



Influence of temperature, anions and size distribution on the zeta potential of DMPC, DPPC and DMPE lipid vesicles



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ABSTRACT

The purpose of the work is to compare the influence of the multilamellarity, phase state, lipid head groups and ionic media on the origin of the surface potential of lipid membranes.

With this aim, we present a new analysis of the zeta potential of multilamellar and unilamellar vesicles composed by phosphatidylcholines (PC) and phosphatidylethanolamines (PE) dispersed in water and ionic solutions of polarizable anions, at temperatures below and above the phase transition.

In general, the adsorption of anions seems to explain the origin of the zeta potential in vesicles only above the transition temperature (T_c). In this case, the sign of the surface potential is ascribed to a partial orientation of head group moiety toward the aqueous phase. This is noticeable in PC head groups but not in PEs, due to the strong lateral interaction between PO and NH group in PE.

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1. Introduction

Electrostatic forces are one of the main interactions taking place in the insertion of peptides and proteins with biological membranes. The fixation and conformational stabilization of proteins and peptides in the membrane has been ascribed to coulombic interactions via charged phospholipids in the membrane and charged amino acids in the protein surface [1–4].

However, most natural membranes are mainly composed of neutral lipids, such as phosphatidylcholines and phosphatidylethanolamines. At the critical lipid concentration, the zwitterionic lipids arrange in a bilayer structure giving a head group lattice on the surface resulting in a surface charge distribution.

Several studies have reported that multilamellar liposomes of these lipids are negatively charged when the electrophoretic mobility of the particles is measured in an ionic media [5–12].

This result has been explained by means of two models. In one of them, it has been argued that the resulting negative charge is due to an exposure of the phosphate groups lying in an outer plane with respect to the choline groups [8,9,13,14]. This proposal is based on the assumption that choline, being partially hydrophobic due

to the methyl groups at the nitrogen end, is buried in the membrane interphase, trying to avoid water. Several experiments with ^2H NMR by Seelig [15] and Seelig et al. [16] support this interpretation.

The problem with this hypothesis is that, as the experimental determinations of zeta potential are done with lipid particles moving in an electric field, the plane of shear is far enough in comparison to the distance of separation of phosphate and choline so that they can be considered as overlapped. Then, surface potential should be near zero. For this reason, the negative surface charge was ascribed to the specific adsorption of counter anions on the surface. In this context, several works showed that negative ions adsorb according to its volume i.e. the degree of polarization of their electron clouds [17,18].

The analysis of the validity of these hypotheses has been limited to phosphatidylcholines in the liquid crystalline state.

Although specific ionic effects have become ubiquitous in both chemical and biological literature, a comprehensive theoretical description of the physical interactions responsible for these effects is still elusive [11].

In this work, we present a new analysis of the origin of the charge on the lipid membranes by comparing multilamellar and unilamellar vesicles composed by phosphatidylcholines and phosphatidylethanolamines dispersed in water and in ionic solutions of polarizable anions, at different temperatures.

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2. Materials and methods

2.1. Materials

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and used as received. Chloroform, KCl and KClO_4 were of analytical grade.

Solutions were prepared by weight in distilled and deionized water (Super Q Millipore system, conductivity lower than $18 \mu\text{S cm}^{-1}$). The pHs of the solutions used for zeta potential measurements were pH 7.4.

2.2. Liposome preparation

Lipids were dissolved in chloroform that was removed by evaporation with N_2 stream to obtain a dry lipid film. The residual amounts of the organic solvent were removed maintaining the films under high vacuum for additional 2 h in Thermo Scientific Speed Vac SPD11V. The resulting dry lipid films were then hydrated with 5 mL of three different media: 1 mM KCl, 1 mM KClO_4 and water, pH 7.4, and homogenized with cycles of vigorous vortexing at around 10°C above the transition temperature of the lipids. This combination of vortexing and heat yields a polydispersed population of MLVs. The final conductivity and pH of the resulting suspension were the same as that found for pure water. These values were not altered during the determination of zeta potential. Then, samples were equilibrated at the working temperatures [19–22] inside the Zetasizer equipment.

Unilamellar vesicles (LUVs) were obtained by a sequential extrusion of the MLVs dispersions in an Avanti Mini-Extruder, through a polycarbonate membrane of 100 nm pore size at around ten degrees centigrades above the transition temperature of each lipid. Then, samples were equilibrated at the working temperatures [19–22] inside the Zetasizer equipment. Sizes were measured before and after temperature stabilization with good reproducibility.

2.3. Methodologies

The size distribution of liposomes before and after extrusion and the zeta potentials (ζ) of DMPC, DPPC and DMPE liposomes were determined in Zetasizer Nano ZS90 equipment (Malvern Instruments Ltd., UK). Measurements with DMPC vesicles were done at 35°C , 18°C and 8°C . In all cases, samples were incubated at each temperature during 1 h before measurement. In the same way,

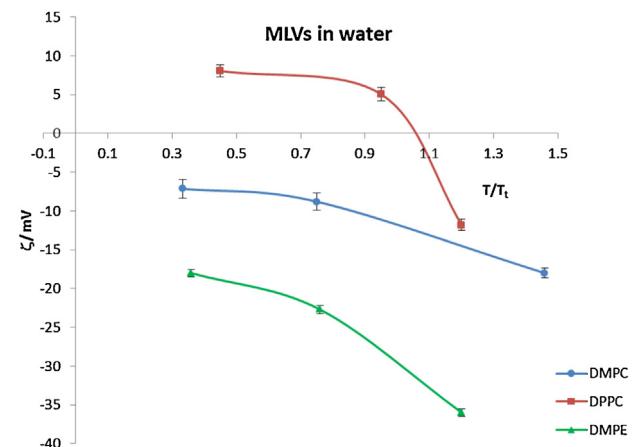


Fig. 1. Zeta potential at different reduced temperatures of MLVs composed by DMPC, DPPC and DMPE prepared and dispersed in water. In all cases, the standard deviation was lower than 10%. Reported data are averaged over three different batches of liposomes.

measurements were made for DPPC at 48 , 38 and 18°C and for DMPE at 18 , 38 and 60°C . Reported data are averaged over three different batches of liposomes.

3. Results

The zeta potential at different reduced temperatures of MLVs of lipids dispersed in pure water is shown in Fig. 1. As an additional data, the size distribution of these preparations is shown in Table 1.

In this condition, it is observed that all lipids show a negative zeta potential at temperatures above the phase transition. This behavior is also found with DMPC and DMPE below the phase transition, while DPPC represents an exception since it yields a positive value.

Similar results were obtained for liposomes prepared with the same lipids dispersed in 1 mM KCl or KClO_4 (Fig. 2). It is immediately evident that zeta potential of DPPC MLVs above the transition temperature is negative in the presence of KCl and becomes more negative in KClO_4 . This is congruent with the proposal that negative charges are due to the adsorption of polarizable anions.

This behavior is also observed in DMPC but the differences between the anionic species are within the experimental error. Also, the size distribution of the lipid dispersions is shown in Table 1.

At temperatures below the phase transition, all dispersions become less negative and in the case of DMPC, the zeta potential is

Table 1
Zeta potential and size particle of MLV and LUVs Of DMPC, DPPC and DMPE dispersed in different anionic media and temperatures.

$T/\text{ }^\circ\text{C}$	H ₂ O			KCl 1 mM			KClO ₄ 1 mM		
	MLVs size \pm SD ^a (μm)	MLVs $\zeta \pm$ SD ^a (mV)	LUVs $\zeta \pm$ SD ^a (mV)	MLVs size \pm SD ^a (μm)	MLVs $\zeta \pm$ SD ^a (mV)	LUVs $\zeta \pm$ SD ^a (mV)	MLVs size \pm SD ^a (μm)	MLVs $\zeta \pm$ SD ^a (mV)	LUVs $\zeta \pm$ SD ^a (mV)
DMPC	8	2.5 ± 0.12	-7.1 ± 0.70	-7.6 ± 0.80	4.3 ± 0.22	7.9 ± 0.70	3.1 ± 0.30	6.2 ± 0.31	15.0 ± 0.90
	18	2.4 ± 0.12	-8.8 ± 0.80	-9.4 ± 0.90	5.1 ± 0.25	6.1 ± 0.50	0.50 ± 0.10	3.1 ± 0.15	11.0 ± 0.80
	35	1.6 ± 0.80	-18.0 ± 1.1	-20.0 ± 1.4	2.5 ± 0.13	-7.5 ± 0.60	-10.0 ± 0.70	2.5 ± 0.13	-10.0 ± 0.70
DPPC	18	9.3 ± 0.50	8.1 ± 0.60	2.7 ± 0.30	7.4 ± 0.37	-3.5 ± 0.30	0.41 ± 0.10	9.3 ± 0.46	0.91 ± 0.10
	38	3.2 ± 0.16	5.1 ± 0.50	2.3 ± 0.20	4.3 ± 0.22	-4.8 ± 0.40	-2.6 ± 0.20	3.3 ± 0.17	-4.1 ± 0.30
	48	0.85 ± 0.04	-11.0 ± 0.80	-10.0 ± 0.70	$1.3 \pm$	-16.0 ± 0.80	-16.0 ± 1.1	0.91 ± 0.05	-23.0 ± 1.1
DMPE	18	4.6 ± 0.23	-18.0 ± 0.90	-29.0 ± 1.7	2.7 ± 0.14	-25.0 ± 1.3	-30.0 ± 1.8	2.5 ± 0.12	-25.0 ± 1.3
	38	2.6 ± 0.13	-22.0 ± 1.1	-33.0 ± 1.6	2.4 ± 0.12	-28.0 ± 1.4	-34.0 ± 1.9	2.1 ± 0.11	-28.0 ± 1.4
	60	0.55 ± 0.03	-36.0 ± 1.8	-39.0 ± 1.8	0.75 ± 0.04	-34.0 ± 1.5	-42.0 ± 2.1	0.86 ± 0.04	-35 ± 1.4

^a SD: standard deviation. $n=3$.

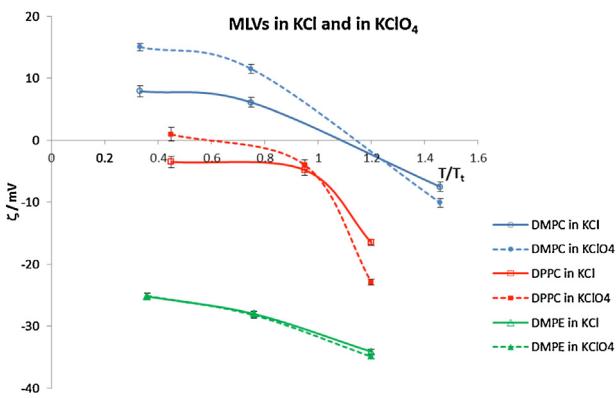


Fig. 2. Zeta potential at different reduced temperatures of MLVs composed by DMPC, DPPC and DMPE prepared and dispersed in 1 mM KCl (—) and KClO_4 (----). In all cases, the standard deviation was lower than 10%. Reported data are averaged over three different batches of liposomes.

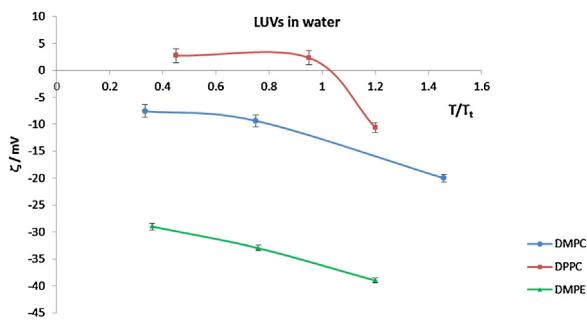


Fig. 3. Zeta potential at different reduced temperatures of LUVs composed by DMPC, DPPC and DMPE prepared and dispersed in water. In all cases, the standard deviation was lower than 10%. Reported data are averaged over three different batches of liposomes.

positive even in the presence of ClO_4^- . Finally, the effect of anions is absent on DMPE preparations within this range of temperatures.

In the next figures, the differences between MLVs and LUVs in water and anionic solutions are shown.

In Fig. 3, it is observed that extrusion does not change the trend observed in MLVs except for DMPE for which LUVs of 100 nm show a more negative potential than MLVs (see Table 1).

The more significant effects of extrusion are observed for DMPC, that shows a displacement to negative values in the sequence, $\text{Cl}^- < \text{ClO}_4^-$ (Fig. 4).

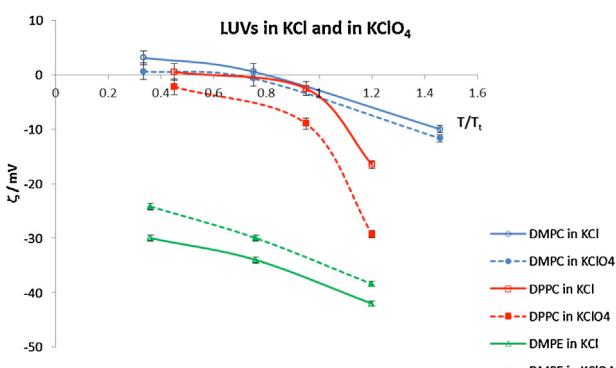


Fig. 4. Zeta potential at different reduced temperatures of LUVs composed by DMPC, DPPC and DMPE prepared and dispersed in 1 mM KCl (—) and KClO_4 (----). In all cases, the standard deviation was lower than 10%. Reported data are averaged over three different batches of liposomes.

4. Discussion

The present results show that the zeta potential strongly depends on the phase state of the lipids and on the lipid species, both in MLVs and LUVs. The zeta potential measurements carried out in water demonstrate the presence of a surface charge, which could be explained by the reorientation of the lipid head groups. A negative value of surface potential would indicate a preferred exposure of the PO_4 group in relation to the choline group, while the opposite would happen when the potential is positive.

The comparison of the changes in size and in zeta potential with temperature in Table 1, indicates that in all cases, the greater the diameter of the particles the more positive the zeta potential. This may be explained at least by two ways considering that below the phase transition the lipids are less hydrated than in the fluid phase [23,24]. One possibility is that when liposomes pass from the liquid crystalline to gel state they aggregate and as a consequence, the measurements correspond to the zeta potential of the aggregates. The other possibility is that with the decrease of temperature, chains condense to form a gel state and this modifies the zeta potential. As a consequence this triggers aggregation of the MLVs. This proposal can be corroborated with the results obtained in LUVs. In this case, the size remains unchanged, within the experimental error, with the shift from gel to liquid crystalline and after the passage of electrical current. In this condition, the zeta potential changes to less negative values from gel to fluid. In conclusion, the size increase in MLVs can be better explained by the second proposal.

In conclusion, changes in the phase state modify the surface properties and this, for reasons that are beyond the purpose of this paper, triggers the aggregation of MLVs, but not of LUVs.

The lipid that appears to be less sensitive to the ionic environment is DMPE, which may be ascribed to its high transition temperature due to the strong phosphate amino interaction at the head groups. In addition, this lipid has a lower propensity to hydrate [5,25], maintaining the ionic interactions found in the solid when dispersed in water. The only change observed in zeta potential with DMPE was when MLVs were extruded to form 100 nm LUVs. In this case, zeta potential of lipid particles prepared in water changes to negative values, being the effect of anions not too pronounced.

The PE groups form a very compact lattice that is stabilized by strong electrostatic interactions and hydrogen bonds between the ammonium nitrogen and the phosphate oxygens. This head-group lattice is largely unaffected by hydration and temperature. It may be possible that extrusion may distort the P–N interaction in PE vesicles affecting the exposure of phosphates groups to the aqueous phase [6].

In contrast, the fully methylated ammonium groups in phosphatidylcholine do not allow such close $\text{PO} \dots \text{N}$ contacts. In consequence, water molecules are incorporated into the head-group lattice linking phosphate groups into ribbons and shielding groups of equal charge. Consequently the number of hydration of PC is much higher than that for PE, resulting in a lower shielding of the negative charge therefore yielding more negative zeta potential values.

For PC vesicles, the hypothesis of anionic adsorption and head group conformational change seems to apply depending on the ions present in the media and the phase state. In general, the adsorption hypothesis seems to apply in vesicles above the transition temperature. In contrast, the adsorption theory does not explain the behavior observed below T_c . The reorientation of the head group directors in PC by phase transitions is already known from the literature, especially in the field of ^{31}P – NMR and X-ray diffraction. [14,26,27] The positive value obtained for DPPC in water can be explained by a protrusion of the choline group out of the membrane plane toward the water phase (Fig. 5A). The addition of Cl^-

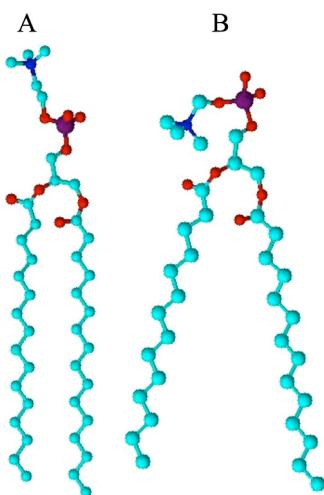


Fig. 5. Diagram representation of the conformational model of lipid in liposome at solid crystalline state in water. Conformational analysis and geometry optimization of phospholipids was performed using a ACD/Labs ChemSketch 12.0 software. (A) DPPC; (B) DMPC.

or ClO_4^- changes the results toward negative values as expected although the sequence does not follow the polarization degree of the anions. In LUVs the behavior is qualitatively comparable being all values displaced to less positive zeta potentials.

The case of DMPC looks more complicated. There is probably a protrusion of the phosphate groups out of the membrane plane toward the water phase (Fig. 5B). It seems that the presence of anions modifies the zeta potential to more positive values in the sequence Cl^- to ClO_4^- . The significant change observed with ClO_4^- , indicates that choline groups are exposed to water in the presence of this anion. The same occurs with Cl^- , but at a lesser extent. This suggests that anions are disfavoring the interaction of the choline group with the membrane phase. This interaction could be considered as hydrophobic, due to the methyl groups. In such case, the chaotropic effect of large anions would disrupt the water structure disfavoring the hydrophobic interaction of cholines.

The possibility that choline groups could emerge from the membrane plane to water is enhanced due to the presence of defects at the gel state. These defects are still present in LUVs since the potential is still positive.

The question is why DPPC liposomes are more positive than DMPC. Other authors also found differences in PCs in other properties. Phospholipid bilayer membranes are well known to change their own structure according to environmental conditions (e.g., temperature). For example, dipalmitoylphosphatidylcholine (DPPC) bilayer membrane undergoes the pretransition from the lamellar gel phase to the ripple gel phase at ca. 34°C and subsequently the main transition from the ripple gel phase to the liquid crystalline phase at ca. 42°C [28]. These kinds of bilayer transitions are generally regarded as thermodynamically first-order transitions, and thus are accompanied by a discontinuous change in apparent molar volume of the phospholipid. According to Tamai [29], the apparent specific volume of DPPC in water is higher than that corresponding to DMPC at all temperatures. For DPPC and DMPC, the temperature dependence curves are very similar to the results reported in the earlier densitometric and dilatometric study by Nagle and Wilkinson [30]. This would mean that DPPC is less packed in comparison to DMPC in spite of the increase in chain length. However, this is obviously inconsistent with the established knowledge on the phospholipid bilayer structure that the increase of the hydrocarbon chain length tends to enhance the

lateral packing of lipid molecules within the membrane due to the stronger van der Waals interaction between the hydrocarbon chains. Furthermore, the larger value of the total specific volume for DPPC in the gel state than for DMPC in the same state is interpreted as due to the fact that the fraction of the headgroup region, corresponding to a higher density region, decreases with an increase in hydrocarbon-chain length. This may be explained as a consequence of the orientation of the polar head group to compensate the high packing imposed by the lateral interactions of the acyl chains. The higher density of DMPC found by Tamai can be due to the location of the hydrophobic moiety of cholines into the lipid interphase thus enhancing the lateral interactions. In order to decrease the density in DPPC, the choline group should be exposed above the plane diminishing the hydrophobic interaction, which is congruent with the positive value observed in Fig. 3.

In conclusion, the present results show that phase changes affect the surface properties of MLVs and LUVs in the same direction, although it triggers aggregation only for MLVs in the gel phase.

The adsorption of anions can explain the negative sign of the surface charge only for membranes in the liquid crystalline state.

Below T_c , head groups may reorient to expose the choline group to water, thus explaining the positive sign of the zeta potential. This is noticeable in PC head groups but not in PEs, due to the strong lateral interaction between PO and NH group in PE.

As a general take home lesson, these results and analysis suggest that sign in the surface lipid membranes cannot be directly predicted from the head group composition since it varies with lamellarity, curvature, phase state and the ionic media.

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References

- [1] S. McLaughlin, Annu. Rev. Biophys. Biophys. Chem. 18 (1989) 113.
- [2] S. McLaughlin, Curr. Top. Membr. Trans. 9 (1977) 71.
- [3] D. Murray, A. Arbuszova, G. Hangyás-Mihályné, A. Gambhir, N. Ben-Tal, B. Honig, S. McLaughlin, Biophys. J. 77 (6) (1999) 3176.
- [4] L. Rusu, A. Gambhir, S. McLaughlin, J. Rädler, Biophys. J. 87 (2) (2004) 1044.
- [5] F. Lairion, A. Disalvo, J. Phys. Chem. B 113 (2009) 1607.
- [6] E.A. Disalvo, A.M. Bouchet, Colloids Surf. A: Physicochem. Eng. Asp. 440 (2014) 170.
- [7] J. Sýkora, P. Jurkiewicz, R.M. Epand, R. Kraayenhof, M. Langner, M. Hof, Chem. Phys. Lipids 135 (2) (2005) 213.
- [8] K. Makino, T. Yamada, M. Kimura, T. Oka, H. Ohshima, T. Kondo, Biophys. Chem. 41 (1991) 175.
- [9] K. Makino, A. Shibata, Advances in Planar Lipid Bilayers and Liposomes, vol. 4, Elsevier Inc., 2006, pp. 1554–4516, Chapter 2.
- [10] H.J. Petrache, I. Kimchi, D. Harries, V.A. Parsegian, J. Am. Chem. Soc. 127 (33) (2005) 11546.
- [11] H.J. Petrache, T. Zemb, L. Belloni, V.A. Parsegian, PNAS 103 (2006) 7982.
- [12] R.J. Clarke, C. Lüpertz, Biophys. J. 76 (1999) 2614.
- [13] R.H. Pearson, I. Pascher, Nature 281 (1979) 499.
- [14] H. Hauser, I. Pascher, R.H. Pearson, S. Sundell, Biochimica et Biophysica Acta (BBA) – Rev. Biomembr. 650 (1981) 21.
- [15] J. Seelig, Q. Rev. Biophys. 10 (3) (1977) 353.
- [16] J. Seelig, P.M. MacDonald, P.G. Scherer, Biochemistry 26 (24) (1987) 7535.
- [17] S.A. Tatulian, Biochim. Biophys. Acta 736 (1983) 189.
- [18] S.A. Tatulian, Eur. J. Biochem. 170 (1987) 413.
- [19] D.D. Lasic, Liposomes in Gene Delivery, CRC Press, Boca Raton, FL, 1997.
- [20] A. Rodriguez-Pulido, F. Ortega, O. Llorca, E. Aicart, E. Junquera, J. Phys. Chem. B 112 (2008) 12555.
- [21] A.E. Rodriguez-Pulido, F. Aicart, O. Ortega, E. Llorca, E. Junquera, J. Phys. Chem. B 112 (2008) 2187.
- [22] A. Rodriguez-Pulido, A. Martin-Molina, C. Rodriguez-Beas, O. Llorca, E. Aicart, E. Junquera, J. Phys. Chem. B 113 (2009) 15648.

- [23] E. Sparr, H. Wennestrom, *Biophys. J.* 81 (2001) 1014.
- [24] H. Binder, *Eur. Biophys. J.* 36 (4–5) (2007) 265.
- [25] J.T.S. Coimbraa, S.F. Sousaa, P.A. Fernandesa, M.M. Rangelb, J. Ramosa, J. Biomol. Struct. Dyn. 32 (2014) 103.
- [26] W.W. Sulkowski, D. Pentak, K. Nowak, A. Sulkowska, *J. Mol. Struct.* 744–747 (2005) 737.
- [27] D.P. Tielemans, S.J. Marrink, H.J.C. Berendsen, *Biochim. Biophys. Acta* 1331 (1997) 235.
- [28] R.N.A.H. Lewis, N. Mak, R.N. McElhaney, *Biochemistry* 26 (1987) 6118.
- [29] N. Tamai, Y. Nambu, S. Tanaka, M. Goto, H. Matsuki, S. Kaneshina, *Colloids Surf. B: Biointerfaces* 92 (2012) 232.
- [30] J.F. Nagle, D.A. Wilkinson, *Biophys. J.* 23 (1978) 159.