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# Cationic lipid membranes—specific interactions with counter-ions

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**Abstract**

Lipids bearing net electric charges in their hydrophilic headgroups are ubiquitous in biological membranes. Recently, the interest in cationic lipids has surged because of their potential as non-viral transfection vectors. In order to utilize cationic lipids in transfer of nucleic acids and to elucidate the role of charged lipids in cellular membranes in general, their complex interactions within the membrane and with the molecules in the surrounding media need to be thoroughly characterized. Yet, even interactions between monovalent counter-ions and charged lipids are inadequately understood. We studied the interactions of the cationic gemini surfactant (2R,3R)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane dibromide (RR-1) with chloride, bromide, fluoride, and iodide as counter-ions by differential scanning calorimetry and Langmuir balance. Chloride interacts avidly with RR-1, efficiently condensing the monolayer, decreasing the collapse pressure, and elevating the main transition temperature. With bromide and iodide clearly different behaviour was observed, indicating specific interactions between RR-1 and these counter-ions. Moreover, with fluoride as a counter-ion and in pure water identical results were obtained, demonstrating inefficient electrostatic screening of the headgroups of RR-1 and suggesting fluoride being depleted on the surface of RR-1 membranes.

(Some figures in this article are in colour only in the electronic version)

## 1. Introduction

All polar lipids bear ionic charges in their hydrophilic headgroups. Phospholipids, such as phosphocholines, phosphoethanolamines, and sphingolipids are zwitterionic, bearing both the

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negatively charged phosphate and positively charged quaternary ammonium moieties in their headgroups. In addition to the above lipids bearing net electric charges are also ubiquitously present in cellular membranes. Introducing charged lipids into model membranes has a profound impact on membrane properties including hydration and thermodynamics, as well as distribution of ions in the vicinity of the surface [1]. Accordingly, deprotonated acidic phospholipids that bear negative charge represent means to regulate various biochemical processes, e.g. by direct electrostatic interaction with macromolecules, changing physical state of the membrane accommodating the functional protein, or electrostatically attracting substrates into the vicinity of an enzyme [2, 3]. Furthermore, it was recently described that acidic phospholipids can complex with a range of cationic proteins to form macroscopic fibrous structures with amyloid characteristics [4, 5]. Also two cationic lipids, namely sphingosine and sleep-inducing lipid oleamide [6–8], are found in cells. Sphingosine, a metabolite of sphingolipids abundant in nuclear membranes, has been shown to modulate a range of cellular and physiological functions such as growth and differentiation, initiation and maintenance of immunological responses, as well as oncogenesis [9–11]. It has been suggested that sphingosine exerts its effects by virtue of its net cationic charge [12]. In keeping with the above, sphingosine affects phospholipid thermal phase behaviour [13], avidly interacts with DNA [6, 7], and forms ternary complexes with DNA and histones [14], as well as inducing lateral ordering into time-averaged superlattices in mixed sphingosine/POPC monolayers [15]. Sphingosine may also be utilized as a cationic lipid for gene delivery [16].

Interest in cationic lipids has increased dramatically after the introduction of 'lipofection', i.e. liposomal transfection that utilizes complexes of cationic lipids and DNA ('lipoplexes') to convey foreign genetic material into eukaryotic cells [17]. Lipoplexes are safe, reliable, and easy to make. However, their major drawback is relatively low gene transfer efficiency compared to techniques employing viruses. To overcome this drawback physical, chemical, and biological properties of lipoplexes have been investigated to establish a rational basis for the design of better lipofection complexes [18, 19]. We have demonstrated that surface charge density ($\rho$) of binary membranes of cationic and zwitterionic lipids profoundly affects model membranes inducing at low $\rho$ reorganization at headgroup level [20, 21] and eventually at high $\rho$ causing a transition of the conventional bilayer to the interdigitated phase [22]. Interestingly, also lipofection efficiency of lipoplexes is dependent on $\rho$ [21, 23], suggesting that the observed changes in the physical properties of membranes containing the cationic lipids are relevant to the biological processing of lipoplexes.

Novel cationic amphiphiles have been synthesized in an attempt to find more efficient chemical compounds for lipofection. Gemini surfactants, composed of two conventional surfactants connected by a spacer, are an interesting class of amphiphilic molecules that demonstrates a number of properties uncommon for conventional surfactants, including very low CMCs, high surface activities, and rich pleomorphic phase behaviour [24–26]. The chemical structure of geminis allows for chemical synthesis of a great variety of different surfactants, thus making these amphiphiles particularly interesting for applications requiring precisely controlled self-assembly, such as lipofection [27].

To better understand the functions of charged lipids in biological membranes and for instance to utilize the full potential of cationic lipids in biological applications, the complex electrostatics of lipids in membranes, their phase behaviour, and interactions with surrounding media, including contained macromolecules, have to be thoroughly characterized. Classically, interactions of charged lipid membranes and counter-ions in the surrounding solution have been described by the Gouy–Chapman approximation, that simplifies the system by treating counter-ions as point charges and confining membrane charges into an infinitely narrow plane [1]. Despite its simplicity Gouy–Chapman theory complies surprisingly well with most

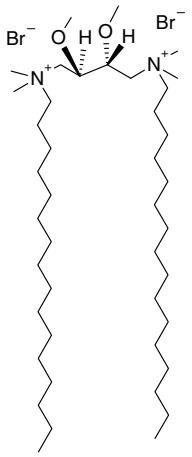


**Figure 1.** Structure of gemini surfactant RR-1.

experimental results. However, it also involves significant oversimplifications. Notably, it neglects the impact of headgroup hydration and structural changes within membrane, e.g. conformational changes of the lipids [1]. Moreover, since the counter-ions are treated as point charges, their geometry is completely omitted, together with their differing tendencies to organize the structure of surrounding water and to form van der Waals interactions with the interface. In brief, Gouy–Chapman theory predicts that ions with equal valences should produce identical effects. Yet, a wealth of experimental evidence, including classical experiments by Hofmeister [28], demonstrates that different equally charged metal counter-ions produce different and specific effects on amphiphile membranes [29].

Interactions of cationic gemini surfactants and their counter-ions are inadequately studied, though these interactions are likely to be important for their self-assembly and phase behaviour. Different salts are ubiquitously present in biological milieu, emphasizing the importance of counter-ion interactions in biological applications of geminis. The cationic gemini surfactant (2R,3R)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane dibromide (RR-1, for structure see figure 1) demonstrates a very complex phase behaviour that is critically dependent on the specific nature and concentration of counter-ions. In this study we present a systematic comparison on the impact of chloride, bromide, fluoride, and iodide on the phase behaviour and self-assembly of RR-1 assessed by differential scanning calorimetry and Langmuir balance.

## 2. Experimental details

### *Materials*

NaCl was from J T Baker, NaBr from Aldrich, and KCl, NaF, and KI from Merck. The gemini surfactant (2R,3R)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane dibromide (RR-1) was synthesized and kindly provided by Professor Giovanna Mancini [32]. Concentrations of RR-1 solutions in chloroform were determined gravimetrically using a high precision electrobalance (Cahn, Cerritos, CA, USA) and confirmed by analysis of compression isotherms for a freshly made RR-1 solution. Freshly deionized filtered water (Milli RO/Milli Q, Millipore Inc., Jaffrey, NH, USA) was used in all experiments.

*Differential scanning calorimetry*

Aqueous suspensions of RR-1 were prepared by mixing appropriate amounts of their stock solutions in dry chloroform to obtain the desired compositions, after which the solvent was removed by evaporation under a stream of nitrogen. For removal of residual amounts of solvent the samples were further maintained under high vacuum for at least 2 h. The resulting dry surfactant films were then hydrated with water with the indicated [salt] and thereafter incubated for 30 min at approximately 60 °C, i.e. above the temperatures of the transition endotherms of the surfactants and their mixtures. Subsequently, the obtained surfactant dispersions were vortexed and immediately loaded into the calorimeter cuvette (final concentration 1 mM). A VP-DSC microcalorimeter (Microcal Inc., Northampton, MA, USA) was operated at a heating rate of 0.5 °C $min^{-1}$. Data were analysed using the routines of the software provided by the instrument manufacturer.

*Monolayer measurements*

A computer controlled Langmuir-type film balance (MicroThrough XS, Kibron Inc., Helsinki, Finland) was used to record compression isotherms ($\pi$–$A$). All glassware was rinsed thoroughly with ethanol and water. The surfactant was dissolved in chloroform and spread in this solvent onto the surface of 14 ml of indicated aqueous subphase at ambient temperature (approximately 21 °C). To ensure complete evaporation of the solvents the films were allowed to settle for 4 min prior to recording the $\pi$–$A$ isotherms. The monolayers were compressed by two symmetrically approaching barriers at a rate of $<4$ $Å^2$/molecule/min, so as to allow for the reorientation and relaxation of the lipids in the course of the compression. Surface pressure was measured by the Wilhelmy technique with a small diameter alloy probe placed in the air/water interface and hanging from a high sensitivity microbalance (KBN 502, Kibron Inc.). Surface pressure $\pi$ is defined as

$$\pi = \gamma_0 - \gamma,$$

where $\gamma_0$ is the surface tension of the air/buffer interface and $\gamma$ is the value for surface tension in the presence of a lipid monolayer compressed at varying packing densities.

## 3. Results and discussion

In the course of our studies on cationic gemini surfactants we noticed that RR-1 and its enantiomer (2S,3S)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane dibromide (SS-1) interact avidly with NaCl in aqueous solutions, producing optically clear dispersions with slowly settling macroscopic aggregates. Investigation under an optical microscope revealed crystalline nature for these aggregates (figure 2(a) [33]). Furthermore, a stereoisomer that differs from these two surfactants only in the conformation of the spacer, and thus with a different distance between the two cationic charges, (2S,3R)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane dibromide (SR-1), spontaneously forms giant vesicles in specific [NaCl] and temperature ranges (figure 2(b), [34]), yet does not induce crystal growth like the above mentioned SS-1. Since no crystal formation was evident for salts other than $Cl^-$ a specific interaction between the cationic gemini headgroup and this anion seems plausible. We explored interactions of RR-1 with various monovalent counter-ions in more detail by means of DSC and Langmuir balance.

DSC traces for 1 mM RR-1 dispersions were measured in water and with varying concentrations of NaCl, KCl, NaBr, and NaF. In pure water and with (from 0.15 up to 1 M) NaF no endotherms were observed within the measured temperature range (10–70 °C, data not shown).

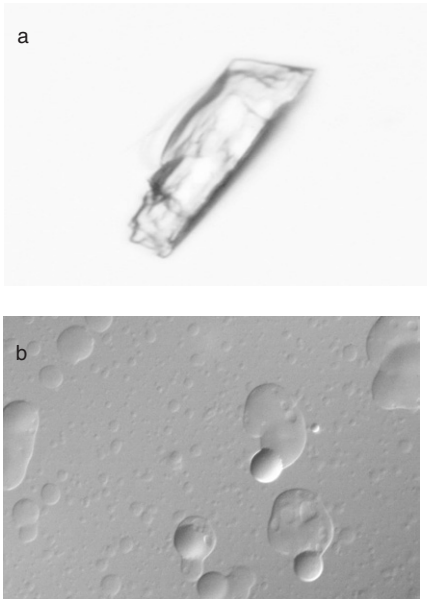


**Figure 2.** (a) A micrograph depicting a typical crystalline aggregate that forms in aqueous solution of 1 mM RR-1 with 2 M NaCl. (b) A micrograph demonstrating population of giant vesicles formed in 1 mM M-1 solution with 2 M NaCl at $T > 31\,^{\circ}\mathrm{C}$. Details of the above experiments will be published elsewhere [33, 34].

This is likely to result from inadequate screening of the highly charged headgroups of RR-1, preventing packing of the surfactant into nanoscale assemblies such as micelles or vesicles producing endothermic transition. Interestingly, a recent study has demonstrated that fluoride ions do not follow the Poisson–Boltzmann distribution in the vicinity of a positively charged lipid interface, but instead the interface is depleted on $F^-$ counter-ions [30], thus suggesting that $F^-$ does not provide efficient screening for RR-1 headgroups and, accordingly, behaviour similar to that seen in pure water can be expected. Alternatively, highly polarizable $I^-$ could intercalate between cationic headgroups of RR-1, thus disrupting its packing into amphiphile aggregates.

In aqueous dispersions of RR-1 with NaCl, KCl, or NaBr, endothermic peaks were evident (figure 3). For NaCl and KCl the shapes of the endotherms (figure 3) as well as transition temperatures ($T_m$) as a function of salt concentration were almost identical (figure 4), thus suggesting that the identity of the cation constituting the secondary screening layer is less important than the anion in the primary layer in determining the thermal phase behaviour of the dicationic RR-1 (figure 5). Accordingly, $T_m$ increased from 38.3 to 51.8 °C when [NaCl] was elevated from approximately 0.15 to 2 M, while for the same [KCl] the value for $T_m$ increased from 38.1 to 52.1 °C. If [NaCl] was further increased, $T_m$ reached 56.2 °C at 3 M. Because of its lower solubility in water, the concentration range studied for KCl was from 0.15 to 2 M. Changing anion to $Br^-$ resulted in significantly higher $T_m$s than observed for chloride salts (figure 4). At [NaBr] = 0.15 M a relatively broad endotherm with $T_m$ at 54.2 °C was observed, and with elevating [NaBr] $T_m$ steadily increased to 64.6 °C at [NaBr] = 2 M (figure 4). The ability of $Br^-$ to elevate $T_m$ compared to $Cl^-$ seems controversial as $Br^-$ does not induce formation of crystalline aggregates and based on our data an adequate explanation for the observed behaviour cannot be suggested. However, the complex interplay of the headgroup geometry and changes in the hydration of the ions as well as headgroup could be involved.

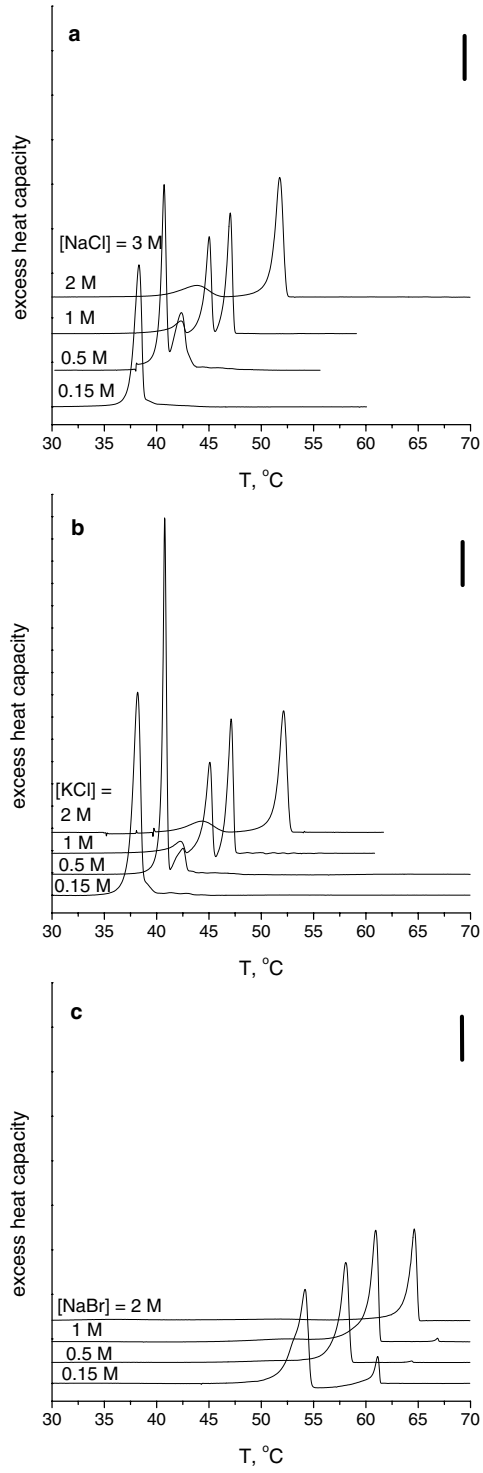


**Figure 3.** DSC traces of 1 mM RR-1 in aqueous solution with indicated concentrations of (a) NaCl, (b) KCl, and (c) NaBr. The calibration bars represent 20 kJ $°C^{-1}$ $mol^{-1}$.

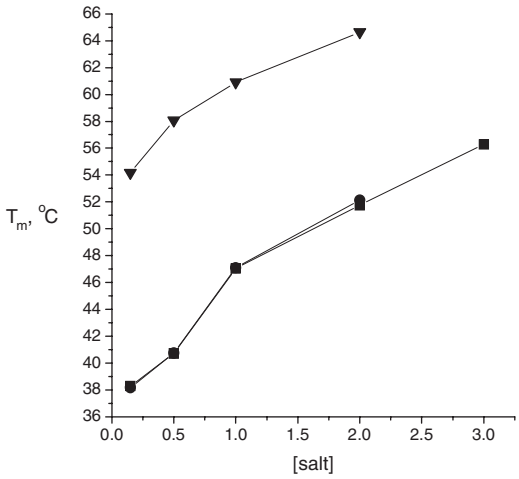


**Figure 4.** Main transition temperatures ($T_m$) of RR-1 dispersions as function of [salt] derived from DSC data recorded with NaCl (■), KCl (●), and NaBr (▲).

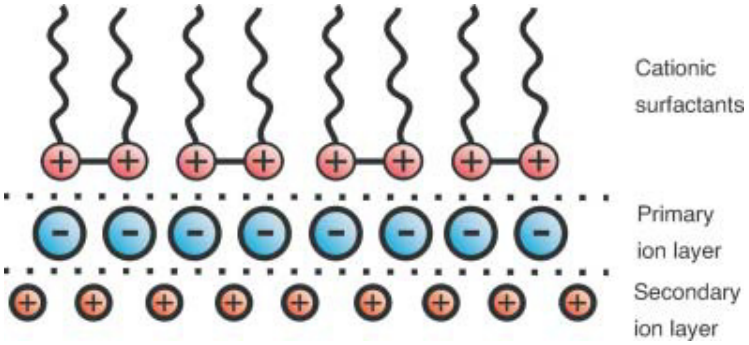


**Figure 5.** Schematic illustration of cationic gemini surfactant membrane and primary and secondary screening ion layers.

Varying anion and its concentration resulted in changes also in the shapes of the endotherms as well as in the enthalpy contained in endotherms ($\Delta H$). While a single and rather co-operative endotherm was observed at $[Cl^-] = 0.15$ M a marked phase separation with two and three peaks was evident at $[Cl^-] = 0.5$ and 1 M, respectively (figures 3(a) and (b)). Further increment in $[Cl^-]$ resulted in a broad endotherm at lower temperatures and a sharp endotherm at relatively high $T_m$. A plausible explanation for the phase separation is given by the formation of two laterally segregated phases in the RR-1 membrane, one with an associated pseudocrystalline counter-ion lattice and the second constituted by 'free' RR-1 molecules. In keeping with the above, phase separation is evident only at $[Cl^-] \geqslant 0.5$, and the enthalpy of the endotherm with higher $T_m$, presumably corresponding to domains with attached crystals, increases as a function of $[Cl^-]$ (figures 3(a) and (b)). For $Br^-$ a broad endotherm was observed accompanied by a small peak at higher temperatures for $0.15 \leqslant [NaBr] \leqslant 1$ M while at [NaBr] = 2 M only a single endotherm was evident. $\Delta H$ as a function of counter-ion concentration revealed somewhat counterintuitive behaviour when considering changes observed in $T_m$. Again for both chloride salts very similar data were obtained. Accordingly, first an increase in $\Delta H$ until a maximum of approximately 104 kJ mol$^{-1}$ at $[Cl^-] = 0.5$ M was

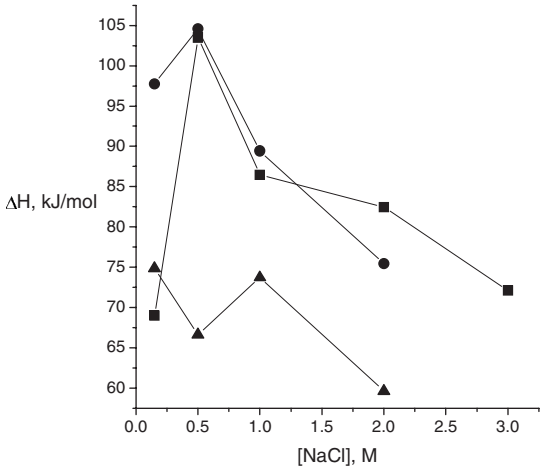


**Figure 6.** Enthalpies contained in transition endotherms ($\Delta H$) of RR-1 dispersions as function of [salt] derived from DSC data recorded with NaCl (■), KCl (●), and NaBr (▲).

evident, after which $\Delta H$ diminished with elevating [$Cl^-$] (figure 6), with $T_m$ increasing. For $Br^-$, enthalpies contained in endotherms were lower, and similarly to chlorides a decreasing trend as a function of [$Br^-$] was evident (figure 6).

Analogously to DSC results Langmuir isotherms recorded for RR-1 with pure water or aqueous NaF solution as a subphase were poorly reproducible (data not shown). This finding is in keeping with the suggested depletion of $F^-$ ions on the surface of RR-1 membrane and, accordingly, poor reproducibility is likely to be due to inadequate screening of the cationic charges of the RR-1, resulting in an unstable monolayer. However, the monolayer became stable in the presence of $Cl^-$, $Br^-$, or $I^-$ as counter-ions. For instance, addition of NaCl into the subphase resulted in reproducible compression isotherms shown in figure 7(a). Elevating [NaCl] from 0.15 to 2 M significantly condensed the monolayer, evident as reduced mean molecular areas at which the isotherms deviate from the baseline ($A_{\text{lift-off}}$) (figure 8). Accordingly, whereas $A_{\text{lift-off}}$ was approximately 128 $Å^2$/acyl chain at [NaCl] $=$ 0.15 M, increasing [NaCl] induced a rapid decrement in $A_{\text{lift-off}}$ with a minimum of approximately 78 $Å^2$/acyl chain at [NaCl] $=$ 2 M. DSC data demonstrating increment of $T_m$ as a function of [$Cl^-$] are compatible with these results, suggesting tighter packing of the acyl chains due to addition of [NaCl]. Interestingly, increasing [NaCl] also resulted in markedly altered behaviour in very densely packed monolayers. More specifically, surface pressures at which the collapse of the monolayer was observed ($\pi_{\text{collapse}}$) diminished abruptly from $>$54 mN $m^{-1}$ at [NaCl] $<$ 0.5 M to approximately 40 mN $m^{-1}$ at [NaCl] $\geqslant$ 0.5 M (figure 8). Accordingly, the RR-1 monolayer is both condensed and relatively 'fragile' (i.e. not withstanding high surface pressures without collapsing) at [NaCl] $\geqslant$ 0.5 M. These findings are particularly interesting when considering the ability of RR-1 to induce formation of macroscopic crystalline aggregates at [NaCl] well below the saturation point in aqueous solution [33]. If we assume that the dicationic headgroup of RR-1 with fixed distance between charges acts as a nucleation centre for crystallization of NaCl onto the surface of the RR-1 membrane, we would expect a NaCl crystal also to organize the RR-1 molecules into a regular lattice (figure 5). In this kind of a lattice the cationic charge of RR-1 headgroups would be efficiently screened by $Cl^-$ counter-ions and RR-1 molecules packed into a tight and regular lattice. This is compatible with our data demonstrating reduced $A_{\text{lift-off}}$ and increased $T_m$ at [NaCl] $\geqslant$ 0.5 M.

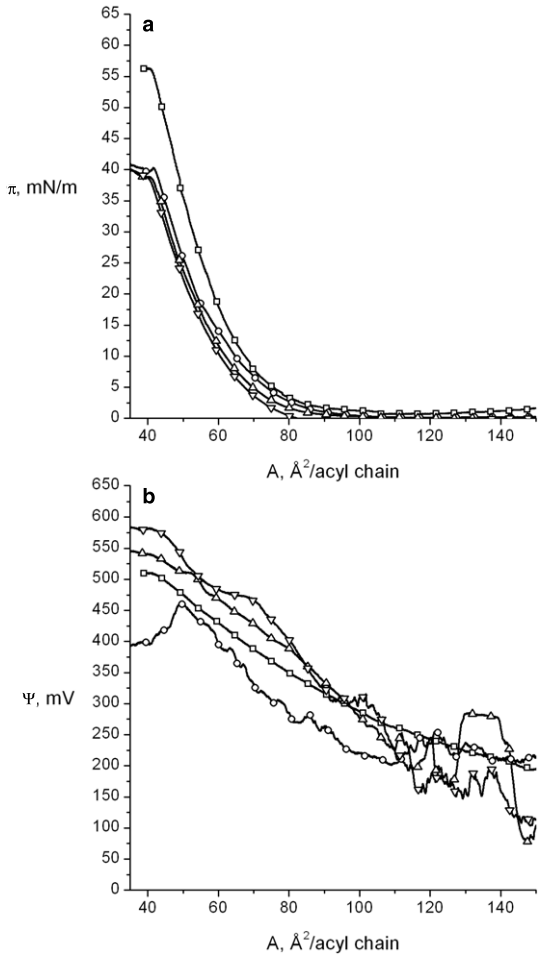


**Figure 7.** (a) Representative $\pi/A$ isotherms and (b) dipole potentials ($\Psi$) as a function of area/molecule recorded for RR-1 monolayers. [NaCl] in the subphase was varied as 0.15 (□), 0.5 (○), 1.0 (△), and 2.0 M (▽).

Yet, an attached pseudocrystalline arrangement on the monolayer interface cannot withstand compression but collapses more easily than a monolayer consisting of RR-1 molecules screened by counter-ions not organized into a crystalline assembly, thus giving a plausible explanation for diminished $\pi_{\text{collapse}}$ at [NaCl] $\geqslant$ 0.5 M. Moreover, formation of a crystalline lattice at the membrane interface markedly decreases the entropy of the system. Accordingly, the observed decrement in enthalpy of transition as function of [$Cl^-$], that seems at first glance to be in contrast with simultaneously elevating $T_m$, becomes (figures 4 and 6) reasonable. More specifically, while higher temperature is required for the disruption of the pseudocrystalline ion lattice because of its augmented coherence and structure extending further from the solution–surfactant interface at higher [NaCl], the entropic gain of the transition also simultaneously increases, thus diminishing the measured enthalpy of the transition.

Dipole potentials ($\Psi$) recorded for RR-1 monolayers with NaCl in the subphase revealed unusual behaviour. At [NaCl] = 0.15 M the dipole potential increased gradually upon the compression of the monolayer, reflecting increasing surface density of the charged RR-1

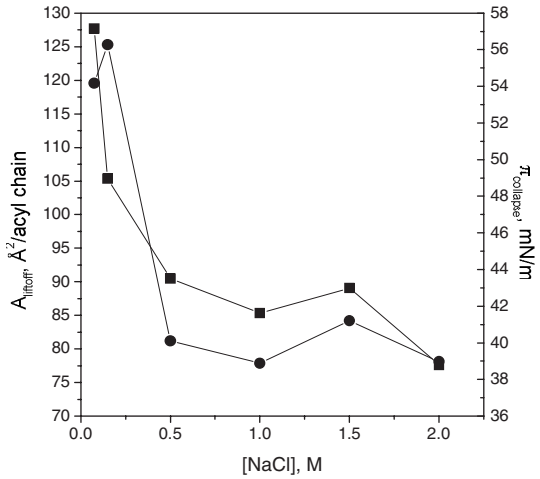


**Figure 8.** Lift-off areas ($A_{\text{lift-off}}$) (■) and surface pressures at the collapse of the monolayer ($\pi_{\text{collapse}}$) (●) as a function of [NaCl] determined from the compression isotherms shown in figure 5(a).

(figure 7(b)). However, at [NaCl] $\geqslant$ 0.5 M different behaviour became evident, with $\Psi$ fluctuating randomly at mean molecular areas ($A$) corresponding to the liquid expanded state (figure 7). Fluctuations ceased upon compression of the films to the liquid condensed state. While the origin of these fluctuations remains uncertain at present, they could be due to formation of clusters of RR-1 with adhering NaCl lattice on the surface of the monolayer, in keeping with phase separation seen upon DSC (figures 3(a) and (b)). The presence of crystalline inhomogeneities in very dilute, uncompressed phospholipid monolayers have been suggested, based on grazing incidence x-ray experiments [31].

Compression isotherms for RR-1 monolayers were also measured with KCl, NaBr, and KI in the subphase. Representative isotherms and the respective $A_{\text{lift-off}}$ as well as $\pi_{\text{collapse}}$ data are compiled in figure 9 and table 1. Replacing molar NaCl by a molar KCl condensed monolayer and decreased $\pi_{\text{collapse}}$ (table 1), suggesting that KCl also forms a crystalline lattice, similarly to NaCl, yet the lattice formed in the presence of KCl organizes the RR-1 monolayer into a more condensed state. Changing the anion in the salt to $Br^-$ expanded the monolayer significantly (figure 9), while $\pi_{\text{collapse}}$ remained essentially at the level observed for the chloride salts (table 1). When KI was added to the subphase the RR-1 monolayers became even more expanded and $\pi_{\text{collapse}}$ decreased to 33 mN m$^{-1}$. These observations are likely to reflect the inability of $Br^-$ and $I^-$ to form commensurate lattices with RR-1. In keeping with the above, for neither NaBr nor KI was the formation of crystalline assemblies observed by optical microscopy [33].

## 4. Conclusions

The cationic gemini surfactant RR-1 and $Cl^-$ interact avidly in aqueous solution, producing crystalline assemblies visible under the microscope [33]. Notably, these aggregates form with both $Na^+$ and $K^+$ as a cation, suggesting that it is the anion identity which is crucial for the process. In keeping with the above, $Cl^-$ demonstrated a clearly distinct behaviour from the other monovalent cations studied (i.e. $Br^-$, $F^-$, and $I^-$) upon interaction with RR-1, as observed by DSC and Langmuir balance. Elevating [$Cl^-$] condensed the RR-1 monolayers and

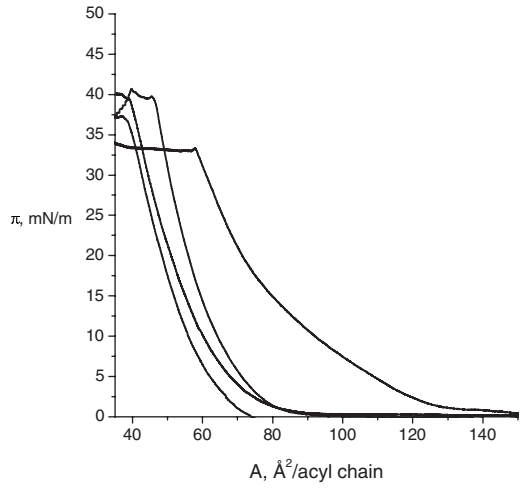


**Figure 9.** Representative $\pi/A$ isotherms for RR-1 monolayers on [salt] = 1 M. Salt in the subphase was (from left to right) KCl, NaCl, NaBr, and KI.

**Table 1.** Lift-off areas ($A_{\text{lift-off}}$), surface pressures at collapse ($\pi_{\text{collapse}}$), and area at collapse determined from compression isotherms shown in figure 8.

| Salt | $A_{\text{lift-off}}$ (Å$^2$/molecule) | $\pi_{\text{collapse}}$ (mN m$^{-1}$) | Area at collapse (Å$^2$/molecule) |
|---|---|---|---|
| NaCl | 81.9 | 39.2 | 39.5 |
| KCl | 70.3 | 37.0 | 38.5 |
| NaBr | 81.9 | 39.5 | 46.0 |
| KI | 140.0 | 33.0 | 58.5 |

simultaneously $T_{\text{m}}$ determined by DSC increased, indicating tighter packing in the hydrocarbon phase of the membrane. Moreover, $\pi_{\text{collapse}}$ for the RR-1 films diminished significantly at $[Cl^-] > 0.15$ M, thus suggesting diminished compressibilities at higher surface pressures. These findings are compatible with the fixed distance between the cationic charges in the RR-1 headgroup constituting a nucleation centre for the formation of a pseudocrystalline salt lattice on the surface of the surfactant membrane. Specificity of the interaction between RR-1 and $Cl^-$ was further emphasized as $Br^-$ and $I^-$ did not condense the RR-1 monolayer as efficiently as chloride salts at equivalent concentrations. Finally, experiments with $F^-$ as a counter-ion for cationic charges of RR-1 resulted in identical results as observed for pure water by both DSC and Langmuir balance. This is in accord with recently published data [30] suggesting depletion of $F^-$ counter-ions on the surface of the positively charged lipid interface.

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