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The relaxation effect as observed on lipid suspensions of low polydispersity

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Lipid suspensions with a low polydispersity ($\Delta = 0.15 \pm 0.05$, as given by photon correlation spectroscopy (PCS)) were used to elucidate the origin of the disagreement between the experimental zeta potential values (ζ_{sm}), obtained from the electrophoretic mobilities through the Smoluchowski equation, and double-layer theory prediction (ζ potential) at low salt concentrations. The values of ζ_{sm}, measured for cardiolipin and phosphatidylserine suspensions in monovalent electrolytes, were compared with the correspondent theoretical values of the ζ potentials corrected for the relaxation effect; the correction was made according to the S.S. Dukhin theory of electrophoresis. It was found, that this correction eliminates the disagreement for cardiolipin in NaCl entirely; it partly solves the problem for cardiolipin in KCl but fails to improve the situation for phosphatidylserine in NaCl.

Introduction

In our previous paper [1] we have discussed some methodological problems arising in the electrophoretic studies of anionic lipid membranes in 1 : 1 electrolyte solution. In particular, we have made an attempt to find out, whether or not the relaxation effect is responsible for the disagreement between the experimental zeta potentials as obtained from the electrophoretic mobility data in combination with the Smoluchowski equation (ζ_{sm}) (see Ref. 2), and those predicted by the Gouy-Chapman-Stern theory (ζ) (see Ref. 3) at low salt concentrations ($C = 10^{-3}$–$5 \cdot 10^{-2}$ M). Using the S.S. Dukhin theory of electrophoresis [4,5] we have shown, that the relaxation effect * may significantly decrease the theoretical analog of ζ_{sm} (ζ_{sm}^t) in the concentration range studied.

We could suppose therefore, that the relaxation effect provides one possible explanation for the observed discrepancy. However, a final conclusion could not be achieved at that stage, because of the wide particle size distribution in the liposome suspensions investigated: quantitative comparison of experimental ζ_{sm} with the appropriate values calculated from the Dukhin theory was justified only for a given paticle size [1,4].

In the present work we make the next step by studying the liposome suspensions of low polydispersity. These suspensions have been obtained by the specially developed two-step centrifugation procedure. Average particle sizes and polydispersities were determined by photon correlation spectroscopy (PCS) technique [6–8] and verified also by electron microscopy; both methods showed, that the lipid suspensions obtained, although not strictly monodisperse, have a narrow enough size distribution to allow the correct comparison of experimental and theoretical values of ζ_{sm}.

Cardiolipin (CL) and phosphatidylserine (PS) suspensions of low polydispersity in either potassium chloride or sodium chloride solution were used for electrophoretic mobility measurements. The measured ζ_{sm} vs. log C curves were compared with the corresponding ζ_{sm}^t vs. log C curves, obtained from the Dukhin theory for a known range of particle sizes found in a given lipid suspension. The theoretical procedure underlying the calculation of ζ_{sm}^t is described in details in Ref. 1.

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* The term 'relaxation effect' commonly used in literature (see Ref. 14) implies the influence of the 'normal' surface conductivity and double-layer polarization on the electrophoretic mobility (see Ref. 2). In this paper we use this term in a more broad sense; apart from the two phenomena mentioned, it comprises also the 'abnormal' surface conductivity, i.e., the whole complex of processes considered in the Dukhin theory (see also Refs. 1, 4 and 15).

Materials and Methods

Cardiolipin from bovine heart was purchased from factory of bacterial preparations (Charkov, Ukraine); phosphatidylserine from bovine brain was obtained from the Laboratory of Bioorganic Chemistry of the Novosibirsk State University (Novosibirsk, Russia). Both lipids gave one spot when checked for purity by TLC on silica gel; additional control was provided by the electrophoretic mobility measurements: samples were used, that gave not more than one peak in the mobility spectrum (see also Ref. 1). Potassium chloride and sodium chloride (both for spectral analysis) were used without further purification. Water was purified by the Millipore-Q System ($\Omega = 2.5$ Mohm).

Multilamellar liposomes were prepared according to Bangham [9] by the evaporation of organic solvent from the initial lipid solution and subsequent addition of aqueous salt solution to the dried lipid film. Lipid suspension in a given salt solution (1 mg lipid per ml) was shaken vigorously for 5–10 min; then pH was adjusted to 7.00 by either potassium or sodium hydroxide (analytical grade purity) and used for the subsequent preparation of low-disperse suspensions. pH measurements were carried out by OP-208 (Radelkis, Hungary) with an accuracy ± 0.03 pH units.

Suspensions of low polydispersity were obtained as follows. 6 ml of the polydisperse suspension of multilamellar liposomes were placed into each of the three teflon test-tubes and centrifuged at $8000 \times$ g for 1 h. Afterwards, the upper fractions (2 ml $\times$ 3) were removed carefully by the sampler and the middle fractions (2 ml $\times$ 3) were placed into a new test-tube that underwent similar centrifugation. After that, the upper fraction (2 ml) was removed again and the middle fraction (2 ml), after a pH control, was used for the experimental purposes. While dividing the lipid suspension into fractions, one had to keep the tip of the sampler all the time just beneath the solution/air interface; the whole operation was performed very slowly, so as to avoid the intermixing of different fractions in the test-tube.

Size control and the electrophoretic mobility measurements were carried out on Zetasizer-2 (Malvern, UK). Average particle size (diameter, 2α) and polydispersity (Δ) of the lipid suspension were determined by means of PCS technique, described in details elsewhere [6,7]. For each sample, the result was obtained as an average value from at least ten measurements; for a given lipid, as much as 5–7 samples were usually studied. For lipid suspensions in 10^{-3} M salt solutions, the vesicle diameters were found to be 0.7 ± 0.1 μm (CL) and 0.5 ± 0.1 μm (PS); for both lipids, $\Delta = 0.15 \pm 0.05$.

The electron microscopy control was fulfilled by the method described in details in Ref. 10; this method had been used successfully for studies of the structure of liquid disperse systems (e.g. Refs. 11 and 12). Aqueous suspension of a given lipid was quickly (within 0.1–0.5 s) frozen by liquid nitrogen in the specially constructed device. The frozen sample was fractured under vacuum. Then V_2O_3 (at 45°) and carbon (at 90°) were deposited successively on the fracture faces. V_2O_3/carbon replica was mounted, carried over to the copper grid and examined in the transmission electron microscope (EMV-100-AK). The micrographs produced from the replica show the arrangement of the particles in the fracture plane [10].

The electrophoretic mobilities were measured in parallel with size determination at 22°C; the standard procedure of the mobility measurements is described in Refs. 8 and 13. Zeta potentials for the peak mobility values were calculated according to the Smoluchowski equation and are denoted here as ζ_{sm}. For each sample, measurements were started in 10^{-3} M salt solution adjusted to pH 7.00 by the corresponding base; the chelating agents were not used, for they had no effect on zeta potentials (see also Ref. 1). Salt concentration was increased stepwisely by the addition of the appropriate amounts of 1 M and 3 M of sodium or potassium chloride solution. To eliminate minor pH changes in the course of experiment [1], the pH was adjusted to 7.00 at each experimental point, that is, at each value of salt concentration. Also the size control by PCS was repeated at several salt concentrations in the range 10^{-3}–10^{-1} M; within the limits of experimental error, average particle sizes were found to be unchanged for the two lipids studied.

Calculation of the theoretical curves

To find out whether the relaxation effect is responsible for the observed behaviour of ζ_{sm} at low salt concentrations we calculated its theoretical analog (ζ'_{sm}) from the S.S. Dukhin theory of electrophoresis. As was shown recently in Ref. 16, the Dukhin approach proves to be more successful in describing the experimental data for polystyrene latexes at low ionic strengths then that suggested by Wiersema [17] and O'Brien and White [18]. Following the way chosen in our previous work [1], we repeated here the calculations based on the Dukhin approach. The simplified form of the Dukhin-Deryaguin equation [5] gives the relationship between the electrophoretic mobility, Stern potential (ψ_d), zeta potential (ζ) and particle radius (α); as was explained in Ref. 1, this equation may be easily presented in the form convenient for calculation of ζ'_{sm}:

$$\zeta'_{sm} = \zeta \cdot \left[\frac{(1 + \mathrm{Rel}) \cdot \frac{z|\tilde{\zeta}|}{4} - \mathrm{Rel}\,\mathrm{lnch}\frac{z\tilde{\zeta}}{4}}{\frac{z|\tilde{\zeta}|}{4}(1 + 2\,\mathrm{Rel})} \right] \qquad (1)$$

where $\tilde{\zeta} = e\zeta/kT$, z is ion valency. The relaxation effect is reflected by the criterion Rel [4]; it was calculated here from the expression:

$$\mathrm{Rel} = \frac{e^{|\psi_d|e/2kT}}{\varkappa a} \qquad (2)$$

where æ is the reciprocal Debye length. Stern potential was identified with surface potential ψ_s; ζ and ψ_s were calculated from the Gouy-Chapman-Stern theory * (see, for details, Refs. 1 and 3); the parameters used for calculation are given in the following section.

As was pointed out in Refs. 1, 4 and 5, the Dukhin theory is valid for the thin double layer ($\varkappa a \gg 1$). In our study, $\varkappa a > 30$ (except for one case indicated below); hence, Eqn. 1 gives an error not exceeding 5% [19].

The calculated curves were found to be practically identical to those obtained according to the original Dukhin-Deryaguin equation [2,4]. For comparison, we calculated also several points using the computations of O'Brien and White [18] available from Hunter [2] (for potassium chloride solutions) and the computations of Wiersema et al. [17] tabulated by Ottewill and Shaw [20] (for sodium chloride solutions). The results are shown, respectively in Figs. 3 and 4.

Results and Discussion

The electron micrograph of CL vesicles in 10^{-3} M NaCl solution is shown in Fig. 1A. Lipid vesicles appear as black spheres (produced by carbon) supplemented with white triangular 'shadows' (produced by V_2O_3). The first indicate the particles' diameters; the second allow to determine the vesicles' heights with respect to the fracture plane. The vesicles look approximately spherical, nonaggregated, and are rather homogeneous in size. Size distribution was determined for the series of micrographs covering the 'vesicular' part of a given replica, with an accuracy $\pm$ 0.05 μm. The resulting histogram (Fig. 1B) reveals that the greater part of the vesicle population (about 60%) has the diameter between 0.5 and 0.7 μm. The good agreement of this result with that obtained by PCS (see Materials and Methods) shows, that the number of large particles is quite small; otherwise, the PCS technique would have given the exaggerated values of the average diameters [6,8].

Figs. 2–4 show the results obtained for CL vesicles in NaCl and KCl solutions and also for PS vesicles in NaCl solution. The experimental points (filled circles) demonstrate the characteristic 'two-slope' behaviour,

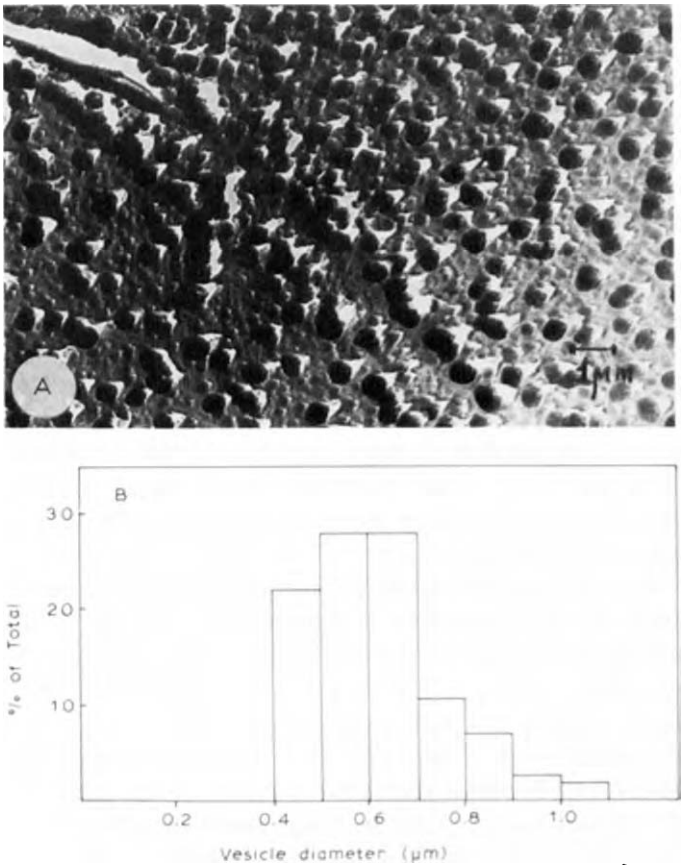

Fig. 1. Cardiolipin suspension of low polydispersity in 10^{-3} M sodium chloride. (A) Electron mircrograph of quickly freezed sample. (B) Size distribution obtained from six electron micrographs of a given replica for the total number of 1160 liposomes.

that has been observed earlier for polydisperse suspensions [1]. Following the way approbated in this previous work, we have divided our fitting procedure into two stages: (1) selection of the theoretical ζ (log C) curve, satisfying the experimental data in the region of positive slope to the concentration axis (higher salt concentrations) and (2) calculation of the correspondent ζ^t_{sm} (log C) curve; the latter allowed us to anticipate the changes of ζ_{sm} in the region of very small, if any, slope of the experimental points to the concentration axis (lower salt concentrations).

At the first stage, ζ potentials were calculated for different sets of three parameters required: maximal membrane charge density (σ^{max}), cation binding constant (K) and shift layer position (L). For the three cases studied, we used $\sigma^{max} = 1e/60$ Å^2; K and L were varied in accordance with the fitting requirements.

At the second stage, the set of three parameters mentioned was used in calculation of ζ^t_{sm} for the particle size determined in the corresponding liposome suspension.

With CL vesicles in NaCl solution (Fig. 2), in the region of positive slope ($C > 5 \cdot 10^{-2}$ M) fitting of

* We used the Gouy-Chapman equation, the Langmuir adsorption isotherm and the $\phi(x)$ dependence for the high potentials [3].

ζ(log C) curve gives either $L = 2$ Å, $K = 0.8$ M^{-1}, or $L = 1.5$ Å, $K = 1.12$ M^{-1} (broken line). For each of these sets of parameters, taking $2\alpha = 0.7 \pm 0.1$ μm found for CL suspensions, we obtained ζ^{t}_{sm} (log C) curves for $\alpha = 0.35 \pm 0.05$ μm (solid lines). Fig. 2 shows the result for $L = 1.5$ Å and $K = 1.12$ M^{-1}; ζ^{t}_{sm} was calculated for $\alpha = 0.3$ μm (upper curve) and $\alpha = 0.4$ μm (lower curve). Hence the shaded region marks the set of experimental values, that may be expected for the sizes considered. As seen from the figure, there is rather good agreement with experimental values of ζ_{sm}; only one point (10^{-3} M) does not follow the theoretical prediction. We may infer that, almost in the whole concentration range investigated, the observed deviation of ζ_{sm} from the double layer theory prediction for ζ potential is likely to be the result of the relaxation effect.

For CL vesicles in KCl solution (Fig. 3), fitting of ζ(log C) at higher salt concentrations ($C > 10^{-1}$ M) results in $K = 0$, $L = 1.5$ Å (broken line). The corresponding ζ^{t}_{sm}(log C) curves for $\alpha = 0.35 \pm 0.05$ μm agree satisfactory with experimental values of ζ_{sm} in the range $6 \cdot 10^{-3}$ M $< C < 10^{-1}$ M. For $C < 6 \cdot 10^{-3}$ M the experimental points lie obviously lower than the theoretical values. Similar observations may be made from the comparison of ζ_{sm} with the corresponding values obtained according to O'Brien and White [18] for $\alpha = 0.35$ μm (triangular symbols). We may conclude therefore, that the relaxation effect really dominates between $6 \cdot 10^{-3}$ M and 10^{-1} M. At lower salt

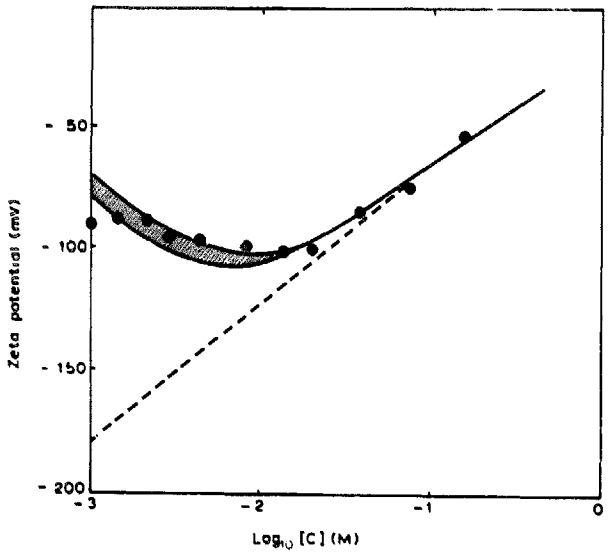

Fig. 2. Zeta potential changes with salt concentration for CL vesicles in sodium chloride solution. Filled circles, experimental values (ζ_{sm}). Here and below experimental error lies within the circle diameter. Broken line, zeta potential (ζ), predicted by the Gouy-Chapman-Stern theory for $\sigma^{max} = 1e/60$ Å^2, $K = 1.12$ M^{-1}, $L = 1.5$ Å. Solid curves, theoretical analog of ζ_{sm}(ζ^{t}_{sm}), calculated from Eqn. 1 for the same σ^{max}, K and L, with $\alpha = 0.3$ μm (upper curve) and $\alpha = 0.4$ μm (lower curve).

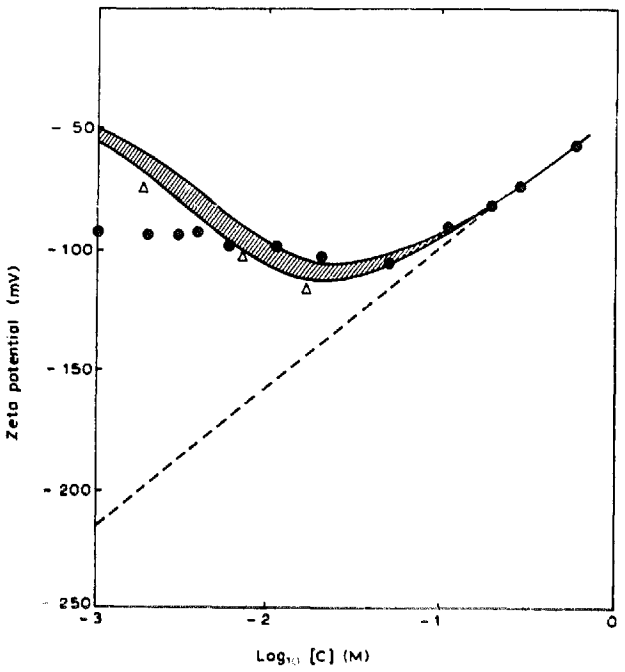

Fig. 3. Zeta potential changes with salt concentration for CL vesicles in potassium chloride solution. Filled circles, ζ_{sm}. Broken line, ζ potential calculated for $\sigma^{max} = 1e/60$ Å, $K = 0$ and $L = 1.5$ Å. Solid curves, ζ^{t}_{sm} obtained for the same σ^{max}, K and L, with $\alpha = 0.3$ μm (upper curve) and $\alpha = 0.4$ μm (lower curve). Triangular symbols, ζ_{sm}, calculated as $E = 2kT/3e$ according to O'Brien and White [2,18] for $\varkappa \cdot \alpha = 150$, 100 and 50.

concentrations, however, some other phenomena are likely to prevail over the double layer relaxation; as a consequence, the observed decrease of the electrophoretic mobility (and hence, of ζ_{sm}) is much less than predicted by the Dukhin theory.

PS vesicles in NaCl solution (Fig. 4) show a positive slope for $C > 4 \cdot 10^{-2}$ M; the ζ(log C) curve is best fitted to the experimental data with $K = 1.25$ M^{-1}, $L = 2$ Å. This binding constant appears to be somewhat higher than that used in [14,21,22] for the same lipid-cation combination; however, this difference may be almost fully eliminated by the change of the maximal charge density: with $\sigma^{max} = 1e/70$ Å^2 (and $L = 2$ Å) we obtain K = 0.85 M^{-1}, quite close to $K = 0.6$ M^{-1} found in [14] for the same σ^{max} and L. ζ^{t}_{sm}(log C) curves were calculated for $\alpha = 0.25 \pm 0.05$ μm. In the range $(1–2) \cdot 10^{-3}$ M, where $\varkappa\alpha < 30$ for $\alpha = 0.2$ μm, the calculation error may exceed 5%; this uncertainty is indicated as the 'pointed' part of the upper curve. Comparison with experimental data reveals the disagreement most obvious in the range $5 \cdot 10^{-3}$–$2 \cdot 10^{-2}$ M. Moreover, the theoretical curves show the minimum, while the experimental curve goes practically in parallel with the concentration axis. Similar discrepancy is observed with the results of Wiersema's [17,20] computations for $\alpha = 0.25$ μm. Thus, in this case the relaxation effect, though undoubtedly present at low

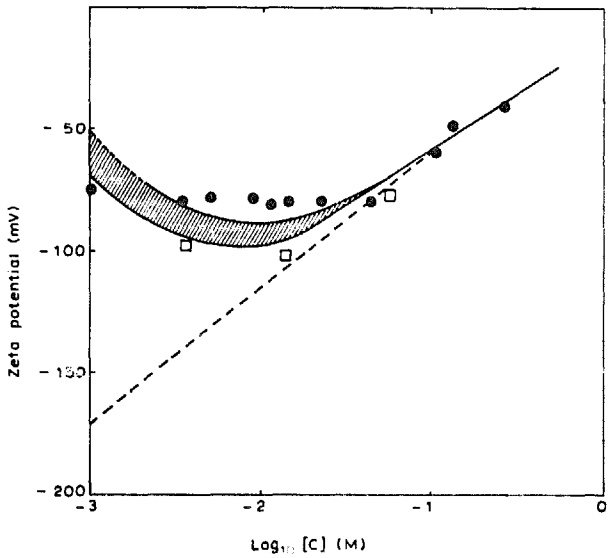

Fig. 4. Zeta potential changes with salt concentration for PS vesicles in sodium chloride solution. Filled circles, ζ_{sm}. Broken line, ζ potential calculated for $\sigma^{max} = 1e/60$ Å^2, $K = 1.25$ M^{-1} and $L = 2$ Å. Solid curves, ζ_{sm}^1 obtained for the same σ^{max}, K and L, with $\alpha = 0.2$ μm (upper curve) and $\alpha = 0.3$ μm (lower curve). Square symbols, ζ_{sm}, calculated according to Wiersema et al. [17,20] for $ae \cdot \alpha = 200$, 100 and 50.

salt concentrations, can hardly be regarded as the only reason of ζ_{sm} deviation from the ζ potential values, predicted by the double layer theory. It may be seen also from Figs. 3 and 4, that the Dukhin theory is unlikely to have the principal advantages in its ability to describe the deviation mentioned, in comparison with the approaches of O'Brien and White and Wiersema et al.

The results presented above allow some conclusions concerning the applicability of the Gouy-Chapman-Stern double layer model to the description of ζ vs. log C curves, obtained for acidic lipid dispersions in 10^{-3} to 1 M solutions of monovalent electrolytes. Firstly, it seems clear, that this classical model is quite successful at relatively high salt concentrations, namely, for (2–5) 10^{-2} M $< C <$ 1 M, the lower limit being dependent on the lipid species. This is in agreement with previous findings of McLaughlin and his colleagues for various lipids in the same concentration range (see, for example, Refs. 21 and 22). Secondly, at lower salt concentrations (10^{-3} M $< C <$ (2–5) $\cdot$ 10^{-2} M) the ability of double-layer model to describe real processes going on at the membrane surface depends both on the type of electrolyte and on the nature of the lipid studied. For a given lipid (CL), the model works relatively well for the sodium chloride solution (Fig. 2) but it is only partly suitable for the potassium chloride solution (Fig. 3). For a given salt solution (sodium chloride), this model is quite good for CL (Fig. 2) and not satisfactory for PS (Fig. 4). This evidence implies the inadequacy of the classical model at low ionic strengths; similar conclusion has been made earlier in the surface potential measurements on planar lipid membranes [23].

A few words should be added about the possible reasons of this discrepancy. Apart from the invalidity of the known assumptions lying at the ground of double layer theory equations (see, for example, Ref. 3), one may suppose here the existence of some 'non-classical' phenomena that may contribute to the measured potential changes. The latter idea originates from the well documented peculiarities of lipid membranes (e.g., formation–destruction of the hydrogen bonding between the lipid polar headgroups [24]) and model lipid systems (e.g., the molecular exchange between the water solution and outer liposome monolayer [25–27]. When the salt concentration is changed in the diluted solutions, these processes may easily produce quite appreciable changes of the membrane charge density (see, for example, Ref. 24) and hence, of the membrane surface potential. Some experimental evidence in favour of this explanation will be presented in our forthcoming paper.

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