Effects of Monovalent Anions of the Hofmeister Series on DPPC Lipid Bilayers Part I: Swelling and In-Plane Equations of State

A. Aroti,* E. Leontidis,* M. Dubois,[†] and T. Zemb[†]

*Department of Chemistry, University of Cyprus, Nicosia, Cyprus; and [†]Laboratoire Interdisciplinaire sur l'Organisation Nanomé trique et Supramolé culaire at Département de Recherche sur l'État Condensé, les Atomes, et les Molécules Service de Chimie Moléculaire at Commissariat à l'Energie Atomique, Saclay, F91191 Gif sur Yvette, France

ABSTRACT   Aiming to improve understanding of the mechanisms behind specific anion effects in biological systems we have studied the effects of sodium salts of simple monovalent anions belonging to the Hofmeister series on the bilayers of the zwitterionic lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine using small-angle x-ray scattering and the osmotic stress technique. NaCl, NaBr, NaNO$_3$, NaI, and NaSCN were used in this investigation. The electrolytes were found to swell the bilayers and to increase the area per lipid headgroup at each value of the osmotic pressure, suggesting the association of anions with the bilayer-lipid interfaces. The effects follow the Hofmeister series with SCN$^-$ inducing the most pronounced changes. "Ion competition" experiments with mixed NaI/NaCl solutions at total salinity 0.1 and 0.5 M revealed that the effect of ions on the lipid equation-of-state is roughly linear at low concentrations, but strongly nonlinear at high concentrations. The experimental results are fitted in a companion article to provide "binding" or "partitioning" constants of anions in the lipid bilayers.

INTRODUCTION

Ion specificity and the Hofmeister series

The influence of aqueous electrolytes on various physico-chemical and biological phenomena has been widely studied since the 19th century. More than 100 years ago, Hofmeister (1) published experimental results showing the effect of various salts on the aqueous solubility of proteins. Since then, numerous experimental studies have shown the importance of specific ion effects in a multitude of biological and physicochemical phenomena (2,3). On the basis of the magnitude of their effects, ions have been ordered into sequences (one for anions and one for cations), which are called the Hofmeister series. For anions, based on increasing salting-in potency for proteins from left to right the series is as follows:

$$SO_4^{2-}, HPO_4^{2-}, OH^-, F^-, HCOO^-, CH_3COO^-, Cl^-, Br^-,$$
$$NO_3^-, I^-, SCN^-, ClO_4^-.$$

Despite the fact that the Hofmeister series plays a significant role in a broad range of phenomena, the precise origin of action of the ions in the series has not yet been clarified and no generally accepted explanation exists at the molecular level (2–4). Several different ideas about the nature of specific salt effects have been proposed to date. A favorite explanation of specific salt effects for a long time was that ions modify the structure and properties of water. The character of water as a solvent for biomolecules would thus change in specific ways in the presence of electrolytes. It has become standard practice to call ions on the left of Cl$^-$ in the Hofmeister series "cosmotropes" or "structure makers", whereas ions on the right of Cl$^-$ are called "chaotropes" or "structure breakers".

Cavity models for the salting-out effect on biomolecules are based on such ideas (5,6). Biologists often refer to the presence of low-density and high-density water close to biological interfaces and the way that ions affect the two water regions (7–9). Consistent with this idea is the proposition by Chandler (10), that an extended hydrophobic surface is "dry" to a certain extent, in the sense of a reduced water density found in its proximity. This dryness would be enhanced by hydrophilic ions and reduced by hydrophobic ions. A related model was put forward by Collins and Washabaugh (2), who postulated that water at the interface between a hydrophobic surface and an electrolyte solution can be divided into three layers, according to the extent that the structure of water is affected by the ions. The disadvantage of such models is their complexity and their relative lack of predictive capability. In addition, considerable recent evidence supports the idea that the structure of water is not heavily perturbed by monovalent ions, and that there is no direct correlation between a solute's impact on water structure and its effect on biomolecule stability (11–13).

An entirely different approach, which has a long history in colloid science, assumes that ion interactions with specific groups on surfaces can explain the Hofmeister series (6,14). Although this approach may be successful for a variety of phenomena to a certain extent, it cannot be the only explanation of specific salt effects, since these are also observed in the absence of specific surface groups, as in the case of the surface tension of electrolyte solutions (3,15) and in the salting-out of small organic solutes or gases (3,6,16,17).

According to a proposition by Ninham and Yaminsky (18) the origin of ionic specificity could be due to the usually neglected dispersion interactions between ions and surfaces. It was proposed that an ionic dispersion potential acting between ions (or ions and water or ions and interfaces) must be included in the usual electrostatic theory to explain specific

salt effects (18–21). However, the dispersion-forces-based calculation of the surface tension of electrolyte solutions by Boström et al. (22–24) predicts a layer free of ions close to the water surface, in qualitative disagreement with recent experiments and molecular simulations of electrolyte solution surfaces. These show that ions like Cl^- have a higher affinity for the free water surface than Na^+ ions, and that large polarizable anions, such as I^-, prefer interfacial solvation sites and have significant concentration peaks at the air/water interface (25–37).

An alternative qualitative model argues that ion specificity arises as a result of the fine balance between ion-water and water-water interactions (38). Recent computer simulation studies on clusters giving emphasis to hydration interactions support this idea (39–41). Kjellander et al. (42) and Marcelja (43) have demonstrated that a way to account for ion specificity in statistical mechanical models of double layers is to use effective ion-ion and ion-surface potentials that indirectly account for ion-water interactions and the water structure. The related concept of an ''active interface'' was put forward recently by Aroti et al. (44,45). The presence of an active interface in an ion-containing system allows the reduction of the system free energy by ''expelling'' those ions that are easily dehydrated to the interfacial region and liberating water molecules that are reincorporated into the bulk water network. Two requirements must be fulfilled by an active interface: a), it must have a considerable degree of disorder, and b), it must be capable of accommodating ions, at a free-energy gain.

It can be concluded from the above broad spectrum of models that no general consensus exists today for the mechanism of specific ion effects, and it remains largely unclear whether ions act through precisely defined, specific, local interactions, or through more delocalized collective interactions. Elucidation of the mechanism of specific ion effects in a particular experimental situation will provide valuable insights for a multitude of ion-specific phenomena, and will have a strong impact on biology and chemistry.

Lipid model systems for the investigation of specific salt effects

Advances in the understanding of specific salt effects can be achieved by choosing appropriate model systems that allow discrimination between the many possible modes of ion-interface interaction. Phospholipids offer significant advantages as model systems: a), They are major constituents of biological membranes. b), Some are charged and others uncharged (e.g., serine versus choline headgroups), and some may become charged as the pH changes (e.g., lipids with ethanolamine headgroups). Zwitterionic phospholipids in particular do not interact strongly with the ions and thus may potentially render ''visible'' weaker specific interactions. c), They can be examined as bilayers (in the form of vesicles or lamellar phases), as monolayers at the air-water interface, and even as micelles in the case of single-tail phospholipids.

Point c above was explored by Aroti et al., who have used lipids with phosphatidylcholine headgroups in all possible geometries (micelles, monolayers, and bilayers), examining the effects of anions on all these systems with a goal to combine information and methods ((44–46); E. Leontidis, L. Belloni, and A. Aroti, unpublished data). The work on 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) monolayers at the air-water interface yielded two useful results. First, it was found that lipids affect the disordered liquid-expanded phase of the DPPC monolayer, but not the ordered liquid-condensed phase (46). Second, it was found that classical electrostatic binding models cannot explain ion-monolayer interactions, but a model of ions partitioning within the monolayer was much more fruitful, supporting the active-interface idea ((45); E. Leontidis, L. Belloni, and A. Aroti, unpublished data).

Besides monolayers, phospholipid bilayer systems offer a range of experimental parameters that can be measured to quantify their interactions with anions such as: a), Structural changes like the maximum swelling, the bilayer thickness, and the area per headgroup of the lipid. b), Equation of state (osmotic pressure versus interbilayer distance) curves, which provide information about many important interactions mediated by the ions. c), Surface potential values, affected by conformational changes of the phospholipids, changes in the average tilt of the headgroup dipole, and the formation of an electrostatic double-layer in the presence of ions.

In what follows we will concentrate on bilayers of zwitterionic lipids. The interaction of zwitterionic phospholipid bilayers (either vesicles or bilayer stacks) with ions has been extensively studied in the past (47–76). Many studies originally concentrated on cations, given the biological function and importance of Na^+, K^+, Ca^{2+}, and Mg^{2+} (47–57). Anion effects on the structural properties of lipid bilayers have been examined in a rather limited and nonsystematic way. Methods such as ^{1}H-NMR, ^{2}H-NMR, or ^{31}P NMR, Raman spectroscopy, electron paramagnetic resonance (EPR) spectroscopy, x-ray diffraction and neutron diffraction, ζ-potential measurements, and differential scanning calorimetry have been used to study specific anion effects on the structural properties of lipid bilayers (58–76).

^{1}H-NMR studies have shown that the strength of interaction of anions with zwitterionic lipids follows the Hofmeister series. I^- and SCN^- were found to interact strongly enough with phosphocholines, but the interaction could not be characterized as localized binding (58). Chaotropic anions caused splitting of the choline ^{1}H resonances of EPC bilayers, whereas no changes could be detected in the glycerol and phosphate headgroup region, indicating that the ions do not bind to or affect the phosphate moiety of the lipid molecule (59). Using Raman spectroscopy Loschilova and Karvaly also concluded that the interaction of anions with PC lipids follows a Hofmeister series, and that electrostatics alone cannot explain the spectral changes observed in the presence of anions (60). Considerable insight was obtained from ^{2}H-NMR experiments,

since the deuterium quadrupolar splitting of deuterated cho-
lines can be used to quantitate the degree of ''binding'' of
ions to the lipid headgroups. An investigation of the influ-
ence of anions on 1-palmitoyl-2-oleoyl-phosphatidylcholine
bilayers has shown that the chaotropic anions produce the
most significant changes of the deuterium quadrupolar split-
tings (61,62). Assuming that the ions are adsorbed on the
bilayers and using the DLVO theory, it was possible to es-
timate the surface potential of the bilayers and the binding
constants of the ions to the lipids.

The influence of anions on the surface potential of lipid
bilayers (PE and PC vesicles or PC bilayers) has been mea-
sured by McLaughlin et al. (64), Tatulian (65), and Clarke
et al. (67) using either electrophoretic mobility or fluores-
cence spectroscopy with fluorescent dyes. They have observed
that the lipid membrane potential becomes more negative
through adsorption of the anions following the Hofmeister
series. Tatulian (65) used the DLVO theory to calculate sur-
face potentials and binding constants of anions to the lipids,
whereas Clarke et al. (67) has used the fluorescence shift of
specific dyes to obtain values of the intrinsic binding constant
of ClO_4^- on dimyristoylphosphatidylcholine. A collection of
binding constants of anions on phospholipids unfortunately
shows significant variability between different experimental
methods, or even between experiments of different groups
using similar methods (see companion article).

The effect of anions on the phase transition temperatures
of lipids has also been studied repeatedly with the general
result being that the chaotropic ions have pronounced effects
on the main phase transition of lipids ($L_\beta \rightarrow L_\alpha$) (63,68–73).

Structural information for zwitterionic lipid bilayers in the
presence of electrolytes with emphasis on the anions has
been obtained using x-ray diffraction or neutron diffraction
measurements, but is surprisingly scarce (66,69,70,73,74).
The influence of monovalent anions on the structural prop-
erties of DPPC bilayers has shown that the DPPC bilayers
swell continuously in 1 M potassium salt solutions until a
limiting bilayer repeat distance is obtained. In the presence of
SCN^- ions, the existence of an interdigitated structure was
postulated (69,70,73). EPR measurements also suggested that
the chaotropic anions I^- and SCN^- may induce an inter-
digitation of the DPPC hydrocarbon chains (75,76). Tatulian
(66) performed neutron diffraction on bilayers and observed
that addition of NaCl does not affect the DPPC lamellar
structure whereas $NaClO_4$ drastically influences the lamellar
repeat spacing.

In recent years useful information at the atomic level on
local interactions of anions with lipid bilayers has been ob-
tained by molecular dynamic simulations (77–84). Sodium
was found to create complexes with more than one lipid mol-
ecules (80–84) whereas anion penetration into zwitterionic
lipid bilayers was found mostly for large anions (chaotropic
anions) that can penetrate deeply into the bilayers (78,79).
None of these studies was based on polarizable models for
water and ions.

Where we stand

From the previously published work it can be concluded that
a very visible interaction between anions and zwitterionic
lipids exists, but it has not been studied very systematically
to date, and the actual interaction was only semiquantified
using chemical binding constants. In this work we reevaluate
the effects of anions on DPPC bilayers in the fluid phase by
applying the osmotic stress method in combination with
small-angle x-ray scattering (SAXS) using a range of sodium
salts to obtain an extensive experimental database for the
application of models. The experimental results (equation of
state curves of osmotic pressure versus interbilayer distance
and area per lipid headgroup) are fitted in the companion
article, using different theoretical models, especially regard-
ing the electrostatic repulsion due to ion adsorption. In spirit,
this work is related to the recent article by Petrache et al.,
who investigated the effects of KCl and KBr on dilaur-
oylphosphatidylcholine (DLPC) bilayers (74). However, in
this work we are using anions known to interact more strongly
with the lipids and consequently observe effects not observed
in previous investigations.

MATERIALS

DPPC was obtained either from Sigma-Aldrich (St. Louis,
MO) or from Avanti Polar Lipids (Alabaster, AL), and used
without further purification. All sodium salts were purchased
from Sigma-Aldrich with purity >99%, with the exception
of NaSCN, the purity of which was >98%. Salt solutions
were prepared using ultrapure water (specific resistance of
18.2 MΩ cm) produced by a three-stage Millipore (Billerica,
MA) Milli-Q Plus 185 purification system. Polyethylene-
glycol (PEG) 20,000 was purchased from Fluka (Milwaukee,
WI) and used without further purification. The pH value of
the aqueous solution of PEG 20,000 was equal to 6.2.

METHODS

Small angle x-ray scattering

The x-ray diffraction method was used to determine the lipid bilayer struc-
tural parameters in the presence of sodium salt solutions of concentrations
0.05, 0.1, and 0.5 M. Absolute scaled SAXS experiments have been per-
formed using a laboratory built High Flux camera with pinhole geometry
and high sensitivity at the Service de Chimie Moléculaire, CEA-Saclay
(France) (85,86). A two-dimensional gas-filled detector with a diameter equal
to 0.3 m was used to record the experimental spectra. The effective q-range
of this detector is from 0.02 to 0.4 Å^{-1} where $q = 4\pi/\lambda \sin\theta$. The exposure
time for each sample was 30 min. All experiments have been performed at a
controlled temperature $T = 50 \pm 1°C$. The lamellar repeat spacing D was
obtained experimentally using Bragg's diffraction law, and can be divided into
the bilayer thickness, b_L, and the water bilayer separation, d_w. The bilayer
thickness and water bilayer separation were calculated using the following
expressions:

$$b_L = \phi_L D \qquad (1)$$

$$d_w = D - b_L. \qquad (2)$$

φ_L is the volume concentration of the lipid in the sample, which is related to the weight fraction, c, of lipid in the sample as follows (87):

$$\phi_L = \left[1 + \frac{(1-c)\bar{v}_w}{c\bar{v}_L} \right]^{-1}. \qquad (3)$$

The weight fraction, c, was determined by the Karl Fischer titration method. $\bar{v}_w$ and $\bar{v}_L$ are the partial specific volumes of water and phospholipid, respectively. $\bar{v}_w$ was taken as 1.00 ml/g and $\bar{v}_L$ as 1.0091 ml/g for DPPC with melted hydrocarbon chains (88,89). In addition, the headgroup area, A, of the DPPC molecules can be estimated through the lipid bilayer thickness, b_L, using the following geometric relationship:

$$A = \frac{2 \times MW \times \bar{v}_L}{b_L \times N_{AV}}. \qquad (4)$$

MW in Eq. 4 is the lipid molecular weight and N_{AV} is Avogadro's number. There is an alternative way to partition the lamellar repeat distance into b_L and d_w by assigning the phospholipid headgroups to the water layer (88–90). We have tried this method as well in the theoretical analysis of the data that we report in the companion article. Because we did not observe any significant qualitative differences between the two methods regarding the fitting results, we only report d_w values based on Eqs. 1–3 above.

Osmotic stress

The osmotic stress technique for measuring interbilayer forces has been reviewed in detail by Rand and Parsegian (91). Briefly, the water between the bilayers is allowed to come to thermodynamic equilibrium with an excess solution of a high molecular weight polymer (PEG) of known osmotic pressure, which is in contact with, and competes for, the available water with the lipid multilamellar system. The osmotic pressure applied by the PEG solution is related to its concentration and temperature as follows:

$$\Pi(\text{dyn/cm}^2) = -1.31 \times 10^6 G^2 T + 141.8 \times 10^6 G^2$$
$$+ 4.05 \times 10^6 G, \qquad (5)$$

where $G = w/(100 - w)$ and w is the weight percent of the polymer in solution, and $T = 50 \pm 1°C$ in these experiments. The above equation was modified accordingly for Π in Pa.

This calibration expression has been established by Michel (92,93) for PEG with an average molecular weight of 8000 and is strictly valid in the range $5°C \le T \le 40°C$ and up to $G = 0.8$. We have used this expression with PEG 20,000 because the osmotic pressure is known to be roughly independent of MW in the range of 8000–20,000 for concentrations such that the solution is in the semidilute regime (94). Moreover, Eq. 5 has been used at temperatures exceeding 40°C without further verification because, according to Dubois et al. (95), PEG is not subject to hydrolysis or fragmentation that might modify the applied osmotic pressure under these conditions.

Experimentally, a known amount of PEG was mixed with NaA salt solutions of various concentrations and then added to dry DPPC in weighing bottles. A semipermeable membrane was not used to separate the polymer from the lipid solution, since the PEG 20,000 mixes very poorly with the sample solution. The samples were allowed to equilibrate at room temperature for 48–72 h and then were transferred to an oven that was thermostated at $T = 50 \pm 1°C$ for 18–20 h before using the samples. After reaching equilibration the samples were transferred to aluminum x-ray sample holders, sealed with Kapton, and mounted immediately to a thermostated cell at $T = 50 \pm 1°C$. The time allowed to the samples to equilibrate at $T = 50 \pm 1°C$ was established by reference experiments using DPPC in pure water, which fully reproduced literature results. For DPPC in the presence of electrolytes a number of samples have been allowed to equilibrate for 20 h as well as for 48–72 h before their use. In all cases we obtained the same results for the lamellar spacings as functions of the applied osmotic pressure, indicating that equilibration is already reached at a time of 20 h even in the presence of salts. Repeat spacings were determined by x-ray diffraction as described before.

Equilibration of the samples

Various methods exist for the preparation of the samples to achieve equilibration (96) such as: a), dropping dry DPPC into salt water; b), mixing DPPC with limited amounts of salt water then allowing further equilibration with excess salt solutions; and c), cycling through the main phase transition in excess salt solution. In our experiments, we have used only the first preparation method because it is the most common and also the most often used in the literature (see Fig. 1). Usually the problem of equilibration is confronted by letting the sample overnight at a temperature higher than the chain melting temperature. We followed this procedure without observing any problem to the equilibration of the samples. In addition many researchers have used this method in the past with success for the equilibration of their samples.

Effect of salt on the osmotic pressure

The osmotic pressure of the PEG polymer can be affected by the presence of electrolyte, which may influence the Flory-Huggins interaction parameter as shown in Eq. 6. The osmotic pressure, π_{mix}, due to the mixing of polymer and solvent molecules (e.g., salt solution) can be expressed by the Flory-Huggins equation (97):

$$-\pi_{mix}\bar{V} = RT\ln\phi_1 + RT\left(1 - \frac{1}{n}\right)\phi_2 + RT\chi\phi_2^2, \qquad (6)$$

where φ_1 is the volume fraction of the solvent, φ_2 is the volume fraction of the polymer, n is the number of segments in the polymer chains (proportional to the molecular weight), and χ is the Flory-Huggins interaction parameter, which characterizes the interaction between the polymer segments and the solvent molecules.

However, according to Parsegian et al. (91), the effect of an electrolyte solution on the osmotic pressure of a PEG solution is small, and does not depend strongly on the ionic strength for small electrolyte concentrations. The strongest effect of a salt solution on the osmotic pressure of PEG was reported for the chaotropic salt $NaClO_4$, which lowers the osmotic pressure exerted by PEG 20,000 in 1 M solutions by up to 40% (91).

If we accept that the effect of any salt solution on the osmotic pressure of PEG is the maximum found for $NaClO_4$ (40%), then according to Eq. 5 the change in the osmotic pressure of PEG for two limiting concentrations, e.g., $G = 0.015$ and $G = 0.79$ is as shown in Table 1. From Table 1 we see that even if the effect of a salt solution on the osmotic pressure is considerable, the error in the logΠ scale used in the representation of the experimental results is significantly compressed. In our case, we accepted that the salt solution affects the osmotic pressure of the polymer by ~20% and we took

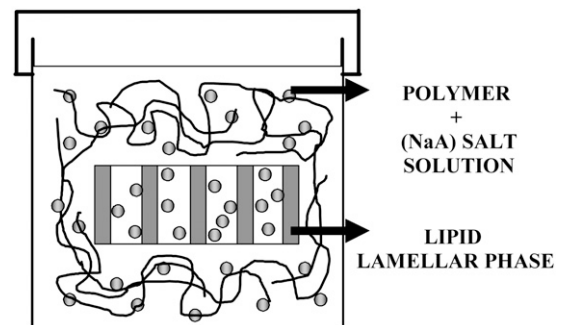FIGURE 1   Equilibrium of the samples.

TABLE 1 Osmotic pressure variation of PEG solutions in the presence of salt

G	Π (dyn/cm^2)	Π (40%) (dyn/cm^2)	$\log\Pi$ (dyn/cm^2)
0.015	77,918	77,918 $\pm$ 31,167	4.89 $\pm$ 0.2
0.79	50,300,335	50,300,335 $\pm$ 20,120,134	7.70 $\pm$ 0.2

this into consideration for calculating the exerted osmotic pressure of PEG 20,000.

Maximum swelling

To determine the maximum swelling of DPPC in pure water and in the presence of sodium salts solutions, a known amount of lipid was added to varied amounts of solution and allowed to equilibrate for 48–72 h at room temperature. Then the samples were transferred to an oven thermostated at $T = 50 \pm 1°C$ for 18–20 h before x-ray measurements.

Karl Fischer

The water content of the samples was determined by Karl Fischer (KF) titrations using a 684 KF Coulometer (Brinkmann Metrohm, Westbury, NY). I$_2$ was generated electrolytically. The KF reagent (Hydranal coulomat) was purchased from Riedel-De Haen (Seelze, Germany).

EXPERIMENTAL RESULTS

In this work we have carried out the following experimental measurements:

1. Determination of the maximum swelling of DPPC in pure water (no salt) and in different sodium salt solutions of concentration 0.05, 0.1, and 0.5 M by small angle x-ray scattering.
2. Determination of the bilayer thickness of DPPC in pure water (no salt) and in different sodium salt solutions of concentration 0.05, 0.1, and 0.5 M using small angle x-ray scattering and Karl Fischer titrations.
3. Construction of osmotic pressure–interbilayer distance curves using the osmotic stress technique in combination with small angle x-ray scattering experiments to obtain an equation of state (EOS) of the zwitterionic (DPPC) bilayer system in the fluid state ($T = 50°C$) in the presence of different sodium salt solutions.
4. Ion ''competition'' experiments, using NaI/NaCl mixtures at constant total salinity of 0.1 and 0.5 M, and varying the relative amount of the two anions. In these experiments the interbilayer distance was measured at constant osmotic pressure.

Maximum swelling

The maximum swelling curves for DPPC bilayers in NaA salt solutions of concentration 0.1 M are presented in Fig. 2 as plots of the repeat distance D versus the inverse of the lipid volume fraction. This way of plotting is suggested by Eq. 1, according to which D should be inversely proportional
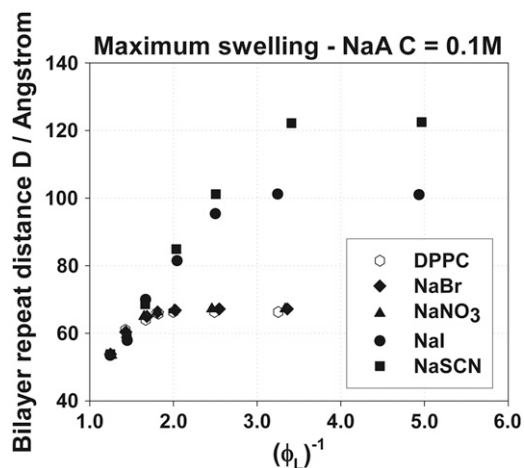
FIGURE 2 Maximum swelling of DPPC in the presence of NaA salt solutions of concentration $C = 0.1$ M.

to φ_L for constant bilayer thickness. From Fig. 2 we can see that the maximum swelling is influenced by the anion type in a significant way. Similar maximum swelling curves were obtained with NaA concentrations of 0.05 and 0.5 M and are not shown here (45). The anions used influence the maximum swelling in the order Br$^-$ < NO$_3^-$ < I$^-$ < SCN$^-$, which is a direct Hofmeister series with the SCN$^-$ having the largest effect on the equilibrium separation. The influence of the concentration is more complex; it depends on the type of the anion used and will be discussed below. Table 2 summarizes the maximum swelling results for DPPC in the presence of the sodium salt solutions of various concentrations whereas in Table 3 the maximum swelling parameters of DPPC in pure water found in this work are compared with those found in the literature, and a good agreement is observed (87,98).

Bilayer thickness

The bilayer thickness was determined using Eq. 1. Fig. 3 shows the dependence of the bilayer thickness on the osmotic

TABLE 2 Maximum swelling of DPPC in NaA salt solutions of different concentrations

Concentration	Parameters	NaBr	NaNO$_3$	NaI	NaSCN
$C = 0.05$M	D_{max} (Å)	–	–	74.8	148.0
	$\varphi_{L(max)}$%	–	–	48.8	26.3
	$d_{w(max)}$ (Å)	–	–	38.3	109.05
$C = 0.1$M		NaBr	NaNO$_3$	NaI	NaSCN
	D_{max} (Å)	67.2	67.2	101	122.5
	$\varphi_{L(max)}$%	52.2	52.2	39.2	33.3
	$d_{w(max)}$ (Å)	32.1	32.1	61.5	81.7
$C = 0.5$M		NaBr	NaNO$_3$	NaI	NaSCN
	D_{max} (Å)	68.0	66.8	77.5	85.6
	$\varphi_{L(max)}$%	52.5	52.1	46.5	42.7
	$d_{w(max)}$ (Å)	32.3	32.0	41.5	49.0

TABLE 3 Comparison of the maximum swelling of DPPC in pure water with values found in the literature

Maximum swelling parameters	DPPC in pure water $T = 50°C$
D_{max} (Å)	67.0*, 67.0[†], 66.3[‡]
$\varphi_{L(max)}$	0.54*, 0.51[†], 0.53[‡]
b_L (Å)	35.9*, 34.2[†], 34.9[‡]
$d_{w(max)}$ (Å)	31.1*, 32.8[†], 31.4[‡]
A (Å^2)	68.1*, 71.2[†], 70.0[‡]

*Values taken from Rand and Parsegian (98).

[†]Values taken from Lis et al. (87).

[‡]Values obtained in this work.

pressure exerted on the DPPC bilayers for NaA solutions of concentration 0.1 M. Similar experimental results were obtained with NaA concentrations of 0.05 and 0.5 M and are presented as supporting information. In general, the bilayer thickness of DPPC is affected by the type and concentration of anions used. The chaotropic anions I^- and SCN^- are those that have the strongest effect on the bilayer thickness of DPPC whereas Br^- and NO_3^- do not seem to have a significant influence. In Fig. 3 it is shown that the bilayer thickness decreases at small osmotic pressures indicating that the presence of anions induces significant membrane thinning. This considerable thinning may be evidence of lipid interdigitation.

Area per headgroup

The headgroup area, A, of a DPPC molecule in the absence and presence of various NaA salt solutions was calculated using Eq. 4. Fig. 4 shows the dependence of the headgroup area on the osmotic pressure exerted on the lipid bilayers in the presence of 0.1 M NaA salt solutions, and proves that the headgroup area is affected by the type of anions used. At small osmotic pressures, the chaotropic anions I^- and SCN^- have the strongest effect on the headgroup area, an effect that
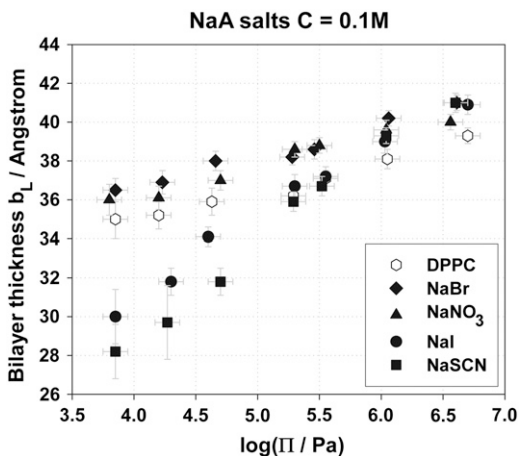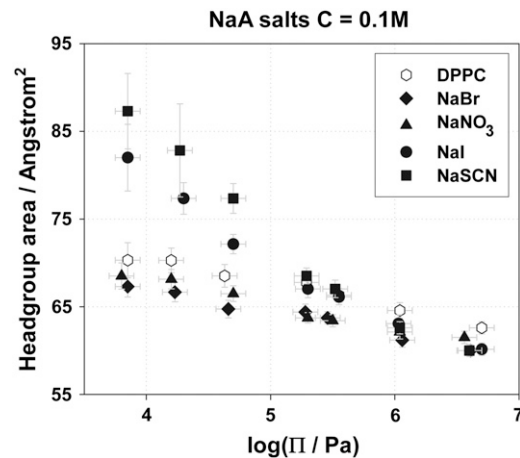

FIGURE 4   Headgroup area, A, versus the osmotic pressure, Π, exerted on DPPC bilayers in the presence of NaA solutions of concentration 0.1 M.

may be attributed to ion binding, although partial lipid interdigitation may not be ruled out. Br^- and NO_3^- dehydrate the DPPC headgroup to some extent, and it appears that the dehydration effect is stronger than ion binding for these less chaotropic ions. In addition, as the osmotic pressure increases, the headgroup area in the presence of NaI and NaSCN decreases in a faster way, and at high pressures it becomes roughly independent of the salt present, indicating that the dehydration of the headgroups with pressure plays a more important role than ion binding. Similar experimental results were obtained with NaA concentrations of 0.05 and 0.5 M and are provided as supporting information.

Pressure-distance isotherms (equation-of-state curves)

Osmotic pressure versus interbilayer distance curves ($\log\Pi - d_w$) have been used by many investigators to provide information on forces acting between lipid bilayers (49,50,53,66, 69,70,73,74,87,91,98). The $\log\Pi - d_w$ curves for DPPC in pure water and in NaBr, NaNO$_3$, NaI, and NaSCN salt solutions of concentrations 0.05, 0.1, and 0.5 M are presented in Fig. 5, a–c, respectively. The experimental points at $\log\Pi = 0$ are the equilibrium separations at maximum swelling of DPPC bilayers in water and in the presence of salt solutions. Assigning maximum swelling values to $\log\Pi = 0$ is an assumption regularly made in the literature, but we conclude in the companion article that it generates some serious problems for the theoretical modeling of the interaction curves.

Generally, we observe that the water bilayer separation d_w for the same osmotic pressure increases when salts are present (at all salt concentrations). The change of d_w is more pronounced at small osmotic pressures and diminishes as the osmotic pressure applied to the bilayers increases. Most experimental curves appear to converge at high osmotic
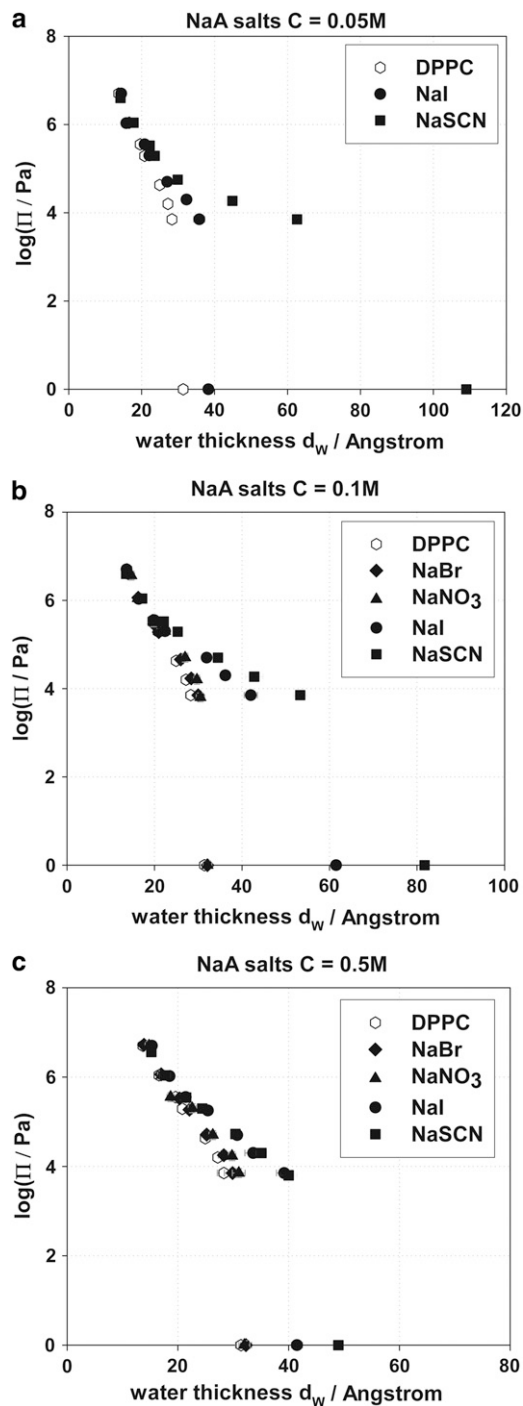

FIGURE 3   Bilayer thickness, b_L, versus the osmotic pressure, Π, exerted on DPPC bilayers in the presence of NaA solutions of concentration 0.1 M.

FIGURE 5 (a) $\log\Pi - d_w$ curves of DPPC in the presence of NaBr, NaNO$_3$, NaI, and NaSCN at concentration $C = 0.05$ M. (b) $\log\Pi - d_w$ curves of DPPC in the presence of NaBr, NaNO$_3$, NaI, and NaSCN at concentration $C = 0.1$ M. (c) $\log\Pi - d_w$ curves of DPPC in the presence of NaBr, NaNO$_3$, NaI, and NaSCN at concentration $C = 0.5$ M.

ing the smallest. The effect of the anions on d_w follows the Hofmeister series. NaCl and NaBr appear to have a much smaller effect on DPPC at 50°C, than that of KCl and KBr observed by Petrache et al. on DLPC bilayers at lower temperatures (74).

The increase of the water bilayer separation depends on the concentration of the sodium salt solutions. Fig. 6 a shows the influence of NaSCN on d_w whereas Fig. 6 b shows the influence of NaI on d_w at three concentrations, 0.05, 0.1, and 0.5 M. It is observed that d_w decreases continuously at fixed osmotic pressure upon increasing the concentration of NaSCN salt from 0.05 to 0.5 M. On the contrary, NaI is more effective at a concentration of 0.1 M whereas 0.05 M provides the smallest effect and 0.5 M gives intermediate results. Similar behavior for DPPC in the presence of Ca^{2+} ions has been observed by Lis et al. (49). This complex behavior may be explained by two contrasting phenomena that determine the electrostatic repulsive force between the bilayers; these are ion binding and electrostatic screening. It can be assumed that the influence of NaI on d_w at a concentration of 0.05 M is weaker because the adsorption of I$^-$ on the DPPC headgroups and hence interfacial charging is still relatively small. In contrast, the influence of NaI on d_w at a concentration of 0.5 M is weak due to high electrostatic screening. As a result, there will be an intermediate concentration, for which the repulsion between the DPPC bilayers due to the adsorbed anions is highest. NaSCN behaves differently; the electrostatic repulsion decreases consistently with increasing salt concentration. This behavior may be explained by assuming a very strong binding of SCN$^-$ on the DPPC headgroups. Even at low concentrations, e.g., 0.05 M, SCN$^-$ binds more strongly to the DPPC molecules than I$^-$, and thus the electrostatic repulsion that is generated is stronger than that observed in the presence of an equal concentration of I$^-$. As NaSCN concentration increases more SCN$^-$ ions bind to the DPPC headgroups but apparently the increased binding is more than counterbalanced by the double-layer screening. It is possible that a maximum repulsive force between the bilayers might also have been observed at NaSCN concentrations lower than 0.05 M, but such concentrations were not examined in our experiments.

On the contrary, the presence of NaBr and NaNO$_3$ salt solutions does not appear to affect significantly the water bilayer separation, d_w, by varying the salt concentration as observed in Fig. 6 c for NaNO$_3$. The KCl and KBr experiments of Petrache et al. demonstrated the opposite ionic effect (74). In those experiments, d_w continuously increased as the salt concentration increased, which must be attributed to the relatively weak binding of these ions, which require high bulk concentrations to reach interfacial saturation.

Cl$^-$/I$^-$ ion competition experiments

To compare the effect of a relatively hydrophilic (Cl$^-$) and a chaotropic (I$^-$) ion on the structure of DPPC bilayers, the

pressures (low d_w), implying that the hydration forces that dominate the interactions at these distances do not depend strongly on salt presence. The increase of the water bilayer separation depends on the type of anion in the sodium salts, with SCN$^-$ having the strongest effect on d_w, and Br$^-$ hav-
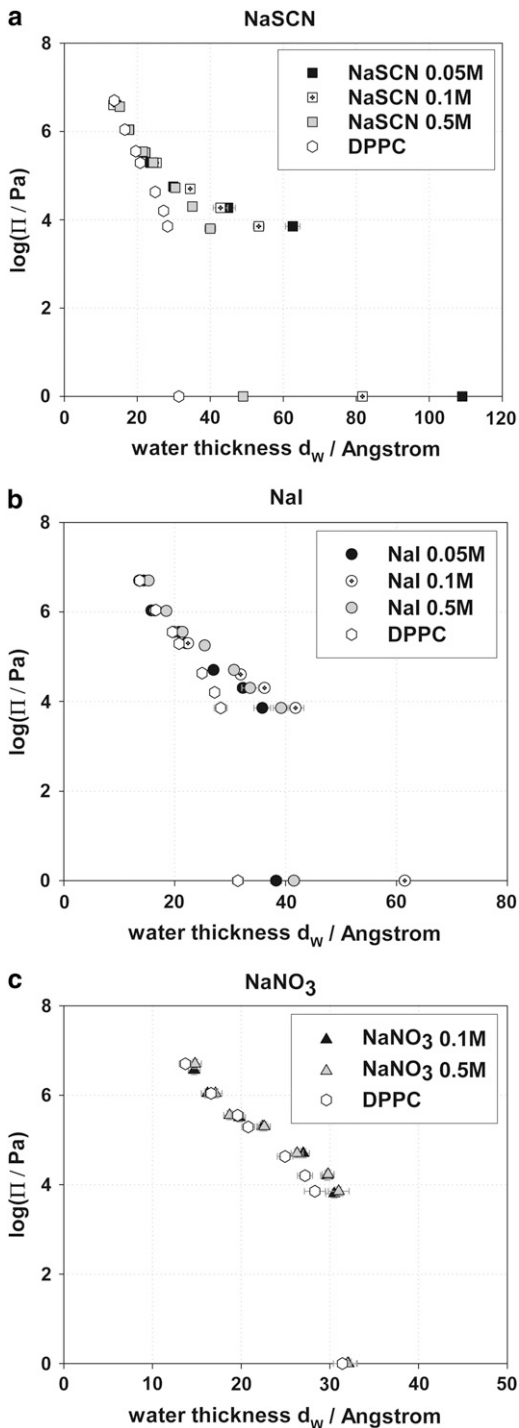
FIGURE 6 (a) $\log\Pi - d_w$ of DPPC in the presence of NaSCN at concentration 0.05, 0.1, and 0.5 M. (b) $\log\Pi - d_w$ of DPPC in the presence of NaI at concentration 0.05, 0.1, and 0.5 M. (c) $\log\Pi - d_w$ of DPPC in the presence of NaNO$_3$ at concentration 0.05 and 0.1 M.

FIGURE 7 Water thickness of DPPC in a mixture of NaCl and NaI salt solution at concentration 0.1 and 0.5 M. Total $[NaCl] + [NaI] = 0.1$ M ($\bullet$) and $[NaCl] + [NaI] = 0.5$ M ($\circ$).

equilibrium spacing was measured in the presence of mixtures of NaCl and NaI salt solutions, as shown in Fig. 7. Two series of experiments were performed at a constant total $[NaCl] + [NaI]$ concentration, changing the percentage 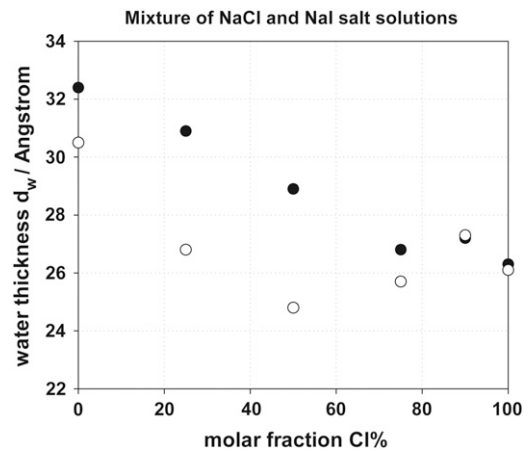of NaI from 0% to 100%. The total salt concentration used in the two cases was 0.1 M and 0.5 M. All spacings were measured at an osmotic pressure of $\log[\Pi / Pa] = 4.6$. Starting at 100% NaCl and adding NaI we observe significant changes in the spacing as soon as NaI becomes >20% of the total salt in the solution. The increase of d_w upon addition of iodide can be explained by the stronger interfacial iodide adsorption, which creates a surface charge on the bilayers; d_w does not change linearly with the percentage of NaI in the mixture, especially at a total salt concentration of 0.5 M, at which it appears to have a minimum value. Nonlinear behavior was also observed by Petrache et al. (74) in their Cl$^-$/ Br$^-$ exchange experiments. In Fig. 7, we see that the effect on d_w strongly depends on the total salt concentration used.

CONCLUSIONS

The experimental results described above show that anions strongly influence the properties of the DPPC bilayers. The swelling effects observed always follow the Hofmeister series and depend on salt concentration. Swelling of the interbilayer distance is linked to an increase of headgroup area (Fig. 4), which can be attributed to lateral electrostatic interactions arising from charging through ionic adsorption. The experimental $\log\Pi - d_w$ curves of DPPC bilayers in the presence of salt solutions show that the water bilayer separation, d_w, (for the same osmotic pressure) increases when salts are present, a fact observed at all salt concentrations. Most of the difference between the force curves ($\log\Pi - d_w$) of DPPC in water and in the presence of NaA solutions is believed to arise from the existence of an electrostatic repulsive force created by the adsorption of the anions to the DPPC headgroups. A lowering of the Hamaker constant between the bilayers is expected in the presence of electrolytes (74,96,99,100) but, as will be discussed in the companion article and in contrast to the conclusion of Petrache et al. (74), it does not suffice to explain the strong effects of

chaotropic anions or the many different structural changes observed in these experiments as functions of salt concentration. The effects of electrolyte concentration on many structural parameters may be explained by two phenomena that determine the electrostatic repulsive force between the lipid bilayers. These are ion binding and ion screening. Interdigitation of the lipids may also occur at low osmotic pressures for the strongly chaotropic SCN⁻ and I⁻ ions.

The maximum swelling experiments in the presence of different NaA electrolytes have been performed for the first time in such a systematic way for DPPC bilayers in the fluid state. The experimental results show that the maximum water uptake by the bilayers is influenced both by the type of the anion and the concentration of the sodium salt solutions with SCN⁻ inducing the greatest effect.

The headgroup area, A, of DPPC molecules at the bilayer surfaces was computed and was found to be affected by the type and concentration of anions used. The DPPC headgroup area in pure water agrees with those reported in the literature determined by the Gravimetric x-ray method with or without a compressibility correction (87,98). The chaotropic anions I⁻ and SCN⁻ have the strongest effect on the DPPC headgroup area, increasing it considerably, especially at small osmotic pressures. This increase of A supports the notion that these chaotropic anions strongly associate with the lipid headgroups.

As stated in the introduction, the goal of this work was to improve understanding of the mechanism of action of Hofmeister anions. The experimental results presented here provide considerable insights, but a more quantitative analysis of the data yielding ion-lipid association parameters is necessary to obtain deeper understanding. The experimental database created in this article allows the application of two completely different theoretical formalisms. One concerns the equation-of-state data and uses a summation of forces between bilayers as is usually done in osmotic stress experiments; it is a ''perpendicular pressure'' model. The second formalism aims to reproduce the lipid headgroup area as a function of salinity, and is a ''lateral pressure'' model. The great challenge is to fit both types of data with the same ionic ''binding constants'' or related association parameters. This extensive theoretical modeling is attempted in the companion article.

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