

The effect of metal cations on the phase behavior and hydration characteristics of phospholipid membranes

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Abstract

To characterize the specificity of ion binding to phospholipids in terms of headgroup structure, hydration and lyotropic phase behavior we studied 1-palmitoyl-2-oleoyl-phosphatidylcholine as a function of relative humidity (RH) at 25 °C in the presence and absence of Li⁺, Na⁺, K⁺, Be²⁺, Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺, Zn²⁺ and Cu²⁺ ions by means of infrared (IR) spectroscopy. All divalent cations and Li⁺ shift the gel-to-liquid crystalline phase transition towards bigger RH values indicating stabilization of the gel state. The observed shift correlates in a linearly fashion with the electrostatic solvation free energy for most of the ions in water that in turn, is inversely related to the ionic radius. This interesting result was interpreted in terms of the excess chemical potential of mixing of hydrated ions and lipids. Calcium, zinc and partially lithium, cause a positive deviation from the linear relationship. IR spectral analysis shows that the carbonyl groups become more accessible to the water in the presence of Mg²⁺, Ca²⁺, Sr²⁺ and Ba²⁺ probably because of their involvement into the hydration shell of the ions. In contrast, Be²⁺, Zn²⁺ and Cu²⁺ dehydrate the carbonyl groups at small and medium RH. The ability of the lipid to take up water is distinctly reduced in the presence of Zn²⁺ and, partially, of Cu²⁺ meaning that the headgroups have become less hydrophilic. The binding mode of Be²⁺ to lipid headgroups involves hydrolyzed water. Polarized IR spectra show that complex formation of the phosphate groups with divalent ions gives rise to conformational changes and immobilization of the headgroups. The results are discussed in terms of the lyotropic Hofmeister series and of fusogenic activity of the ionic species. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Lipid membranes; Hydration; Monovalent and divalent metal cations; Infrared spectroscopy; Lyotropic phase transition

1. Introduction

There is considerable interest in the mode of binding of metal cations to biological membranes

because it affects stability and structure of phospholipid bilayers and modulates the binding and insertion of proteins. Ions interact with charged phospholipids via Coulombic forces. The apparent association of metal cations with lipid membranes is distinctly more intense for anionic lipids than for neutral, zwitterionic ones because the net negative surface charge of membranes of acidic

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lipids increases cation concentration near the lipid–water interface according to the Gouy–Chapman theory of the electrical double layer (McLaughlin et al., 1981). The respective intrinsic association constant of selected ions was, however, shown to be virtually independent of the net surface charge after correction for differences in electric surface potential (Altenbach and Seelig, 1984; Huster et al., 2000). It reflects specific lipid–ion interactions such as orientation dependent charge–dipolar interactions with neutral residues. In addition, molecular orbital effects can make an important contribution to the energetics and coordination of complexes with transition metals (Binder et al., 2001; Christianson, 1991). Also water potentially participates as a ligand in coordination of metal cations with lipid headgroups.

Ions can deeply penetrate of into the polar region of the membrane, which is an interphase rather than an interface because the polar residues are distributed throughout a meshlike region (Cevc, 1991). ‘Chemical’ factors give rise to the binding of ions to individual sites such as the phosphodiester, and possibly also to the carbonyl groups of the lipids. Such headgroup-ion complexes are highly specific and typically involve ions, polar and/or charged moieties of the lipid and hydration water as well (Garidel and Blume, 1999; Garidel et al., 2000). Different, equally charged metal cations can exhibit very different capabilities in affecting membrane properties, such as their ability to induce aggregation and fusion (Arnold, 1995; Ohki and Arnold, 1990; Ohki and Duax, 1986) and their potency to alter the surface and/or dipole potential and the surface pressure acting within the polar interface (Ermakov et al., 2001; McLaughlin et al., 1978, 1981; Papahadjopoulos, 1968). Moreover, transition temperatures between gel and liquid-crystalline phases, and domain formation in mixed lipid membranes are specifically modulated by ion binding to the lipid headgroups (Garidel and Blume, 1999; Huster et al., 2000; Seelig et al., 1987; Silvius and Gagne, 1984).

Specific ion–phospholipid complexes are probably involved in the physiological role of metal

cations. Numerous binding sites can buffer the ion concentration in cells. For example, retinal membranes have been suggested to bind a considerable fraction of the intracellular Ca^{2+} ions (Schnetkamp, 1985). Most likely, the buffer capacity of membranes for ions is an important factor that determines the distribution of ions within living cells (Ichikawa, 1996). Also Beryllium induces a broad spectrum of membranotropic effects in model systems, and *in vivo*. Long known for its high toxicity, the inhaled beryllium (or BeO) dust causes immune-mediated lesions in lungs (chronic beryllium disease), associated with lymphocyte infiltration and aggregation of macrophages (Finch et al., 1998). The specific role of zinc was recently discussed (Binder et al., 2000, 2001).

The present work deals with the specific nature of ion–phospholipid interactions. To this aim, we studied bilayers of the neutral zwitterionic lipid 1,2-palmitoyl-oleoyl-phosphocholine (POPC) as a function of water activity in the presence of chlorides of a series of monovalent alkali metal cations (Li^+ , Na^+ , K^+) and of divalent alkaline earth (Be^{2+} , Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+}) and transition metal (Cu^{2+} , Zn^{2+}) ions, which differ in size and electronic structure. POPC was used because the absence of net charge allows focusing on specific lipid–ion interactions.

We studied lipid–ion interactions as a function of water activity. The investigation of amphiphilic systems at reduced hydration is a well suited technique for this issue because the distribution of ions between the membrane and water strongly shifts towards the lipid phase, and thus net effects of the added ions are intensified compared with highly diluted solutions. Moreover, the interaction of ions with solid and fluid membranes can be judged by their effect on the lyotropic gel-to-liquid crystalline phase transition of the lipid. Finally, studies at variable hydration allow assessing the role of water for ion–lipid interactions and the stability of amphiphilic systems. Relatively strong interactions of divalent cations such as Zn^{2+} with proteins and lipids are paralleled by considerable desolvation effects (Binder et al., 2001; DiTusa et al., 2001).

It was recently shown that the enthalpy of ion–protein interactions mirrors the enthalpy of hydration (DiTusa et al., 2001). Consequently, hydration effects must be considered to interpret the association of ions to macromolecules, lipid membranes and other molecular aggregates in aqueous solution. In this study we combined hydration studies with polarized beam infrared (IR) attenuated total reflection (ATR) measurements, which are capable to characterize the molecular architecture of the polar interface, details of lipid hydration and specific lipid–ion interaction sites (Binder et al., 1997, 1998, 1999, 2001).

The paper is organized as follows, first, we examine the effect of the ions on the lyotropic chain melting transition of POPC. Second, binding of the ions to the phosphate and carbonyl groups and their conformational response were analyzed. Third, we characterize the effect of the ions on the hydration shell of the lipid. In the discussion section the results were correlated to the size and electronic structure of the ions. The results were also discussed in terms of Hofmeister series and fusogenic activity.

2. Materials and methods

2.1. Materials and preparation of lipid vesicles

Vesicles of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, Avanti Polar Lipids, Alabaster, USA) were prepared as follows. The lipid in a chloroform/methanol stock solution (1:3 v/v), was dried and re-suspended by vortexing in water at final lipid concentration of 2–3 mg ml^{−1}, followed by extrusion (Lipex extruder, Biomembranes, Vancouver, Canada) of the suspension through a polycarbonate Unipore membrane (100 nm pore-size, Millipore).

Sample solutions were prepared by mixing appropriate amounts of stock solutions of the lipid and of metal chloride dissolved in water to yield nominal ion-to-lipid mole ratios of $R_{M/L} = 0.8 \pm 0.2$ and 1.6 ± 0.2 for di- and monovalent cations, respectively. Before measurements the sample solutions were stored overnight at a temperature of $T \approx 4$ °C.

2.2. Infrared measurements

Samples were prepared by pipetting 200–300 µl of the respective vesicle dispersion on the surface of a ZnSe ATR crystal and evaporating the water under a stream of warm air. While drying, the material was spread uniformly onto the crystal surface by gently stroking with the pipette tip. The amount of material corresponds to an average thickness of the dry film larger than 3 µm.

The ATR crystal was mounted into a commercial horizontal ATR holder (Graseby Specac, Kent, UK) that had been modified such as to realize a well-defined relative humidity (RH) and temperature within the sample chamber (Binder et al., 1997). We used a flowing water thermostat (Julabo, Seelbach, Germany) and a moisture generator (HumiVar, Leipzig, Germany) to adjust RH (H₂O) to any value between 5 and 95%, with an accuracy of $\pm 0.5\%$ at $T = 25$ °C. Polarized absorbance spectra, $A_{\parallel}(v)$ and $A_{\perp}(v)$ (128 scans, nominal resolution 2 per cm), were recorded by means of a BioRad FTS-60a Fourier transform IR spectrometer (Digilab, MA, USA) at two perpendicular polarizations of the IR beam, parallel (\parallel) and perpendicular (\perp) with respect to the plane of incidence.

The IR order parameter, S_{IR} , of an absorption band is defined as the ensemble averaged second order Legendre polynomial of the angle, θ_{μ} , enclosed between the IR active transition moment and the normal of the surface of the ATR crystal:

$$S_{IR} \equiv P_2(\theta_{\mu}) = \frac{R - K_1^{\infty}}{R + K_2^{\infty}}$$

with $P_2(\theta_{\mu}) \equiv \frac{1}{2} \langle 3 \cos^2 \theta_{\mu} - 1 \rangle$ (1)

It was determined from the dichroic ratio of the polarized absorbances, $R \equiv A_{\parallel}/A_{\perp}$, which were evaluated from $A_{\parallel}(v)$ and $A_{\perp}(v)$ by integration over the spectral range of the band after baseline correction. The constants $K_1^{\infty} = 2$ and $K_2^{\infty} = 2.54$ refer to Harricks ‘thick’ film approximation (Harrick, 1967). Typically, the peak positions and the center of gravity (COG) of the

absorption bands were determined from the weighted sum spectrum $A(v) = A_{\parallel}(v) + K_2^{\infty} A_{\perp}(v)$; Binder and Schmiedel, 1999). The mean position of the antisymmetric phosphate stretching mode, COG($\nu_{as}(PO_4^-)$), was calculated from the $A_{\perp}(v)$ spectrum to minimize the distorting influence of the methylene wagging progression in the gel state of POPC. This series of weak and medium intense bands overlaps with the $\nu_{as}(PO_4^-)$ band (see 4). The wagging bands possess parallel dichroism because their transition moments point roughly along the acyl chains, and thus these modes vanish nearly completely in the $A_{\perp}(v)$ spectrum (not shown).

For pure POPC we have shown that the ratio of the integral absorbance of the $\nu_{13}(H_2O)$ band of water (spectral range: 3700–2700 cm⁻¹) to the integral absorbance of the $\nu(C=O)$ mode of the lipid (1760–1710 cm⁻¹) is proportional to the number of water molecules sorbed onto the lipid (Binder et al., 2000):

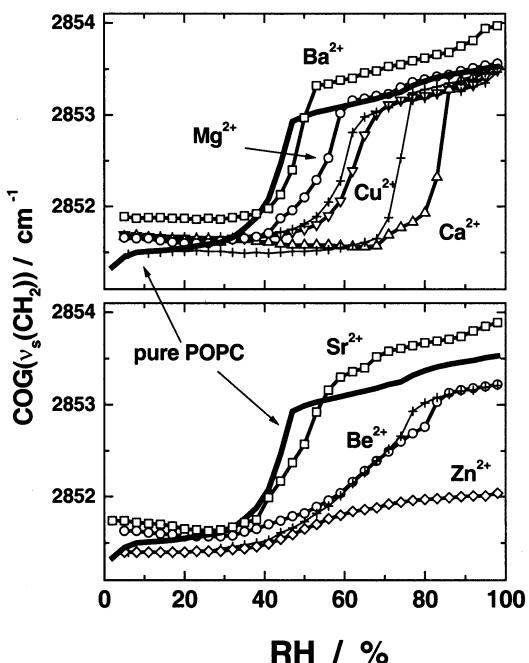


Fig. 1. COG of the symmetric methylene stretching vibrational band of POPC films in the presence of several divalent metal cations (POPC: $M^{2+} = 1: 0.8 \text{ mol mol}^{-1}$) as a function of the RH (hydration scans), at $T = 25^\circ\text{C}$. The crosses indicate dehydration scans of selected samples (see text).

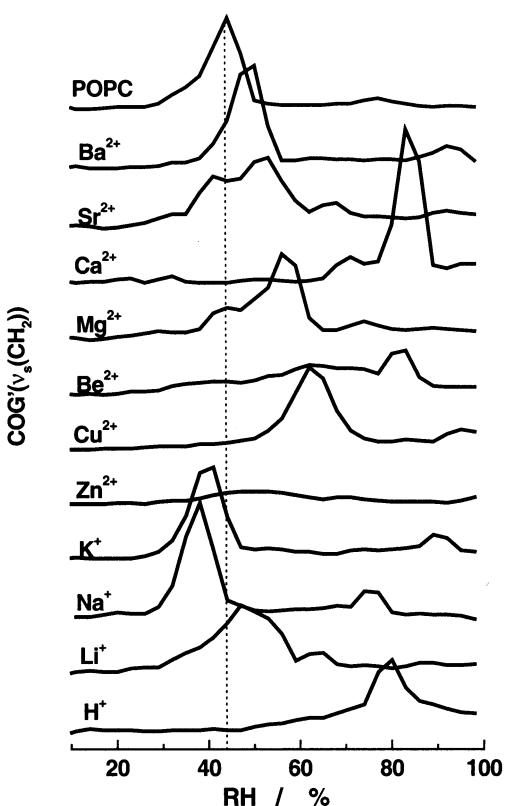


Fig. 2. First derivative of the COG-graphs partially shown in Fig. 1. The peaks refer to the gel-to-liquid crystalline phase transition of the lipid. The respective RH value of pure POPC is indicated by the vertical dotted line.

$$R_{W/L} \approx \frac{KA_W(\nu_{13}(H_2O))}{A_L(\nu(C=O))} \quad (2)$$

$R_{W/L}$ denotes the mole ratio water-to-lipid and K is a constant. For a rough estimation we will assume that this relation holds also for the lipid in the presence of the metal chlorides (see also Appendix A).

3. Results

3.1. The effect of metal ions on the lyotropic chain melting transition of POPC

The chain melting phase transition of phospholipids can be induced in an alternative fashion by increasing temperature, external pressure or hy-

dration (Cevc and Marsh, 1987). We studied the lyotropic phase behavior of the lipid POPC in the absence and presence of various metal chlorides because it gets insight into details of interaction between the components water, lipid and metal chlorides (Binder et al., 1999b). Fig. 1 shows the COG of the symmetric methylene stretching band of the lipid acyl chains, COG($\nu_s(\text{CH}_2)$), as a function of the RH, in the sample chamber. The sigmoidal increase of COG($\nu_s(\text{CH}_2)$) indicates the transformation of the lipid from the gel into the liquid-crystalline phase because the frequency of the methylene stretching vibration sensitively depends on the conformation of the acyl chains.

The position of the phase transition is clearly indicated by a maximum of the first derivative COG' = $\partial \text{COG}(\nu_s(\text{CH}_2)) / \partial \text{RH}$ that is shown in Fig. 2. All divalent metal cations and Li^+ shift the phase transition towards bigger RH values. This tendency indicates the stabilization of the gel state due to strong lipid/ion interactions. In addition one can conclude an increased affinity of the ions

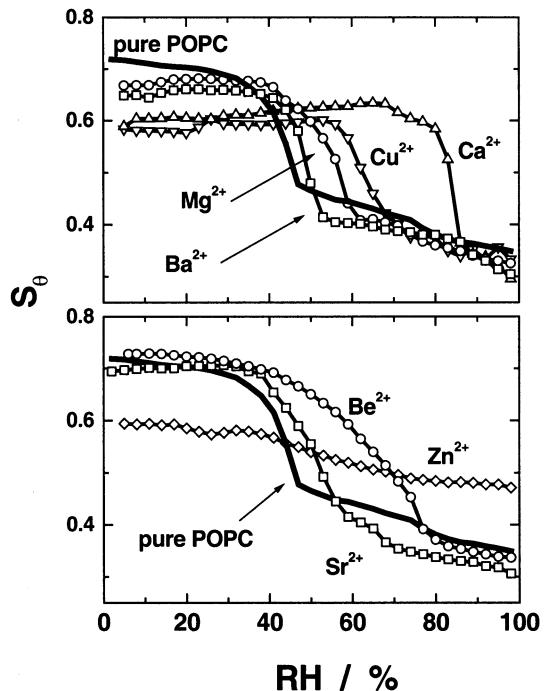


Fig. 3. Chain order parameter, S_0 , of POPC films in the presence of several divalent metal cations as a function of the RH, at $T=25$ °C.

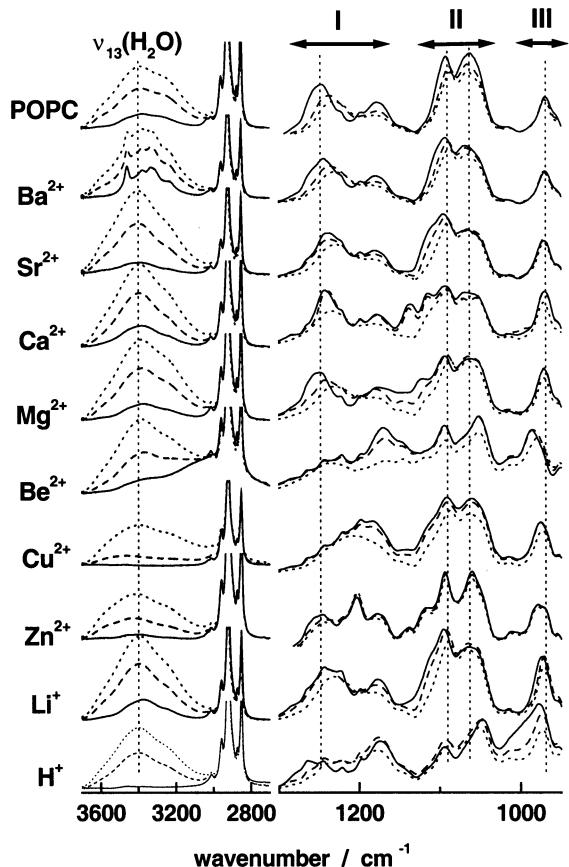


Fig. 4. Selected regions of the IR spectrum of POPC in the absence and presence of various metal chloride species and of HCl at RH = 5% (solid), 50% (dashed) and 80% (dotted). $\nu_{13}(\text{H}_2\text{O})$ denotes the O–H stretching vibration of water adsorbed onto the lipid films in the presence of metal chlorides. The spectral range I contains the antisymmetric PO_2^- stretching vibration, $\nu_{as}(\text{PO}_2^-)$, and the antisymmetric ester-bond stretches of the acyl chains, $\nu_{as}(\text{COC})$. Range II represents a complex superposition of P–O–C and C–O–C stretches with the symmetric PO_2^- stretching vibration, $\nu_s(\text{PO}_2)$ (see text). Range III is characterized by the antisymmetric C–N–C stretching band, $\nu_{as}(\text{CN})$, of the choline group at 970 cm⁻¹ and by a P–O–C vibration near 984 cm⁻¹. The vertical dotted lines serve as a guide for the eye to compare band positions in the different spectra. See also Fig. 8 and (Binder et al., 2001) for assignments.

to the gel state which give rise to their partial desorption at the chain melting transition (see Appendix C). The transition vanishes nearly completely in the POPC + Zn^{2+} system which remains in the gel state in the whole RH range. Zinc ions obviously exert the strongest effect on lipid

phase behavior among the studied ions followed by calcium.

The RH dependences of the first derivative, COG', indicate that a less intense maximum precedes the main transition peak of POPC upon interaction with Sr^{2+} , Ca^{2+} , Mg^{2+} and Be^{2+} and/or it widens considerably when compared with the transition of pure POPC. These effects can be interpreted in terms of a microheterogeneous distribution of membrane-bound ions meaning that an ion-rich gel phase coexist with an ion-depleted fluid phase over a certain transition range of RH. Possibly, ion–lipid complexes of different stoichiometry and also virtually ‘free’ lipid coexist in the gel state. These species melt at a different RHs and one observes a phase transition range. Recently, it was shown that alkali chlorides could induce the formation of discretely different multilamellar lattices in liquid-crystalline multilamellar lipid assemblies, which were referred to as phase separation (Rappolt et al., 1998).

3.2. Chain ordering

The sum of the IR order parameter of the symmetric and antisymmetric CH_2 stretches yields the mean transverse order parameter of the acyl chains, $S_\theta = -(S_{\text{IR}}(\nu_s(\text{CH}_2)) + S_{\text{IR}}(\nu_{\text{as}}(\text{CH}_2))) \equiv P_2(\theta)$ (Binder and Gawrisch, 2001b; Binder and Schmiedel, 1999; Fig. 3). It provides a measure of the average segmental orientation in terms of the angle θ that is enclosed between the normal of the ATR crystal and interconnecting line between the midpoints of adjacent C–C bonds. The positive values of S_θ indicate a predominantly parallel orientation of the chain axes with the normal of the ATR-surface, that means the parallel alignment of the multibilayer stacks with the surface of the ATR crystal. The decrease of S_θ at the gel-to-liquid-crystalline phase transition mainly reflects the increased conformational disorder due to gauche defects in the acyl chains.

Both parameters, the COG of the symmetric methylene stretching band and the chain order parameter are suited estimates of the conformational and macroscopic order of the acyl chains of the lipids. The decrease of S_θ in the gel phase of POPC after addition of most of the divalent ions

probably indicates a more disturbed macroscopic alignment of the multibilayer stacks with the ATR surface and/or the uniform tilt of the chain axes relative to the bilayer normal when compared with the pure lipid (Binder, 1999; Binder and Kohlstrunk, 1999). The virtually constant order parameter of POPC below the phase transition in the presence of Ca^{2+} , Cu^{2+} , Mg^{2+} , Ba^{2+} and Sr^{2+} is compatible with a more rigid packing of the all-trans acyl chains compared with the pure lipid.

3.3. Effect of metal ions on IR absorption bands of the phosphate group of POPC

Fig. 4 shows the range of O–H and C–H stretches and the ‘fingerprint’ region of the IR spectrum of POPC in the presence of metal chlorides at low (5%), medium (50%) and higher (80%) RH. The ‘fingerprint’ region was divided into three ranges, I, II and III, which include several vibrational modes of the lipid headgroups (see Fig. 4 for assignments). The films imbibe water with increasing RH as indicated by the increase of the absorbance of the O–H stretching band of water, $\nu_{13}(\text{H}_2\text{O})$. Progressive sorption of water typically causes the characteristic shift of several stretching vibrations of the phosphate group, which is known as the primary hydration site of phospholipids (Arrondo et al., 1984). This tendency is evidenced by the behavior of the antisymmetric and symmetric PO_2^- stretching bands, $\nu_{\text{as}}(\text{PO}_2^-)$ and $\nu_s(\text{PO}_2^-)$, which protrude near 1240 cm^{-1} in region I and near 1092 cm^{-1} in region II of the spectrum, respectively (see Fig. 4 for assignments).

The COG of the $\nu_{\text{as}}(\text{PO}_2^-)$ band, $\text{COG}(\nu_{\text{as}}(\text{PO}_2^-))$, of POPC in the presence and absence of metal cations is plotted in Figs 5 and 7 as a function of RH to assess the degree of water–phosphate and/or phosphate–ion interactions. For a rough classification, the ions can be divided into three groups with regard to their effect on the phosphate groups as indicated from the ‘fingerprint’ region of the spectrum I–III and from the behavior of $\text{COG}(\nu_{\text{as}}(\text{PO}_2^-))$:

(A) Ba^{2+} , Sr^{2+} (Fig. 5) and the monovalent alkali metal cations Na^+ , K^+ and Li^+ (Fig.

- 6) only weakly affect the shape of the spectra. At low RH the COG($\nu_{as}(PO_2^-)$) slightly shifts to smaller values after interaction of POPC with Li^+ , Ba^{2+} and Sr^{2+} possibly due to direct and/or water mediated phosphate–ion interactions in the gel phase. In the fluid membranes, at a higher degree of hydration, none of the species significantly affects the position of phosphate band, and thus the hydration the PO_2^- moiety.
- (B) The alkali earth metal ions Mg^{2+} and Ca^{2+} increase COG($\nu_{as}(PO_2^-)$) in the gel phase, and to a smaller extent, also in the L_α phase (only Ca^{2+}) when compared with pure POPC. Obviously these species partially dehydrate the phosphate groups. The most marked effect on the spectrum is observed at the left flank of range II. The appearance and sharpening of bands near 1115 and 1137 cm^{-1} especially in the POPC + Ca^{2+} system indicate a conformational change and/or immobilization of the headgroups because these modes can be tentatively as-

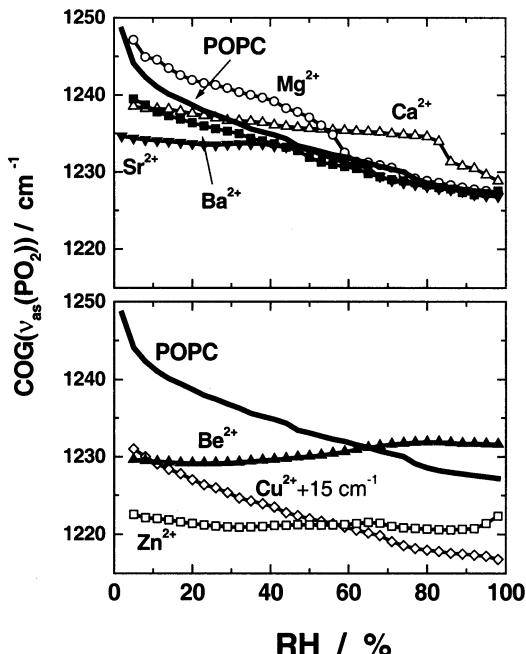


Fig. 5. COG of the antisymmetric PO_2^- stretching band, $\nu_{as}(PO_2^-)$, of POPC in the presence of various divalent metal ions as a function of the RH.

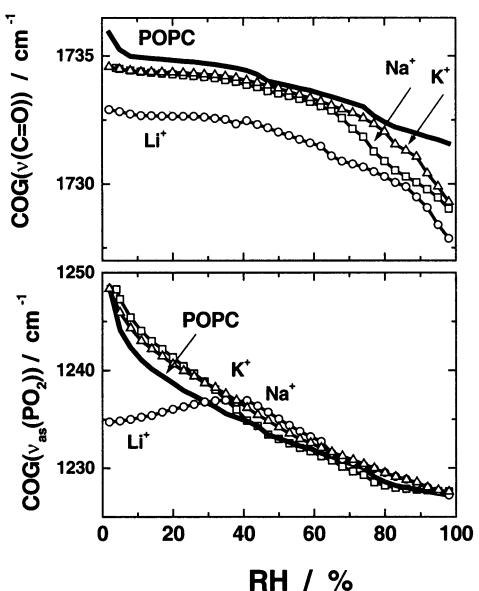


Fig. 6. COG of the antisymmetric PO_2^- stretching band, $\nu_{as}(PO_2^-)$, and of the C=O stretching band, $\nu(C=O^-)$, of POPC in the presence of monovalent metal ions as a function of the RH.

signed to $C^G-(OP)$ and $(PO)-C^C$ stretches, $\nu(C^G-(OP))$ and $\nu((PO)-C^C)$, respectively (the superscripts ‘G’ and ‘C’ refer to the carbons in the glycerol and choline residues, respectively) (Binder et al., 2001). The effect of the ions decreases in the fluid phase indicating desorption of ions.

- (C) The ions Be^{2+} , Cu^{2+} and Zn^{2+} can be collected into a relatively heterogeneous group, which is characterized by a considerable modification of the ‘fingerprint’ region of the spectrum. In all cases the mean position of the $\nu_{as}(PO_2^-)$ band distinctly shifts to smaller wavenumbers indicating relatively strong specific interactions of the nonesterified oxygens with the ions. Upon interaction with Cu^{2+} the $\nu_{as}(PO_2^-)$ band represents a broad feature centered at $1205 cm^{-1}$. This feature sharpens into a narrow, intense peak near $1204 cm^{-1}$ after addition of Zn^{2+} . It is known that Zn^{2+} has an appreciable affinity to phosphates (Mustafa et al., 1999), and thus, this new absorption band was assigned to $\nu_{as}(PO_2^-)$ stretches of

phosphate groups that are involved into complex formation with Zn^{2+} (Binder et al., 2001). A second band near 1245 cm^{-1} originates probably from essentially dehydrated phosphate groups not directly involved into complex formation. The COG, $\text{COG}_{v_{as}}(\text{PO}_2^-)$, is virtually insensitive to RH after binding of Zn^{2+} and Be^{2+} probably because of the screening of the phosphodiester groups from the water.

Two spectral effects should be mentioned which are common for the ions of group C, firstly, the stretching bands of the phosphodiester bonds, $v(P-(OC^C))$ and $v(P-(OC^G))$, distinctly sharpen and increase in intensity at the right-hand-side of region II (1050 – 1040 cm^{-1}) and at the left-hand-side of the $v_{as}(C-N)$ mode (988 – 980 cm^{-1} , region III), respectively. Secondly, the shape and position of the antisymmetric $P-(OC)_2$ stretching band near 830 cm^{-1} , ($v_{as}(P-(OC)_2)$), considerably changes (Fig. 8). Hence, Be^{2+} , Cu^{2+} , Zn^{2+} and,

to a less extend, also Li^+ (group (A)) obviously affect the esterified oxygens by direct interactions and/or by a conformational change. It is known that the anionic phosphinyl portion of the phosphate group prefers to interact with metal ions with syn coordination stereochemistry with a general tendency of the ions to lie out of the plane of the PO_2^- group (Alexander et al., 1990; Christianson, 1991). A symmetrically bidentate phosphinyl–metal ion interaction is not preferred. We suggest that divalent metal cations form bridges between neighboring phosphate groups, which involve strong interactions with the free and esterified oxygens.

3.4. Effect of metal ions on IR absorption of the carbonyl group of POPC

The carbonyl groups represent a second polar moiety, which is strongly involved into hydration of lipid membranes. The COG of the $C=O$ stretching band near 1735 cm^{-1} represents a sensitive marker of H-bond formation between the carbonyls and the water (Blume et al., 1988). Ions of group A and B cause a systematic shift of $\text{COG}(v(C=O))$ towards smaller wavenumbers (Figs. 6 and 7). One could conclude that the carbonyl groups become more accessible to the water possibly because of their involvement into the hydration shell of the ions. On the other hand, a conformational change in the carbonyl region can also explain the observed effect. Note that the shift of $\text{COG}(v(C=O))$ after addition of ions correlates roughly with the respective alteration of the RH value of the phase transition induced by the presence of ions. This result shows that alterations of interactions in the carbonyl region have the potential to affect structure and phase behavior of lipid aggregates to an extraordinary extent because of its pivotal location near the polar interface.

The $v(C=O)$ band shifts clearly towards higher frequencies after addition of ions of group C contrarily to the effect of ions of groups A and B. The respective species obviously dehydrate the carbonyl groups at small and medium RH. The difference with respect to pure POPC, however, decreases or even vanishes at higher RH values.

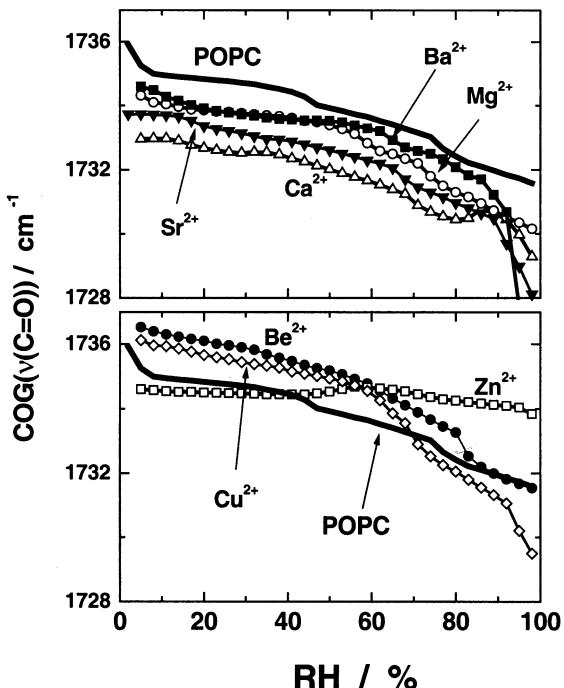


Fig. 7. COG of the $C=O$ stretching band, $v(C=O^-)$, of POPC in the presence of various divalent metal ions as a function of the RH.

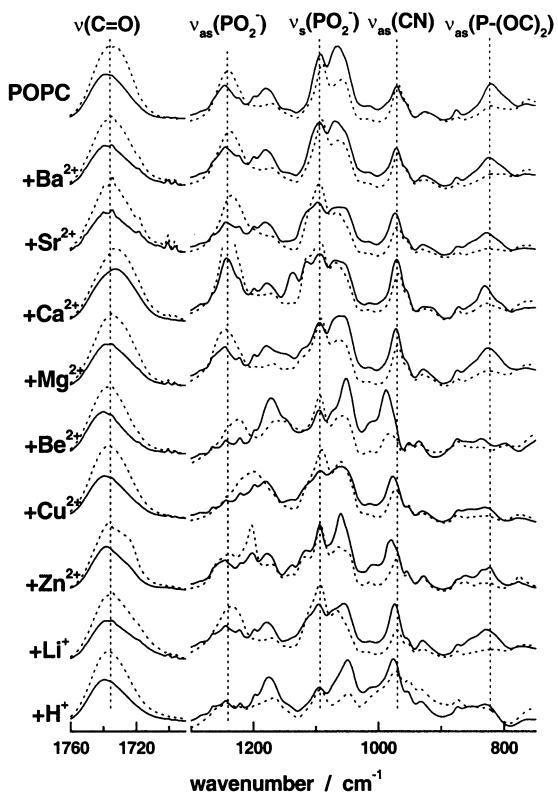


Fig. 8. Polarized IR spectra, $A_{\parallel}(v)$ (solid line) and $2A_{\perp}(v)$ (dotted line), of POPC in the presence of metal chlorides at $T = 25$ °C and RH = 50%. Note that the condition $A_{\parallel}(v) = 2A_{\perp}(v)$ refers to the absence of molecular order of the respective transition moment, or alternatively, to its orientation with the magic angle ($\sim 54.7^\circ$) relatively to the normal of the ATR surface. Selected absorption bands of the PC headgroup and the C=O stretching vibration are assigned above.

In the presence of zinc ions the mean position of $\nu(\text{C=O})$ band becomes much less sensitive to hydration. We suggest that direct interactions between zinc ions and the carbonyl groups at least partially prevent their hydration.

3.5. IR linear dichroism and headgroup ordering

The polarized IR spectra report about modifications of the ordering and orientation of the polar groups of the lipid, which are caused by interactions with metal cations (Fig. 8). For example, the $\nu_{\text{as}}(\text{P}-(\text{OC})_2)$ band in the 830–810 cm^{-1} range possesses clearly parallel linear dichroism

($A_{\parallel} > 2A_{\perp}$) for pure POPC and for POPC interacting with ions of group A and B. The change of the position and shape of this band in the presence of ions of group C is accompanied by a distinct decrease of the relative absorbance A_{\parallel} . This tendency can be explained by a more parallel orientation of the $\text{C}^{\text{G}}-\text{O}-\text{P}-\text{O}-\text{C}^{\text{C}}$ fragment with respect to the membrane surface because the respective transition moment points roughly along the backbone of the phosphate group. On the other hand, the transition moment of the sharp $\nu(\text{P}-(\text{OC}^{\text{G}}))$ band near 985 cm^{-1} which appears after addition of Be^{2+} , Cu^{2+} , Zn^{2+} and Li^+ tends to orient parallel with the bilayer normal as indicated by the relation $A_{\parallel} > 2A_{\perp}$. This effect has been interpreted in terms of a ‘stretched’ trans/trans conformation of the $\text{C}^{\text{G}}-\text{O}-\text{P}-\text{O}-\text{C}^{\text{C}}$ fragment induced by the binding of ions to the phosphate group (Binder et al., 2001).

Note the pronounced parallel linear dichroism of the sharp feature at 1137 cm^{-1} , which appears in the gel phase of POPC after addition of Ca^{2+} . This mode has been assigned to the $\nu((\text{PO})-\text{C}^{\text{C}})$ vibration the transition moment of which is assumed to point approximately along the $\text{O}-\text{C}^{\text{C}}$ bond (Binder et al., 2001). The existence of this band and its strong linear dichroism is compatible with immobilized phosphate groups possessing a uniform oblique orientation with respect to the membrane plane.

3.6. Hystereses

All systems were studied by means of increasing and decreasing RH. The absence of hystereses in most cases (except Be^{2+} , Cu^{2+} and Ca^{2+} , data were not shown for other ions) indicates that the hydration/dehydration regime guarantees thermodynamic equilibrium. Be^{2+} and Cu^{2+} ions cause small hystereses (Fig. 1, crosses). The RH of the chain melting transition of the hydration scan shifts by less than $\Delta\text{RH} \approx 5\%$ upwards compared with the respective RH value of the dehydration scan. This relatively small effect does not affect the basic conclusions drawn from the phase transition data (vide supra).

Calcium induces a more pronounced hysteresis of $\Delta\text{RH} \approx 10\%$ (Figs. 1 and 9). The melting (and

freezing) of the acyl chains clearly correlates with the spectral shift of the $\nu_{as}(PO_2^-)$ band (Fig. 9). This effect can be attributed to an increase and decrease of hydration of the phosphate groups at the phase transition upon melting and freezing of the acyl chains, respectively. Also the characteristic pattern in region II with bands at 1115 and 1137 cm^{-1} disappears and reappears in the same RH range parallel to chain melting and freezing, respectively. Hence, hydration and dehydration scans reveal a reversible behavior of the acyl chains and of the phosphate groups except a certain horizontal shift of the respective graphs each to another. Contrarily, the mean frequency of the $\nu(C=O)$ band shifts in a different fashion at the phase transition upon increasing and decreasing RH (Fig. 9). We suggest that specific binding of Ca^{2+} ions to POPC is accompanied by com-

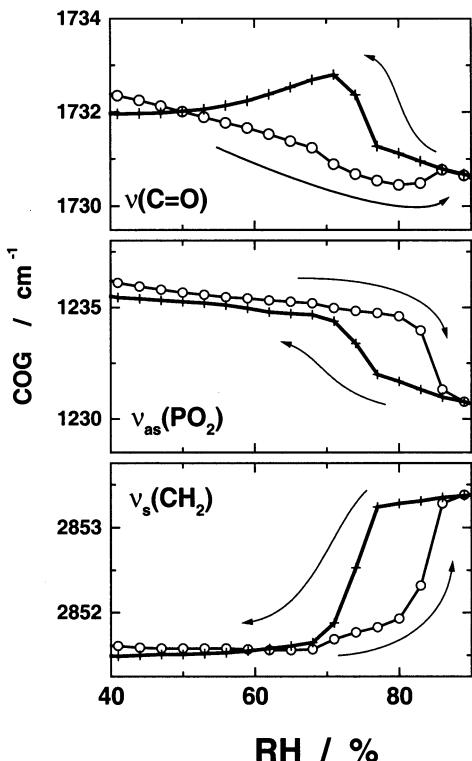


Fig. 9. Hysteresis of hydration and dehydration scans of $POPC + Ca^{2+}$. COG of carbonyl, phosphate and methylene vibrational bands. Directions of the scans are indicated by arrows.

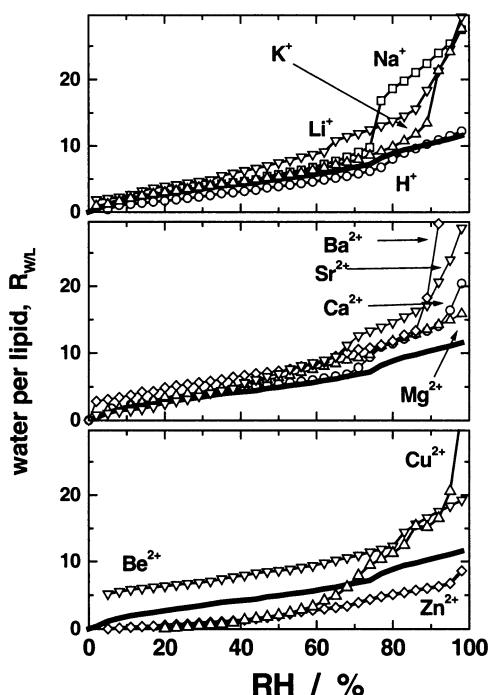


Fig. 10. Sorption isotherms of water onto POPC in the absence (thick line) and presence of metal chlorides. The molar ratio water-to-lipid was estimated from the ratio of the integral absorbances of the $\nu_{13}(H_2O)$ band of water and the $\nu(C=O)$ band of the lipid (see ref. (Binder et al., 2000) for details).

plex alterations in the carbonyl region, which involve the hydration degree (hydrogen bonding) and conformational changes as well. Upon dehydration the $C=O$ frequency relaxes to its equilibrium value only when the sample is equilibrated at small RH in the gel phase.

3.7. Water sorption isotherms of POPC in the presence of metal chlorides

Fig. 10 shows the water sorption isotherms of POPC in the presence and absence of metal chlorides, which were measured by means of IR spectroscopy. The pure salts only solvate at water activities above the water activity of the respective saturated salt solution, a_w^s (see Appendix A). The sorption isotherms of $(POPC + NaCl)$ and of $(POPC + KCl)$ stepwisely increase exactly at the water activities of the respective a_w^s values (see Fig. 10, $a_w^s(NaCl) \approx 0.75$ and $a_w^s(KCl) \approx 0.84$).

This behavior clearly shows that the samples partially possess the hydration characteristics of the pure metal chlorides. In other words, the salts are obviously present in excess. Also the chlorides of divalent alkali earth metals of group A, BaCl_2 ($a_w^s(\text{BaCl}_2) \approx 0.90$) and SrCl_2 ($a_w^s(\text{SrCl}_2) \approx 0.70$), and of Cu^{2+} ($a_w^s(\text{CuCl}_2) \approx 0.60$) imbibe water just at $a_w > a_w^s$ because of the same reason.

Some of the dried salts represent crystalline metal chloride hydrates of the form $\text{MeCl}_2 \cdot x\text{H}_2\text{O}$. The crystal water of pure $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ and of its mixture with POPC gives rise to a characteristic IR-absorption pattern which is characterized by a typical fine structure (Fig. 4). Four O–H stretches and two H_2O bending modes (not shown) are observed as relatively narrow peaks. The sorption isotherms of $\text{POPC} + \text{BaCl}_2$ and of pure POPC run essentially parallel at $\text{RH} < 50\%$. The vertical shift between both isotherms corresponds to $\Delta R_{w/L} \approx 1.7\text{--}2.5$. This value roughly agrees with the number of $\sim 1.6 \pm 0.4$ crystal water molecules which are expected per lipid in the mixture $\text{POPC} + \text{BaCl}_2 \cdot 2\text{H}_2\text{O}$. Also the slight vertical shift of the sorption isotherms in the presence of Mg^{2+} and of Ca^{2+} in the low RH range are probably caused by crystal water of free metal chloride.

The adsorption isotherms provide insight into the hydration potency of POPC in the presence of ions compared with pure POPC. Most of the metal cations have obviously no significant effect on the amount of water bound to the lipid if one takes into account the effect of crystal water at $a_w < a_w^s$ and of hydrated excess salt at $a_w > a_w^s$ (vide supra) and also the uncertainty of the used method (error of $R_{w/L} \sim \pm 1$). The monovalent cations Na^+ and K^+ only weakly interact with the lipid. Hence, the essentially similar isotherms of POPC and of $(\text{POPC} + \text{NaCl})$ and $(\text{POPC} + \text{KCl})$ indicate unperturbed hydration of the lipid in the presence of these ions. In contrast, the divalent ions Sr^{2+} , Mg^{2+} and especially Ca^{2+} clearly bind to the lipid headgroups. The respective isotherms show that the gel-state mixtures of POPC with these ions similarly hydrate as the pure lipid. Hence, there is no additional water in the hydration shell of the lipids owing to separate hydration of the ions. We conclude that the ions

and the lipid headgroups form a complex in which both compounds either share their hydration shells to some degree, or part of the water is even removed due to direct lipid–ion interactions (see also next section). In the fluid phase the considered mixtures bind more water than the pure lipid because a certain fraction of the ions desorbs from the lipid and hydrates independently. Considerable dehydration of the lipid was observed in the presence of Cu^{2+} and Zn^{2+} . The lipid loses about 50% of its hydration shell after interaction with these ions at $\text{RH} < 60\%$. The polar part of the lipid and the ions obviously form an inner-sphere complex in which the hydration shell of both compounds is partly removed owing to direct lipid–ion interactions (for a quantitative analysis of the effect of Zn^{2+} see Binder et al., 2001). With increasing hydration the interaction between Cu^{2+} and POPC obviously weakens.

3.8. IR spectral characteristics of the primary hydration shell

The position and shape of the $\nu_{13}(\text{H}_2\text{O})$ band of water depend on several factors such as the distribution of hydrogen bonds of variable strength over a variety of binding sites and/or the degree of intramolecular and intermolecular vibrational coupling within the particular water structure (Bertie and Whalley, 1964a,b; Walrafen, 1967). We calculated the difference spectra of the lipid films hydrated at $\text{RH} = 38\%$ and at 5% to characterize the primary hydration shell of 1–5 water molecules which bind to the lipid in the presence and absence of metal chlorides (Fig. 11). This procedure completely eliminates, for example, the typical fine structure of $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ (compare Figs. 4 and 11). Independently measured IR spectra of pure $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ show the same fine structure as $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ in the presence of lipid. We conclude that the spectral ‘background’ due to crystal water of metal chlorides was eliminated in this way. Moreover, most of the pure metal chlorides do not solvate at $\text{RH} \leq 38\%$, and thus the hydration of excess salt does not contribute to the spectra. Hence, the difference spectra are expected to report about the energetic and structural prop-

erities of water in the polar region of the membrane.

The IR difference spectrum of the primary hydration shell of pure POPC shows two distinct peaks near 3400 and 3240 cm^{-1} which are due to water molecules in an effective H-bonded structure ('lattice water'; Walrafen, 1967). The right-hand, more intense band can be assigned to a mode where symmetric ν_1 stretches of neighboring water molecules couple out of phase (Bergren et al., 1978; McGraw et al., 1978). The left-hand band is assumed to originate from a complex mixture of different modes with a major fraction of antisymmetric H_2O stretches (ν_3). A weak shoulder near 3600 cm^{-1} was assigned to non- or weakly hydrogen-bonded water (Walrafen, 1967). The primary hydration shell of water that binds to pure POPC obviously forms well structured H

bonds, probably preferentially to the phosphate groups as indicated by the relatively intense band at 3240 cm^{-1} (Fig. 11) (Binder et al., 1999b). With addition of the salts the relative absorbance of this feature markedly decreases. A similar tendency was observed in aqueous solutions of alkali halides (Walrafen, 1962). It can be explained by the partial breakdown of water structure in the polar part of the membrane owing to the presence of ions.

A completely different behavior was observed for ZnCl_2 and CuCl_2 . The maximum of the respective $\nu_{13}(\text{H}_2\text{O})$ bands are distinctly shifted towards higher frequencies owing to weak hydrogen bonding characteristics of the very few water molecules, which adsorb to the lipid. Here, specific interactions with the ions make a considerable fraction of the free oxygens of the phosphate groups inaccessible for H-bonds of the water.

3.9. Hydrolysis

Besides specific ion–lipid interactions the effect of ions may be attributed to their hydrolytic activity, and thus to their potency to increase the H^+ concentration in the headgroup region of the membrane. For sake of comparison we studied the spectrum of POPC at acidic conditions. HCl solution was added to an aqueous vesicle suspension (<10 mM lipid) to yield pH 2.4. Then, an appropriate amount of the solution was dried on the surface of the ATR crystal and subsequently rehydrated at definite RH (see Section 2). The intensity of both PO_2^- stretches, $\nu_{\text{as}}(\text{PO}_2^-)$ and $\nu_s(\text{PO}_2^-)$, considerably decreases in the respective spectra due to the protonation of a considerable fraction of the phosphate groups (Figs. 4 and 8). A relatively weak feature near 1260 cm^{-1} can be assigned to the P=O stretching vibration. It was recently shown that the stretching modes of phosphates which strongly interact with delocalized protons shift towards wavenumbers near 1200 cm^{-1} and considerably broaden that they become hardly detectable in the IR spectra (Brzezinski et al., 1997; Stoyanov, 1999). One can deduce that a certain fraction of H bonds with ionic H^+ species in the headgroup region of the membranes is delocalized between the phosphate oxygens with

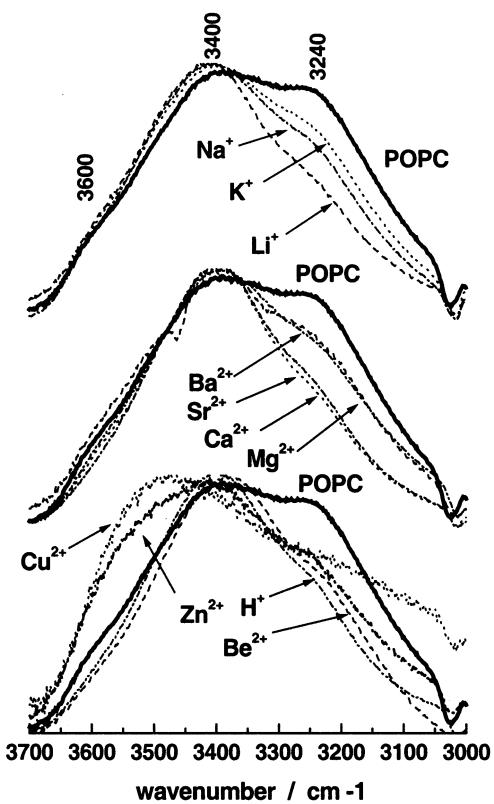


Fig. 11. Normalized difference spectra of the $\nu_{13}(\text{H}_2\text{O})$ band of the water sorbed onto POPC at RH = 38 and 5% in the absence and presence of various metal chlorides and of HCl (pH 2.4).

proton polarizability. These spectral properties were not observed in the POPC + metal chloride systems except POPC + BeCl₂ (see below). Hence, hydrolysis can be excluded as the predominant effect that exert the ions on the lipid in the respective systems. Note that the phase transition of POPC shifts considerably upwards at acidic conditions (Fig. 2). The delocalized protons obviously stabilize headgroup packing and thus the gel state of POPC.

Beryllium ions modify the IR spectrum of lipid bound water in a specific manner. At low RH, the $\nu_{13}(\text{H}_2\text{O})$ band is considerably shifted towards smaller wavenumbers in the presence of BeCl₂ when compared with its position in the other studied systems (Fig. 4). In general, this behavior is indicative for the existence of a network of relatively strong H-bonds (Zundel, 1969). Beryllium salts are known to form Be₃(OH)₃⁺ ions in aqueous solution because of their hydrolytic activity. Ionic H bonds such as (H₂O···H···OH₂)⁺ and (HOH···OH)⁻ are much stronger than neutral ones (Scheiner and Cuma, 1996). Hence, the small frequencies of the O–H modes indicate that Be²⁺ causes the formation of ionic water species in the sample film. The $\nu_{13}(\text{H}_2\text{O})$ difference spectrum in the presence of BeCl₂, however, resembles that of the other salts discussed above (Fig. 11). We conclude that the additionally sorbed water is not or only weakly involved into hydrolysis.

4. Discussion

4.1. Lipid–ion complexes

Previous investigations on interactions of divalent cations such as Ca²⁺ and Zn²⁺ with phospholipid membranes indicated the formation of a well-defined chemical complex (Altenbach and Seelig, 1984; Binder et al., 2001; Huster et al., 2000). Its stoichiometry depends on the type of metal cations, the lipid, its phase state (liquid-crystalline or gel) and on the water activity. The stoichiometry refers to the molar ratio ion-to-lipid at saturation, $R_{\text{M/L}}(\text{sat})$ (see Appendix A). For example, one Ca²⁺ ion coordinates with two POPC molecules in the fluid phase at excess water

conditions ($R_{\text{M/L}}(\text{sat}) = 0.5$; Altenbach and Seelig, 1984). For POPC + ZnCl₂ we found $R_{\text{M/L}}(\text{sat}) = 0.5 – 1.0$ (Binder et al., 2000). Note that Zn²⁺ and Ca²⁺ exert the strongest effect on the lyotropic phase behavior of POPC among the ions studied (vide supra). We, therefore, suggest that the used molar ratio $R_{\text{M/L}} = 0.8 \pm 0.2$ ensures conditions near saturation of the lipid with bound ions.

There is no doubt that the primary binding sites of the cations are the anionic phosphodiester groups (Altenbach and Seelig, 1984; Garidel et al., 2000; Huster et al., 2000). Conclusive evidence has been reported from neutron diffraction (Herbette et al., 1984), luminescence (Herrmann et al., 1986), and NMR investigations (McLaughlin et al., 1978; Petersheim et al., 1989) supporting a specific interaction of metal cations with the phosphate group of phosphatidylcholine (PC). Chapman et al. found a rough correlation between the effect of various metalchlorides on the phase transition of hydrated DMPC and DPPC and the order of the association constants of metal ions with simple phosphates, which decrease according to Li⁺ > Na⁺ > K⁺ for monovalent ions, and Zn²⁺ > Cu²⁺ > Ca²⁺ > Mg²⁺ for divalent ones (Chapman et al., 1977). This order agrees with the effect of these ions on the lyotropic phase transition of POPC reported here (see below). Also the sequence of the intrinsic binding constant of alkaline-earth metal cations onto egg PC membranes, Ca²⁺ > Mg²⁺ > Sr²⁺ > Ba²⁺, agrees with the observed effect of these ions on the lyotropic phase transition of POPC (see Table 1 and (Tatulian, 1987)).

4.2. The lyotropic phase transition of POPC as a function of the solvation free energy of the ions

The lipid–ion complexes involve hydration water. The adsorption isotherms clearly show that the hydration degree of the systems increases with the water activity. Progressive hydration is accompanied by the decompression of the lipid bilayers. At any critical value of the RH, RH^{tr}, the system undergoes the lyotropic phase transition from the gel into the liquid-crystalline phase (Binder and Gawrisch, 2001a). The critical hydration number,

Table 1

Properties of metal cations and of Cl^- and their effect on the carbonyl and phosphate bands of POPC

Ion	$\nu(\text{C=O})^{\text{a}}$	$\nu_{\text{as}}(\text{PO}_2^-)^{\text{a}}$	R_{ion} (nm) ^b	ΔG_{hyd} (kJ mol ⁻¹) ^b	cf ^c	$n_{\text{hydr}}^{\text{d}}$	K_i (M ⁻¹) ^e
K^+	0	0	0.138	295	1.08	6.6	
Na^+	0	0	0.102	364	1.40	5.9	0.15
Li^+	—	—	0.074	474	1.55	4.5	
H^+			0.03	1049			
Ba^{2+}	(—)	(—)	0.136	1249	1.60	9.1	10 (0.28)
Sr^{2+}	—	—	0.125	1379		8.5	16 (0.36)
Ca^{2+}	--	+	0.1	1504		8.0	40 (1.0)
Mg^{2+}	—	+	0.072	1828	2.10	6.0	30 (1.0)
Zn^{2+}	+ +	— —	0.075	1953	2.50		
Cu^{2+}	+	— —	0.073	2008	2.55		
Be^{2+}	+	—	0.04	2393			
Cl^-			0.181	340			0.20

^a Effect of ions on the mean frequencies of the $\nu(\text{C=O})$ and $\nu_{\text{as}}(\text{PO}_2^-)$ vibrations, ‘+’/‘-’ increase/decrease, ‘+ +’/‘- -’ strong increase/decrease, ‘(+)'/‘(-)’ weak increase/decrease, ‘0’ no effect.

^b Pauling ionic radius and the experimental hydration free energy from (Marcus, 1994).

^c Cooperativity factor, cf, which considers the polarization of O–H bonds due to interactions with the cations (see (Luck et al., 1997) and references cited therein); cf = 1.0 refers to non-cooperative interactions.

^d Coordination number of hydrated cations. Data were taken from a molecular dynamics simulation (Babu and Lim, 1999).

^e Intrinsic binding constant characterizing the binding of ions to egg PC taken from (Tatulian, 1987; McLaughlin et al., 1978; in parentheses). Both data sets were obtained from the electrophoretic mobility of lipid vesicles using different models. Note the very similar tendency of both data sets.

$R_{\text{W/L}}^{\text{tr}}$, can be taken from the respective adsorption isotherm. Note that the relation between $R_{\text{W/L}}^{\text{tr}}$ and RH^{tr} is virtually linear in the intermediate RH range. The critical number of water molecules per lipid increases from $R_{\text{W/L}}^{\text{tr}} \approx 3$ (pure POPC) up to about 7 for (POPC + Ca^{2+}) and also for (POPC + Be^{2+}) if one corrects the isotherm of the latter system for the vertical shift relatively to pure POPC. Interestingly, (POPC + Zn^{2+}) showing no phase transition in the studied RH range merely adsorbs 7–8 water molecules at the highest RH value studied (98%).

The relative shift of the lyotropic phase transition of POPC after addition of ions, $\Delta\text{RH} = \text{RH}^{\text{tr}} - \text{RH}_0$ correlates in a linear fashion with the excess solvation free energy, $\Delta G_{\text{hydr}}^{\text{exp}}$, and thus also with the theoretical value $\Delta G_{\text{E}}^{\text{solv}}$ (see Appendix B) according to:

$$\frac{\Delta\text{RH}}{\text{RH}_0} = C + D \times \Delta G_{\text{E}}^{\text{solv}} \quad (3)$$

except for Ca^{2+} , Zn^{2+} and, to a much smaller degree for Li^+ (see part a of Fig. 12; C and D are constants). This interesting result implies a linear relation between the excess chemical potential of

mixing of the respective ions and lipid of the form $\Delta\mu_{\text{E}} = E + F\Delta G_{\text{E}}^{\text{solv}}$ because $\Delta\text{RH}/\text{RH}_0$ is directly related to $\Delta\mu_{\text{E}}$ (see Appendix C, Eq. (C.4)), E and F are constants). The binding of ions to the lipid obviously increases the absolute value of free energy proportional to $\Delta G_{\text{E}}^{\text{solv}}$. In other words, the ions gain free energy upon transfer from a pure aqueous environment to the membrane-bound state. An interpretation of this tendency will be given below. The constant ‘E’ refers to contributions to $\Delta\mu_{\text{E}}$ originating from alterations of the free energy of the system which are virtually independent of the solvation of the ions such as changes of the packing, molecular ordering and conformations in the chain and headgroup region of the lipid, $\Delta\mu_{\text{E}}^{\text{chain}}$ and $\Delta\mu_{\text{E}}^{\text{head}}$, respectively, and deviations from the random distribution of the components, $\Delta\mu_{\text{E}}^{\text{mix}}$. Comparison of Eqs. (3) and (C.4) provides the intercept and slope of the linear relation between $\Delta\text{RH}/\text{RH}_0$ and $\Delta G_{\text{E}}^{\text{solv}}$ in terms of molecular parameters, $C = K((1+q)\Delta R_{\text{M/L}}/(1+(1+q)R_{\text{M/L}})) - (\Delta\mu_{\text{E}}^{\text{chain}} + \Delta\mu_{\text{E}}^{\text{head}} + \Delta\mu_{\text{E}}^{\text{mix}})/RT$ and $D = -KF/RT$, respectively.

The shift of the lyotropic phase transition requires the change of composition between the gel

and fluid phases according to Eq. (A.2). The IR results indicate that the effect of ions on the lipid spectra decreases in the fluid phase. This tendency can be explained by the partial or complete desorption of ions from the lipid in the fluid phase with $0 < \Delta R_{M/L} = R_{M/L}^1 - R_{M/L}^2 = R_{M/L}^{\text{gel}} - R_{M/L}^{\text{fluid}} \leq R_{M/L}^{\text{gel}}$. In other words, ion binding and molecular order of the membrane affect each other. The hydration level of pure POPC increases from $R_{W/L}^{\text{gel}} \approx 3.0$ to $R_{W/L}^{\text{fluid}} \approx 3.5$ at lyotropic chain melt-

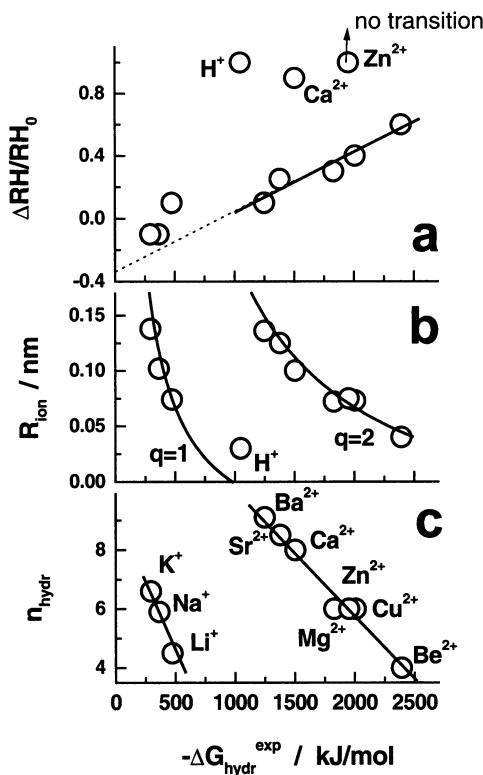


Fig. 12. Shift of the RH of the phase transition, $\Delta RH/RH_0$ (part a), ion radius, R_{ion} (part b), and coordination number of the first hydration shell, n_{hydr} (part c), of the studied ions as a function of the experimental hydration excess free energy, $\Delta G_{\text{hydr}}^{\text{exp}}$, from ref. (Marcus, 1994). $\Delta RH = RH - RH_0$ represents the difference of the RH values of the lyotropic phase transition of POPC in the presence and absence of ions, respectively. The RH values correspond to the maximum of the respective COG' graph shown in Fig. 3. The lines in part a are linear regression according to (Eq. (3)). The straight lines in part c are drawn as a guide for the eye. The curves in part b were calculated according to $R_{\text{ion}} = (aq^2/\Delta G_E^{\text{solv}} - 0.07 \text{ nm})$ with $a = 68.5 \text{ nm} \cdot \text{kJ mol}^{-1}$ which corresponds to $\varepsilon_s = 81$ (see (Eq. (B.1))).

ing transition (Binder et al., 1999b). No of the adsorption isotherms of POPC shows a drastic increase of hydration at this event in the presence of metal chlorides (Fig. 10). With $R_{W/L}^1/R_{W/L}^2 = R_{W/L}^{\text{gel}}/R_{W/L}^{\text{fluid}} \approx 0.8-0.9$ and $R_{W/L}^2 = R_{W/L}^{\text{fluid}} \approx 3-6$ one obtains a rough estimation of the proportionality constant $K = 0.1 - 0.25$ (see Eq. A.2). Let us use $\Delta R_{M/L} = R_{M/L} \approx R_{M/L}(\text{sat}) < 0.8$ (vide supra) and $-0.4 < C < -0.3$ (see Fig. 12). With these values one obtains $(\Delta \mu_E^{\text{chain}} + \Delta \mu_E^{\text{head}} + \Delta \mu_E^{\text{mix}})/RT > 1.5$ for the contribution of non-solvation effects to the excess chemical potential of mixing. That means, extrapolation of the effect of the ions on the phase transition of POPC to $\Delta G_E^{\text{solv}} = 0$ reveals a loss of free energy of at least $1.5 \times RT$ owing to the unfavorable, in terms of free energy, arrangement of the chains and/or headgroups and, possibly, owing to the nonrandom distribution of the ions among the lipids. The factor $F > 4 \times 10^{-3}$ was estimated from the slope of the regression line in part a of Fig. 12 ($D = -3.6 \times 10^{-4} \text{ mol kJ}^{-1}$). This estimation shows that the gain of free energy of the system owing to the insertion of divalent cations into the polar region of the membrane corresponds roughly to the energy of 2–4 hydrogen bonds.

Details of ionic hydration were studied on a molecular level by molecular dynamics simulations (Babu and Lim, 1999). Three factors that influence the solvation excess free energy of ions in an aqueous environment can be extracted from these investigations, (1) the ionic radius determines the distance of closest approach between of negatively charged oxygens and the cations. (2) The first-shell coordination number together with factor (1) determines the minimum value of the (negative) distance-dependent electrostatic solvation energy. It typically reaches absolute values greater than 6000 kJ mol^{-1} for divalent alkaline earth cations. That means that the maximum of the absolute value of the distance-dependent electrostatic solvation energy exceeds the respective values at infinite distance ($\sim |\Delta G_E^{\text{solv}}|$) by a factor of about three. (3) The arrangement of positively charged water-hydrogens around the first shell of water-oxygens adjacent to the ions decreases the absolute value of the distance-dependent electrostatic solvation energy to values close to the Born-

solvation excess free energy (Eq. (B.1)). These properties give rise to the conclusion that slight changes in the first hydration shell around the ions will affect the respective solvation free energy to an extraordinary extent.

The oxygens of the first-coordination shell of metal cations buried in the polar region of the membrane were potentially donated by the phosphodiester groups, by the carbonyl moieties and by water that residues in the headgroup region. Consequently, a more effective arrangement of oxygens around the ions represents one possible factor to decrease the (negative) electrostatic solvation excess free energy. A second reason can be viewed in the fact that the neutral methylene and methyl groups of the phosphocholine moieties occupy a certain volume near the ions, which becomes inaccessible for water. Therefore, sterical restrictions in the headgroup region of the lipid possibly decrease the compensating effect of water-hydrogens around the first-oxygen shell on the overall electrostatic solvation energy.

The water molecules in the interfacial region of the membrane are turning preferentially toward the membrane interior with their dipole moments due to the distribution of the charged groups of the lipid molecules (Binder and Peinel, 1985; Jedlovszky and Mezei, 2001). In particular, the water-hydrogens point preferentially towards the negatively charged phosphate groups and the water-oxygens towards the positively charged trimethylammonium groups. In this way, the orientation of water considerably contributes to the membrane dipole potential (Gawrisch et al., 1992). The electrostatic potential of the membrane interferes with the radial Coulomb potential of the ions. As a result the charged groups of the lipid disturb the arrangement of water around the ions which residue near the phosphate groups and vice versa, the ions alter the orientation of water near the membrane. The modification of water structure is expected to provide an entropic contribution to the excess chemical potential of mixing $\Delta\mu_E^{\text{mix}}$. It was shown that the binding of alkali and alkaline metal cations to lipid phospholipid membranes is endothermic indicating that the adsorption of the ions to the membrane is mainly driven by entropy (Garidel and Blume, 1999;

Lehrmann and Seelig, 1994). The effect of these species on the right-hand flank of the $\nu_{13}(\text{H}_2\text{O})$ band of water presumably indicates the partial disruption of H-bonds between the water molecules and thus a loss of water structure in the primary hydration shell of the lipid after addition of ions, which is compatible with the expected change of entropy.

Interestingly, a linear relationship between the solvation free energy of alkali and alkaline earth metal ions and the dipole potential of the membrane was recently established using a fluorescent probe technique (Clarke and Lüpertz, 1999). According to this study the investigated cations reduce the dipole potential by up to 20%. This effect can be explained by observations, which were made in this study, namely the modification of water structure, the partial compensation of the charge of the anionic phosphate by the metal cations and the reorientation of headgroups.

Alkali earth ions are found to affect the phase behavior of the acidic phospholipid dimyristoyl phosphatidylglycerol (DMPG) in the order $\text{Ca}^{2+} > \text{Mg}^{2+} > \text{Sr}^{2+}$ (Garidel et al., 2000). These species can induce the conversion of DMPG into a crystalline-like gel phase accompanied by the dehydration of the lipid headgroups and deep penetration of the ions into the headgroup region of the membranes. The effect of these ions on the anionic lipid DMPG closely resembles their effect on the neutral lipid POPC in our study. Additional negative charges of lipid headgroup as in DMPG obviously have only a small effect on the specific binding of ions but was essential for recruiting cations to the lipid–water interface in highly diluted systems (Huster et al., 2000). The effect of metal cations on the main phase transition of zwitterionic dipalmitoylphosphatidylcholine is, therefore, very small in diluted aqueous systems, except for Be^{2+} because of unknown reasons (Minami and Inoue, 1998).

4.3. Specific effects

The spectral parameters of the phosphate and carbonyl groups reflect specific interactions of ions with these moieties, which affect phase behavior, headgroup structure and hydration. The

effect of cations on lipid phase behavior and frequency shift of phosphate and carbonyl vibrations agrees with the cation effectiveness reported previously (Akutsu and Seelig, 1981; Seelig et al., 1987) for the influence on the ^2H NMR quadrupole splitting of DPPC vesicles. It increases with decreasing size according to the dependence of solvation free energy on the ionic radius. Medium-sized Ca^{2+} ions, however, shift the POPC phase transition more distinctly than the smaller Mg^{2+} , in contrast to this rule. In bulk water, calcium ions, rather than the smaller Mg^{2+} , have the most favorable first-shell solvation energy among alkaline earth dications due to its larger first-shell coordination number of oxygens ($n_{\text{coord}} \approx 8$) compared with Mg^{2+} ($n_{\text{coord}} \approx 6$) (Babu and Lim, 1999). This tendency correlates with the observed strong effect of Ca^{2+} on POPC membranes. We conclude that the effective arrangement of electronegative oxygens around Ca^{2+} present in bulk water is maintained in the polar part of the membrane. Here, the oxygens were donated by the phosphodiester groups, by the carbonyls and by the water as well. The distinct changes observed for several IR absorption bands of the phosphate group indicate that the C–O– PO_2^- –O–C fragments change their conformation, presumably from a gauche/gauche into a trans/trans conformation to interact favorably with Ca^{2+} (Akutsu, 1981). Sharp absorption bands and considerable linear dichroism reflect rigid headgroup structure. The relatively high frequency of the $\nu_{\text{as}}(\text{PO}_2^-)$ mode suggests that water is partly removed from the phosphate groups. The ions obviously concur with the water of the hydration shell that usually forms H-bonds to the phosphate-oxygens. Calcium and the phosphate groups possibly form a so-called inner sphere complex where water is removed in the region of contact. Magnesium ions affect the spectral parameters in a similar fashion but to a weaker extent. Their smaller size can be thought to fit less effectively between the headgroups.

The smaller beryllium ions considerably modify lipid phase behavior and IR spectral characteristic of the headgroups. The IR spectrum of POPC in the presence of Be^{2+} indicates that ionic water species exist in the headgroup region. We suggest that Be^{2+} ions partially hydrolyze the hydration

shell of the lipid. Beryllium ions, ionized water species and the phosphate groups seem to form a stable ternary complex, which is virtually insensitive to alterations of the chemical potential of water as indicated by the nearly constant position of the $\nu_{\text{as}}(\text{PO}_2^-)$ band (Fig. 5). On the other hand, the relatively high C=O stretching frequency indicates only weak hydrogen bonds to the carbonyl-oxygens which are obviously to some degree shielded from hydration (Fig. 7). Our results partly confirm a recent hypothesis that tentatively explains the ability of beryllium to shift the main phase transition of DPPC by the Be^{2+} -induced dehydration of the membrane (Minami and Inoue, 1998). It seems that Be^{2+} does, however, not dehydrate the phosphate groups but 'fixes' ionized water species near the headgroups. Beryllium ions probably form a so-called outer sphere complex with the phosphate groups where both the phosphates and the ions remain hydrated to some degree. The observed effect of Be^{2+} correlates with the broad spectrum of membranotropic effects in model systems. It induces membrane rigidification and phase separation with the potency comparable to that of trivalent metal cations (Verstraeten et al., 1997). Note that the charge density of Be^{2+} is similar to that of the trivalent cations owing to its small size.

Zinc appears to be the most potent divalent cation among the investigated ones in altering POPC phase behavior. The conformation of the C–O–P–O–C-backbone of the lipid headgroup clearly changes upon Zn^{2+} binding. The ability of the Zn^{2+} -bound phosphate groups to take up water is distinctly reduced, meaning that the headgroups have become less hydrophilic. Molecular orbital effects can make an important contribution to the energetics and coordination of complexes with transition metals (Binder et al., 2001; Christianson, 1991). Zinc ions have, therefore, a higher affinity to electronegative groups compared with other divalent metal cations with similar ionic radius such as Mg^{2+} (Gresh and Sponer, 1999). Zn^{2+} binds more tightly because the interaction with Zn^{2+} possesses partly the characteristics of a covalent bound than of Coulombic interactions between charged groups. This prop-

erty is due to the attractive $3d^{10}$ electron–lone pair interaction of zinc, which is absent for divalent alkali earth metal ions.

Theoretical calculations demonstrate a high flexibility in the modes of binding of hydrated Zn^{2+} because the hydration shell around the ion is highly variable (Gresh and Sponer, 1999; Sponer et al., 1998). In aqueous solution zinc chloride forms several ionic zinc-chlor complexes such as $ZnCl^+$ and $ZnCl_4(H_2O)_2^-$, $ZnOH^+$ and also uncharged linear $ZnCl_2$ molecules in considerable amounts (Irish et al., 1963; Mustafa et al., 1999). The variability in charge, size and polarity of the species can be thought to enable efficient complexation between the lipid and zinc species causing the considerable dehydration of the system. Details of Zn^{2+} interactions with POPC have been recently published (Binder et al., 2001).

The weaker effect of Cu^{2+} on POPC compared with Zn^{2+} can be attributed to the fact that the $3d^9$ orbital of Cu^{2+} is less flexible with respect to coordination number than the $3d^{10}$ orbital of Zn^{2+} (Christianson, 1991).

4.4. Hofmeister series and fusogenicity

Ions destroy the natural hydrogen bonded network of water, having effects similar to increased temperature or pressure (Leberman and Soper, 1995). Small metal cations are strongly hydrated, with small or negative entropies of hydration, creating local orientational order and higher local water density. Ions that have the greatest such effect is known as water-structure-makers whereas ions having the opposite effect are known as structure-breakers.

The lyotropic or Hofmeister series originates from the ranking of various ions toward their ability to precipitate proteins (Hofmeister, 1888). Nowadays the Hofmeister series have been shown to have a much more general utility (Collins and Washabaugh, 1985) reflecting the graduated effect on the structuring or denaturation of biological macromolecules. It is usually given in terms of the water structuring capacity of the ions, which typically increases with decreasing (negative) electrostatic excess free energy of hydration. In addition, cations may induce strong cooperative hydrogen-

bonding around them due to the polarization of O–H bonds by cation–lone pair interactions ($cation^+ \cdots O-H \cdots O-H$), which strongly affect water structure. Luck et al. introduced a cooperativity factor for this effect, which varies as the Hofmeister series from K^+ ($cf = 1.08$) to Zn^{2+} ($cf = 2.5$) ($cf = 1.0$ refers to no cooperativity effect, see Table 1 and (Luck et al., 1997)). This parameter, for example, indicates the stronger effect of Zn^{2+} on water structure compared with Mg^{2+} and it correlates with the effect of these ions on POPC. Hence, the specific water structuring efficiency due to O–H bond polarization obviously intensifies the electrostatic effect of the cations on lipid phase behavior. The turning point between structure-making and structure-breaking ability of the ions with only small net effect on water structure is about Na^+ and K^+ which only little affect the lyotropic phase transition of POPC.

Lipid vesicles aggregate and fuse when multivalent metal cations attain at a certain concentration in the vesicle suspension (see Ohki and Arnold, 2000, 1990 for a review). The general process of membrane fusion must involve a sequence of events, namely vesicle aggregation, surface dehydration and membrane destabilization, before the final merging of the bilayers can proceed. Hydration of polar interfaces and the repulsive forces acting between them are closely related to the ability of lipid bilayers to fuse because the hydration force between polar interfaces serves as a major repulsive interaction which prevents close approach and fusion of vesicles. (Arnold, 1995). Consequently, one molecular mechanisms proposed for fusion of lipid vesicles suggests an increase in membrane dehydration which results in a strong adhesion of two interacting membranes causing the deformation and destabilization of lipid bilayers (Ohki and Arnold, 2000). Ions that hydrate strongly are expected to compete with the lipid headgroups for the water, and make water molecules less available for the headgroups. Moreover, complex formation of metal ions with the phosphate groups may be responsible for the increase in surface hydrophobicity of the membrane. These tendencies would tend to reduce the range of the repulsive hydration force, and thus they promote fusion.

It has been concluded from the swelling behavior of multilamellar PC vesicles that alkali halides decrease the range of the hydration force following the Hofmeister series $\text{Li}^+ > \text{Na}^+ > \text{K}^+$ (Korremann and Posselt, 2001). Divalent cations induce fusion and increase the chain melting transition of mixed PC and phosphatidylserine (PS) vesicles with increasing effectiveness according to $\text{Zn}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+}$ (Barfield and Bevan, 1985) in agreement with the observed effect on POPC. The fusogenic capacity of divalent metal cations for pure anionic PS vesicles decreases in the order $\text{Ba}^{2+} > \text{Ca}^{2+} > \text{Sr}^{2+} > \text{Mg}^{2+}$ (Ohki and Arnold, 1990) which indicates the preference of PS for the larger Ba^{2+} and Sr^{2+} ions when compared with their effect on PC. This conclusion is confirmed by the respective association constants of Ba^{2+} and Sr^{2+} to PS, which exceed those of Ca^{2+} and Mg^{2+} (McLaughlin et al., 1981). In general the effectiveness of metal cations to induce the fusion of vesicles follows the Hofmeister series with a few exceptions, which are probably caused by sterical factors that affect effective insertion of cations in the polar region of the membrane.

5. Summary and conclusion

Insertion of most of the ions into the polar region of lipid membranes of zwitterionic POPC cause the shift of the lyotropic chain melting transition towards higher water activities. Consequently, ion–lipid interactions stabilize the solid phase of the lipid. The effect of the ions on the phase transition POPC correlates in a linearly fashion with the electrostatic solvation free energy of the ions in water that in turn, is inversely related to the ionic radius. This interesting result was interpreted in terms of the excess chemical potential of mixing of hydrated ions and lipids, which obviously mirrors the solvation free energy of the ions in water. Deviations from this rule were observed for Ca^{2+} and Zn^{2+} . Calcium possesses an effective first coordination shell of oxygens in bulk water and, probably, in the membrane as well. Zinc strongly interacts with the free oxygens of the phosphate group due to the attractive $3d^{10}$ electron-lone pair. Ion binding is

partially driven by desolvation of the lipid and the ions. This result reinforces the notion that solute association with water is at least as important as solute–solute, i.e. lipid–ion interactions.

IR spectroscopic results suggest a deep penetration of divalent cations into the polar region of the phospholipid bilayer. Comparison of the effect of different ions reveals a high degree of specificity of ion–lipid interactions, which affect phase behavior, headgroup structure and hydration.

For a rough classification, the investigated ions have been divided into three groups:

- (A) Ba^{2+} , Sr^{2+} and monovalent Na^+ , K^+ and, partly, Li^+ only weakly affect hydration and structure of the polar interphase. The carbonyl groups of POPC become slightly more accessible to hydration.
- (B) Mg^{2+} and Ca^{2+} cause partial dehydration, a conformational change and immobilization of the phosphodiester groups. A complex rearrangement of the carbonyl region accompanies ion binding, which involves hydration and conformational changes. The carbonyl groups become more accessible to the water possibly because these moieties interact with the hydration shell of the ions. The water of the hydration shell seems to be less structurated compared with pure hydrated lipid.
- (C) Be^{2+} , Cu^{2+} and Zn^{2+} and, to a less extend, Li^+ (group (A)) affect the esterified oxygens of the phosphate groups by direct interactions and/or by a conformational change in this region. Cu^{2+} and Zn^{2+} strongly interact with the nonesterified oxygens of the phosphate moieties. Interactions between the lipid and these ions cause considerable dehydration of the phosphate and carbonyl groups. Therefore, they can be classified as inner sphere complexes. Hydrogen bonds of residual water molecules are relatively weak. Beryllium ions cause hydrolysis. Ionized water species, Be^{2+} and the phosphate groups form a stable ternary complex.

Clearly, the biological effect of metal cations on phospholipid membranes depends on two events, namely accumulation near the membrane surface

and specific interactions with the headgroups. Membranes of anionic lipids are typically more strongly affected by metal cations than membranes of zwitterionic lipids because of stronger attractive Coulombic forces. The new data presented here illustrate the specific effect which exert the ions on lipid properties. This high degree of specificity is obviously weakly related to the net charge of the lipid.

The different capabilities of metal cations to bind to lipid headgroups and to affect lipid hydration may be responsible for their different fusogenic activity. The effectiveness of metal cations to affect membrane properties follows the Hofmeister series.

Appendix A. Hydration of lipids in the presence of metal chloride

Let us consider the hydration of lipid in the presence of metal chlorides in terms of a binding model, which appropriately describes the interaction of zinc ions with POPC membranes (Binder et al., 2001). According to this model the ions completely bind to the lipid below a saturation limit, $R_{M/L}(L) = R_{M/L} < R_{M/L}(\text{sat})$, where $R_{M/L}$ denotes the total amount of ions in the sample in terms of the mole ratio ions-to-lipid. $R_{M/L}(L)$ is the respective mole ratio of ions bound to lipids and $R_{M/L}(\text{sat})$ its maximum (saturation) value. At $R_{M/L} \geq R_{M/L}(\text{sat})$ the lipid becomes saturated with ions ($R_{M/L}(L) = R_{M/L}(\text{sat})$). The excess of ions remains ‘free’, that means not interacting with the lipid ($R_{M/L}(\text{free}) = R_{M/L} - R_{M/L}(\text{sat})$). The samples hydrate in a humid atmosphere according to:

$$\begin{aligned} R_{W/L} &= R_{W/L}(L) \quad \text{for } R_{M/L} \leq R_{M/L}(\text{sat}) \quad \text{and} \\ R_{W/L} &= R_{W/L}(L) + R_{W/M}(M)(R_{M/L} - R_{M/L}(\text{sat})) \\ &\quad \text{for } R_{M/L} > R_{M/L}(\text{sat}) \end{aligned} \quad (\text{A.1})$$

Here, $R_{W/L}$ is the total amount of water sorbed onto the sample in terms of the mole ratio water-to-lipid. $R_{W/L}(L)$ denotes the respective amount of water, which adsorbs to the lipid phase, and $R_{W/M}(M)$ is the mole ratio water-to-ions of water which solvates the excess salt. The pure salt solvates only at water activities above the water

activity of the respective saturated salt solution, a_w^s , i.e. $R_{W/M}(M) = 0$ at $\text{RH} < a_w^s \cdot 100\%$.

Appendix B. The Born model of ion solvation

Divalent alkaline earth and monovalent alkali cations can be viewed in a simplified fashion as positively charged spheres of radius R_{ion} , which tend to fit into the polar region of the membrane owing to attractive Coulombic charge–charge interactions with the anionic phosphate groups. In a continuum solvent of dielectric constant ϵ_s , the electrostatic excess solvation free energy of a spherical ion possessing an effective radius R_{eff} and a charge qe is $\Delta G_E^{\text{solv}} = -(qe)^2(1 - \epsilon_s^{-1})N_A/(8\pi\epsilon_0 R_{\text{eff}})$ according to the Born model (where e is the magnitude of the electronic charge, ϵ_0 and N_A denote the absolute dielectric constant and Avogadro's number, respectively). The effective Born radius of hydrated cations, R_{eff} , has been identified as $R_{\text{eff}} \approx 0.5(R_{\text{ion}} + R_{\text{gmax}})$ where R_{gmax} is the first peak position of the ion–oxygen radial distribution function, which can be determined by means of molecular dynamics simulations (Babu and Lim, 1999). Gross coincidence of the distances between selected metal ions and oxygen nuclei in crystals and in aqueous and nonaqueous solution has been established experimentally despite different ligand sizes (Schmid et al., 2000), and thus one can use $R_{\text{gmax}} \approx R_{\text{ion}} + R_{\text{oxygen}}$ to a good approximation ($R_{\text{oxygen}} \approx 0.14$ nm is the atomic radius of oxygen). Finally, one obtains the excess solvation free energy as a function of ionic radius:

$$\begin{aligned} \Delta G_E^{\text{solv}} &= -\frac{aq^2}{R_{\text{ion}} + 1/2R_{\text{oxygen}}} \quad \text{with} \\ a &= \frac{e^2(1 + \epsilon_s^{-1})}{8\pi\epsilon_0} \end{aligned} \quad (\text{B.1})$$

It corresponds to the transfer of one mole of ions from vacuum into a dielectric medium at infinite dilution. Experimental solvation (hydration) excess free energies, $\Delta G_{\text{hydr}}^{\text{exp}}$, of the studied ions in water can be well reproduced by means of (Eq. (B.1); compare lines and symbols in Fig. 12, part b).

Appendix C. Lyotropic phase transition of lipids in the presence of ions

The Gibbs free energy of a ternary lipid–water–metal chloride mixture per mol of lipid is $G = \mu_L + R_{W/L}\mu_W + R_{M/L}\mu_M$ for $R_{M/L} \leq R_{M/L(\text{sat})}$, where μ_L , μ_W and $\mu_M = \mu_{\text{Me}} + q\mu_{\text{Cl}^-}$ denote the chemical potentials of lipid, water and metal chloride (MeCl_q), respectively. The complete differential, $dG = d\mu_L + R_{W/L}d\mu_W + \mu_W dR_{W/L} + R_{M/L}d\mu_M + \mu_M dR_{M/L}$, can be set equal to the alteration of G at isobaric and isothermal conditions, $dG = \mu_W dR_{W/L} + \mu_M dR_{M/L}$, to obtain the Gibbs–Duhem relation:

$$d\mu_L = -R_{W/L}d\mu_W - R_{M/L}d\mu_M \quad (\text{C.1})$$

The chemical potentials of the components in two coexisting phases are equal at the phase boundary, i.e. $\mu_i^{\text{tr}} = \mu_i^1 = \mu_i^2$, or equivalently, $d\mu_i^{\text{tr}} = d\mu_i^1 = d\mu_i^2$ ($i = L, W, M$; the superscripts ‘1’ and ‘2’ refer to the two phases which coexist at the transition, ‘tr’). With (Eq. (C.1)) one obtains:

$$d\mu_W^{\text{tr}} = K d\mu_L^{\text{tr}} \quad \text{with}$$

$$K = \frac{(R_{M/L}^1 - R_{M/L}^2)}{(R_{M/L}^1 - R_{M/L}^2) \times R_{W/L}^1/R_{W/L}^2} \quad (\text{C.2})$$

The chemical potential of water is $\mu_W = \mu_W^b + RT \cdot \ln(RH/100\%)$ because the sample hydrates in equilibrium with water vapor of RH ($\mu_W^b = \text{constant}$ is the chemical potential of bulk water). Insertion into Eq. (C.2)) gives:

$$\frac{dRH^{\text{tr}}}{RH^{\text{tr}}} = -K \frac{d\mu_L^{\text{tr}}}{RT} \quad (\text{C.3})$$

The chemical potential of the lipid splits into the standard chemical potential and the mixing entropy, $\mu_L = \mu_L^0 + RT \ln(x_L)$. The mole fraction of lipid is $x_L = (1 + (q+1)R_{M/L})^{-1}$ in the presence of dissociated salt. The standard chemical potential can be written in the form $\mu_L^0(x_L) = \mu_L^0(1) + \mu_E(x_L)$ where $\mu_L^0(1) = \text{constant}$ denotes the chemical potential of pure lipid and $\mu_E(x_L)$ is the excess chemical potential, which considers deviations from ideal mixing. Consequently, the differential in (Eq. (C.3)) refers to the change of excess chemical potential of the lipid and of the mixing entropy, $d\mu_L = d\mu_E(x_L) + RT dx_L/x_L$. In-

sertion into (Eq. (C.3)) and approximation of the differentials by differences give:

$$\frac{\Delta RH}{RH_0} \approx K \left(\frac{(q+1)\Delta R_{M/L}}{(1+(q+1)\Delta R_{M/L})} - \frac{1}{RT} \Delta \mu_E \right) \quad (\text{C.4})$$

where RH_0 denotes the transition value of the pure lipid and $\Delta RH = RH - RH_0$ is the shift of the transition owing to the addition of ions.

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