techpan, Poland). Sonication was applied five times for 90 seconds. Since, during the process heat is liberated, cooling the suspension was necessary. It was carried out by using an ice bath (container with a mixture of ice and dry sodium chloride). Phosphatidylcholine liposome sizes determined using a Zetasizer Nano ZS (Malvern Instruments, UK) apparatus exhibited a size distribution profile, with one population (representing approximately 90% of all particles) with a diameter 160 nm and the other (representing about 9% of the particles) with a diameter 30 nm. The size and distribution of the liposomes have been evaluated from the intensity of the dispersed light.

### Electrophoretic mobility measurements

Electrophoretic mobility measurements of liposomes were carried out as a function of *pH*. A Malvern Instruments,

Zetasizer Nano ZS was used. The reported values were the average of six measurements performed at a given *pH*. All experiments were performed at least three times.

The zeta potential  $\zeta$  values were calculated from the electrophoretic mobilities by using the Henry correction of Smoluchowski's equation

$$\zeta = \frac{3\mu\eta}{2\varepsilon\varepsilon_0 f(\kappa a)}, \quad (1)$$

where  $\mu$  is the electrophoretic mobility,  $\eta$  is the viscosity of the aqueous solution,  $a$  is the particle radius,  $\kappa^{-1}$  is the Debye length,  $\varepsilon_0$  and  $\varepsilon$  are the permittivity of free space and the relative permittivity of the medium, respectively.

The surface charge density values were calculated from the electrophoretic mobilities using the equation [35]

$$\delta = \frac{\mu\eta}{d}, \quad (2)$$

where  $d$  is the diffuse layer thickness

The diffuse layer thickness was determined from the formula

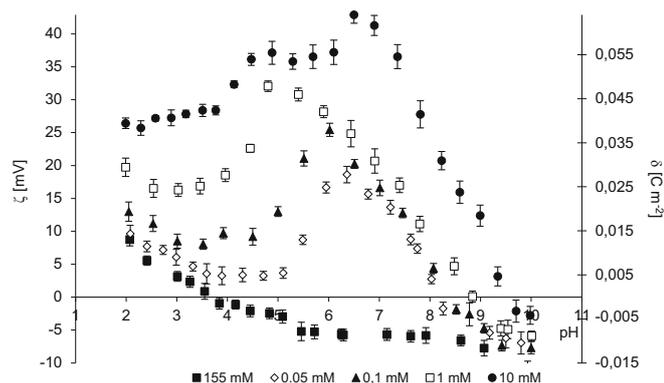
$$d = \sqrt{\frac{\varepsilon\varepsilon_0 RT}{2F^2 I}}, \quad (3)$$

where  $R$  is the gas constant,  $T$  is the temperature,  $F$  is the Faraday number and  $I$  is the ionic strength of the electrolyte.

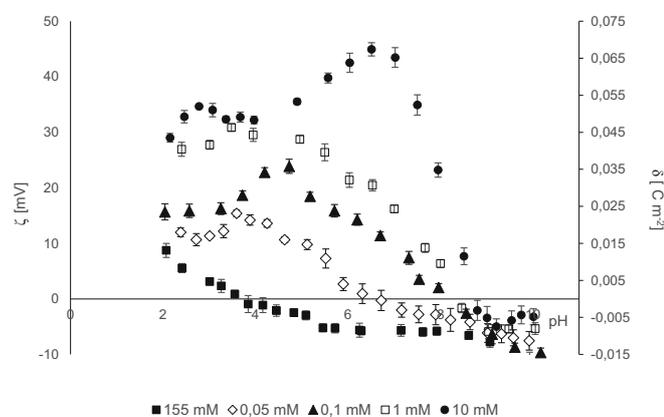
## Results and discussion

The effect of different trivalent cations (Al<sup>3+</sup>, In<sup>3+</sup> and La<sup>3+</sup>) in chloride electrolytes on zeta potential and surface charge density of phosphatidylcholine liposomal membranes as a function of electrolyte concentration was studied. A series of electrophoretic mobility measurements of sonicated phosphatidylcholine liposomes were performed (*pH* range from 2 to 10). From the electrophoretic mobilities, the zeta potential and the membrane surface charge densities were calculated, using eqs. (1) and (2), respectively. The obtained results allowed us to evaluate the impact of both the concentration and the type of trivalent metal ion on the PC membrane.

Figure 1 shows the influence of the trivalent metal ion concentration and *pH* value on the zeta potential (left axis) and on the phosphatidylcholine membrane surface charge densities (right axis). Additionally, on each of the figures, data obtained in 155 mM NaCl (in absence of trivalent ions) are plotted. The increase of the positive surface charge and the slight decrease of the negative surface charge with the increase in aluminium ion concentration (at a given *pH* value) is observed (fig. 1). The binding of trivalent metal ions with zwitterionic phosphatidylcholine surface causes a change in the magnitude and sign of the zeta potential/the membrane surface charge density. The isoelectric point of the membrane, which is one of the most important parameters used to describe variable-charge surfaces, showed a shift towards higher *pH* values for Al<sup>3+</sup> compared Na<sup>+</sup> (from *pH*  $\sim 4$  to *pH*  $\sim 8-9$ ).



**Fig. 1.** Zeta potential (left axis) and the corresponding surface charge density (right axis) of phosphatidylcholine liposomal membranes in 155 mM NaCl +  $x$  mM  $\text{AlCl}_3$  (where  $x = 0.05, 0.1, 1, 10$ ) as a function of  $pH$ .

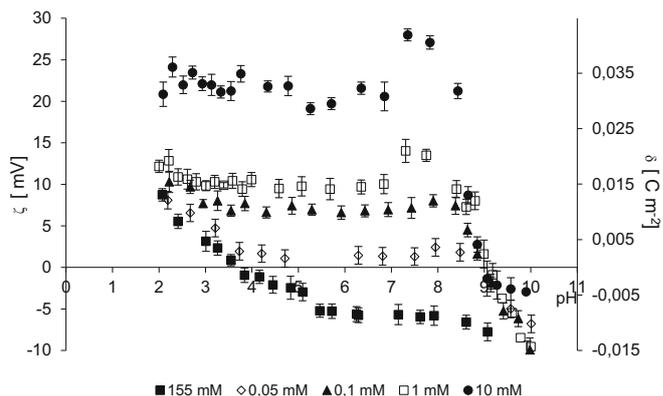


**Fig. 2.** Zeta potential (left axis) and the corresponding surface charge density (right axis) of phosphatidylcholine liposomal membranes in 155 mM NaCl +  $x$  mM  $\text{InCl}_3$  (where  $x = 0.05, 0.1, 1, 10$ ) as a function of  $pH$ .

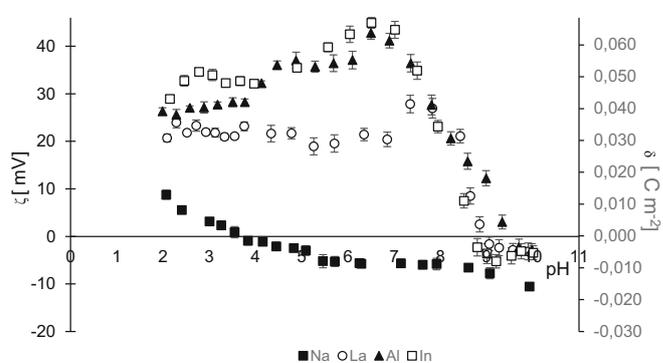
A similar behaviour was observed for the other two systems (figs. 2 and 3). The results indicate a strong association between trivalent ions and PC membrane, for which Coulomb attraction is responsible.

Figure 4 presents curves of the zeta potential (left axis) and phosphatidylcholine membrane surface charge density (right axis) as a function of  $pH$  for all electrolytes obtained by the same concentration (10 mM). Since an analogous tendency was observed for all studied concentrations (1, 0.1, 0.05 mM) only the data in 10 mM electrolytes solutions are shown. At low  $pH$  values, the highest surface charge density values were obtained for  $\text{In}^{3+}$ , whereas the lowest for  $\text{La}^{3+}$ . At high  $pH$  values, no statistically significant changes were observed.

Phosphatidylcholine contains a phosphate and a choline group. The positive charge of the choline group and the negative charge of the phosphate compensate each other such that phosphatidylcholine has a zero net charge. On the other hand, the polar character of the head group is the reason for the interaction of PC with ions [36]. The strength of ion-ion interactions is dependent on the charge densities of the ions. The ion pairs formed by cations and



**Fig. 3.** Zeta potential (left axis) and the corresponding surface charge density (right axis) of phosphatidylcholine liposomal membranes in 155 mM NaCl +  $x$  mM  $\text{LaCl}_3$  (where  $x = 0.05, 0.1, 1, 10$ ) as a function of  $pH$ .



**Fig. 4.** Zeta potential (left axis) and the corresponding surface charge density (right axis) of phosphatidylcholine liposomal membranes in 155 mM NaCl + 10 mM chloride electrolytes as a function of  $pH$ . Filled square: NaCl; open circle:  $\text{LaCl}_3$ ; filled triangle:  $\text{AlCl}_3$ ; open square:  $\text{InCl}_3$ .

anions with higher charge densities is energetically favorable because the attraction force is stronger [37]. As can be seen from the figures, the surface charge density of egg phosphatidylcholine near neutral  $pH$  in the presence of 155 mM NaCl without addition of trivalent ions is equal to  $-0.0085 \text{ C m}^{-2}$  which corresponds to a zeta potential equal to  $-5.72 \text{ mV}$ . This finding is consistent with Sabin *et al.*, who demonstrated that egg phosphatidylcholine liposomes at  $pH 7.4$  possess negative charge and for concentration around 155 mM NaCl, the zeta potential is equal to around  $-5 \text{ mV}$  [38]. On the other hand, our data seem to disagree with previous reports by a number laboratories, for example Stuart McLaughlin's research group, that phosphatidylcholine liposomes in the presence of sodium chloride solutions, at neutral  $pH$ , have a zeta potential near zero (and no net surface charge density) [39]. Winiski *et al.* reported the zeta potentials of PC vesicles, in both 10 mM and 100 mM NaCl solutions ( $pH = 7.5, 0.1 \text{ mM}$  MOPS buffer), as zero [40]. According to Klasczyk *et al.*, the zeta potential of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) in 150 mM NaCl is around  $-1.5 \text{ mV}$  [36]. We suppose that the calibration of the

equipment as well as the sample preparation may be the cause for the considerable negative surface charge density of PC liposomes reported in our paper. We used the sonication method for liposomes preparation and the exposure of phosphatidylcholine to ultrasound could facilitate hydrolysis and oxidation.

All of the analyzed ions ( $\text{Al}^{3+}$ ,  $\text{In}^{3+}$ ,  $\text{La}^{3+}$ ) adsorb onto phosphatidylcholine liposomes, leading to a change in the membrane electrical properties. The tendency of the change of the surface charge density in a function of  $\text{pH}$  is similar for all studied electrolytes. The greatest change has been observed for indium and aluminium ions, and the smallest for lanthanum ions, compared to the data obtained for monovalent ions (sodium). Our data are in accordance with the literature, *i.e.* adsorption in the electrical double layer at the membrane-electrolyte solution interface increases with the valence of the adsorbing cation [41]. Besides, even if the valency of the cations is the same, the affinity to phospholipids may be different, for example, the affinity of  $\text{La}^{3+}$  is lower than that of  $\text{Ce}^{3+}$  [37].

Al and In are closely related in their chemical and physical properties, having similar ionic radii, charge densities and coordination numbers [42]. And similarly La, although not closely related to both metals, is also trivalent and influences the phospholipid membranes properties. All these metals have strong affinity and bind electrostatically to phosphate and carboxyl groups of phosphatidylcholine. On the basis of measurements performed on phosphatidylcholine monolayers by the Langmuir method, Sabin *et al.* demonstrated that lanthanum cations specifically adsorb onto charged groups of the zwitterionic lipid molecules and induce orientation changes of the headgroup [21]. According to the authors,  $\text{La}^{3+}$  may bind with the headgroup of the PC membrane near  $-(\text{PO}_4)^-$  group which induces an electrostatic attraction between the phosphate groups of neighboring lipids. A lot of studies of the interactions between lanthanide ions and artificial membranes have employed NMR spectroscopy, which emerged as one of the most important tools used for the examinations of a large variety of issues related to changes in the structural and dynamical properties of membranes [2, 43–47]. It has been demonstrated by NMR of model membranes [44, 45] that phosphatidylcholine bind divalent cations as well as trivalent cations, such as  $\text{Pr}^{3+}$  and  $\text{Eu}^{3+}$  although the binding of the lanthanides is stronger than that of the alkaline metal cations [44]. Brown and Seelig used  $^2\text{H}$  and  $^{31}\text{P}$  NMR measurements to detect conformational changes, orientation and flexibility of the choline headgroups in phosphatidylcholine bilayers. According to the authors, the polar headgroups are bent at the position of the phosphate group so the PC dipoles are aligned parallel to the membrane surface. Addition of trivalent ions (shift reagents) to DPPC induced large changes in the spectral parameters, that is, the  $^2\text{H}$  quadrupole splittings and, depending on the ion added, also in the  $^{31}\text{P}$  chemical shift anisotropy, which must be attributed to specific ion-induced changes in the choline head group conformation of DPPC [46].

Trivalent ions interact strongly with membranes, both biological and model and cause changes in a number of membrane physico-chemical properties [20, 48, 49]. We experimentally showed that the studied ions adsorb on PC membranes causing changes in the membrane surface charge densities values. Based on the experimental data, we proposed a mathematical description of the equilibria associated with the adsorption of trivalent ions on the phosphatidylcholine membrane (see appendix). However, we were unable to determine the numerical values of the parameters characterizing the equilibria. We have shown mathematically that a large number of equilibria, which occurs in the analyzed systems considerably complicates the considered model and makes it impossible to determine the searching parameters. Making a complete interpretation of the changes occurring on the surface of lipid membranes caused by adsorption of trivalent ions will be possible after the re-analysis of the studied system, both physicochemically and mathematically.

## Conclusions

The effect of different trivalent metal ions ( $\text{Al}^{3+}$ ,  $\text{In}^{3+}$ ,  $\text{La}^{3+}$ ) in chloride electrolytes on the surface charge of phosphatidylcholine liposomal membranes was investigated. The analysis of the obtained results demonstrated a significant increase in the surface charge density with increasing cation concentration and a slight decrease in the negative charge (analogous relations were observed for all trivalent cations). The observed, at the appropriate concentration, effect of charge compensation in the phosphatidylcholine membrane, resulting in a change of the sign of the surface charge, proves a strong association of trivalent ions with the membrane, caused by the strong Coulomb attraction.

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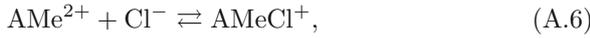
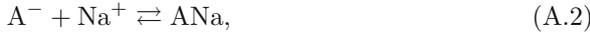
## Author contribution statement

JK carried out the experiment, analyzed the data and wrote the manuscript. ZAF did the theoretical analysis. All the authors have read and approved the final manuscript.

## Appendix A. Theoretical considerations

A ten-equilibrium model has been proposed to describe mathematically the adsorption of trivalent ions on the zwitterionic phosphatidylcholine membrane surface. In the considered system, in which mono- and trivalent

ions are present, the following interactions were assumed (eqs. (A.1)–(A.10)):



where  $A^-$  is the group  $-(PO)_4^-$ ,  $B^+$  is the group  $-N^+(CH_3)_3$  of phosphatidylcholine.

Surface concentrations of the membrane components and volume concentrations of the ions present in the solution are determined by association constants according to the equations

$$a_{AH} = K_{AH} \cdot a_{A^-} \cdot a_{H^+}, \quad (A.11)$$

$$a_{ANa} = K_{ANa} \cdot a_{A^-} \cdot a_{Na^+}, \quad (A.12)$$

$$a_{AMe^{2+}} = K_{AMe^{2+}} \cdot a_{A^-} \cdot a_{Me^{3+}}, \quad (A.13)$$

$$a_{AMe+OH} = K_{AMe+OH} \cdot a_{AMe^{2+}} \cdot a_{OH^-}, \quad (A.14)$$

$$a_{AMe(OH)_2} = K_{AMe(OH)_2} \cdot a_{AMe+OH} \cdot a_{OH^-}, \quad (A.15)$$

$$a_{AMe+Cl} = K_{AMe+Cl} \cdot a_{AMe^{2+}} \cdot a_{Cl^-}, \quad (A.16)$$

$$a_{AMeCl_2} = K_{AMeCl_2} \cdot a_{AMe+Cl} \cdot a_{Cl^-}, \quad (A.17)$$

$$a_{AMeOHCl} = K_{AMeOHCl} \cdot a_{AMe+OH} \cdot a_{Cl^-}, \quad (A.18)$$

$$a_{BOH} = K_{BOH} \cdot a_{B^+} \cdot a_{OH^-}, \quad (A.19)$$

$$a_{BCl} = K_{BCl} \cdot a_{B^+} \cdot a_{Cl^-}, \quad (A.20)$$

where  $K_{AH}$ ,  $K_{ANa}$ ,  $K_{BOH}$ ,  $K_{BCl}$ ,  $K_{AMe^{2+}}$ ,  $K_{AMe+OH}$ ,  $K_{AMe(OH)_2}$ ,  $K_{AMe+Cl}$ ,  $K_{AMeCl_2}$ ,  $K_{AMeOHCl}$  are association constants;  $a_{A^-}$ ,  $a_{Me^{3+}}$ ,  $a_{B^+}$ ,  $a_{AMe^{2+}}$ ,  $a_{AMe+OH}$ ,  $a_{AMe^{2+}}$ ,  $a_{AMe+Cl}$  are the surface concentrations of particular groups on the membrane surface [ $\text{mol m}^{-2}$ ],  $a_{H^+}$ ,  $a_{OH^-}$ ,  $a_{Na^+}$ ,  $a_{Cl^-}$  are the volumetric concentrations of the solution ions [ $\text{mol m}^{-3}$ ].

It is possible to write one more equilibrium:



Nevertheless, eq. (A.21) and eq. (A.8) are mutually dependent and therefore eq. (A.21) can be neglected in further considerations. Below a brief mathematical reasoning in order to prove this thesis was carried out.

Association constant of eq. (A.4):

$$K_{AMe+OH} = \frac{a_{AMe+OH}}{a_{AMe^{2+}} \cdot a_{OH^-}}.$$

Association constant of eq. (A.8):

$$K_{AMeOHCl} = \frac{a_{AMeOHCl}}{a_{AMe+OH} \cdot a_{Cl^-}}.$$

After the multiplication of both equilibria

$$K_{AMe+OH} \cdot K_{AMeOHCl} = \frac{a_{AMeOHCl}}{a_{AMe^{2+}} \cdot a_{OH^-} \cdot a_{Cl^-}}$$

and then dividing by eq. (A.6) leads to eq. (A.21):

$$\frac{K_{AMe+OH} \cdot K_{AMeOHCl}}{K_{AMe+Cl}} = \frac{a_{AMeOHCl} \cdot a_{AMe^{2+}} \cdot a_{Cl^-}}{a_{AMe^{2+}} \cdot a_{OH^-} \cdot a_{Cl^-} \cdot a_{AMe+Cl}}.$$

The surface concentrations of PC are given by  $C_{PC}$ :

$$a_{A^-} + a_{AH} + a_{ANa} + a_{AMe^{2+}} + a_{AMeCl_2} + a_{AMe(OH)_2} + a_{AMe+OH} + a_{AMe+Cl} + a_{AMeOHCl} = C_{PC}, \quad (A.22)$$

$$a_{B^+} + a_{BOH} + a_{BCl} = C_{PC}. \quad (A.23)$$

The surface charge density of the PC membrane is given by the equation

$$\delta = (a_{B^+} + a_{AMe^{2+}} + a_{AMe+OH} + a_{AMe+Cl} - a_{A^-})F. \quad (A.24)$$

Treating eqs. (A.11)–(A.20) and eqs. (A.22)–(A.24) as a system of equations enables the elimination of the following surface concentrations:  $a_{AH}$ ,  $a_{ANa}$ ,  $a_{BOH}$ ,  $a_{BCl}$ ,  $a_{AMe(OH)_2}$ ,  $a_{AMeOHCl}$ ,  $a_{AMeCl_2}$  (from eqs. (A.22), (A.23)) or  $a_{A^-}$ ,  $a_{B^+}$ ,  $a_{AMe^{2+}}$ ,  $a_{AMe+OH}$ ,  $a_{AMe+Cl}$  (from eq. (A.24)); the final result is the following:

$$a_{A^-} + K_{AH}a_{A^-}a_{H^+} + K_{ANa}a_{A^-}a_{Na^+} + K_{AMe^{2+}}a_{A^-}a_{Me^{3+}} + K_{AMe+OH}a_{A^-}a_{OH^-} + K_{AMe+Cl}a_{A^-}a_{Cl^-} = C_{PC}, \quad (A.25)$$

$$a_{B^+} + K_{BOH}a_{B^+}a_{OH^-} + K_{BCl}a_{B^+}a_{Cl^-} = C_{PC}. \quad (A.26)$$

Finally, eq. (A.25) has the following form:

$$a_{A^-} (1 + K_{AH}a_{H^+} + K_{ANa}a_{Na^+} + K_{AMe^{2+}}a_{Me^{3+}} + (K_{AMe^{2+}}a_{A^-}a_{Me^{3+}})(K_{AMe+OH}a_{OH^-} + K_{AMe+Cl}a_{Cl^-}) + (K_{AMe+OH}a_{OH^-} - K_{AMe^{2+}}a_{A^-}a_{Me^{3+}}) \times (K_{AMe(OH)_2}a_{OH^-} + K_{AMeOHCl}a_{Cl^-}) + (K_{AMe+Cl}a_{Cl^-} - K_{AMe^{2+}}a_{A^-}a_{Me^{3+}} + K_{AMeCl_2}a_{Cl^-}) = C_{PC}. \quad (A.27)$$

Pulling out  $a_{A^-}$  before the parenthesis leads eq. (A.27) to form

$$a_{A^-} [(1 + K_{AH}a_{H^+} + K_{ANa}a_{Na^+} + K_{AMe^{2+}}a_{Me^{3+}}) + (K_{AMe^{2+}}a_{Me^{3+}} + K_{AMe+OH}a_{OH^-}) + (K_{AMe^{2+}}a_{Me^{3+}} + K_{AMe+Cl}a_{Cl^-}) + (K_{AMe+OH}a_{OH^-} - K_{AMe^{2+}}a_{Me^{3+}} + K_{AMe(OH)_2}a_{OH^-}) + (K_{AMe+OH}a_{OH^-} - K_{AMe^{2+}}a_{Me^{3+}} + K_{AMeOHCl}a_{Cl^-}) + (K_{AMe+Cl}a_{Cl^-} - K_{AMe^{2+}}a_{Me^{3+}} + K_{AMeCl_2}a_{Cl^-})] = C_{PC}. \quad (A.28)$$

The insertion of eqs. (A.26) and (A.28) into eq. (A.24) gives the equation

$$\frac{\delta}{F} = \frac{C_{PC}}{K_{BOH}a_{OH^-} + K_{BCL}a_{Cl^-}} - \frac{C_{PC}}{[\dots]}, \quad (\text{A.29})$$

where [...] is the long parenthesis of eq. (A.28).

These mathematical operations resulted in the elimination of all unwanted parameters:  $a_{AH}$ ,  $a_{ANa}$ ,  $a_{AMe^{2+}}$ ,  $a_{AMeCl_2}$ ,  $a_{AMe(OH)_2}$ ,  $a_{AMeOHCl}$ ,  $a_{AMe+Cl}$ ,  $a_{BOH}$ ,  $a_{BCL}$ , but the obtained final eq. (A.29) is extremely complex and, in this form, impossible to solve.

The proposed quantitative description of equilibria associated with the adsorption of trivalent ions on the phosphatidylcholine membrane surface is, in our view, valid, however, it requires improvement. The large number of equilibria that exist in the analyzed system determines the presence of a large number of parameters characterizing the equilibria, whose quantitative determination, as demonstrated by the foregoing considerations, is not possible. Is therefore necessary to adopt certain simplifications, aimed at reducing the number of these parameters, only then it will be possible to design all the searched values. In our view, the acquisition of data being a combination of theoretical considerations and experimental studies, may significantly expand the knowledge about phenomena in which biological membranes in living cells are involved.

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