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The use of zeta potential as a tool to study phase transitions in binary phosphatidylcholines mixtures

M.B. Sierra[a], V.I. Pedroni[a], F.E. Buffo[b], E.A. Disalvo[c], M.A. Morini[a,*]

[a] Laboratorio de Fisicoquímica, Dpto. de Química, INQUISUR, Universidad Nacional del Sur, Argentina
[b] Dpto. de Matemática, Universidad Nacional del Sur, Argentina
[c] Laboratorio de Biointerfases, CITSE, Universidad Nacional de Santiago del Estero, Argentina

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ABSTRACT

Temperature dependence of the zeta potential (ZP) is proposed as a tool to analyze the thermotropic behavior of unilamellar liposomes prepared from binary mixtures of phosphatidylcholines in the absence or presence of ions in aqueous suspensions. Since the lipid phase transition influences the surface potential of the liposome reflecting a sharp change in the ZP during the transition, it is proposed as a screening method for transition temperatures in complex systems, given its high sensitivity and small amount of sample required, that is, 70% less than that required in the use of conventional calorimeters. The sensitivity is also reflected in the pre-transition detection in the presence of ions. Plots of phase boundaries for these mixed-lipid vesicles were constructed by plotting the delimiting temperatures of both main phase transition and pre-transition vs. the lipid composition of the vesicle. Differential scanning calorimetry (DSC) studies, although subject to uncertainties in interpretation due to broad bands in lipid mixtures, allowed the validation of the temperature dependence of the ZP method for determining the phase transition and pre-transition temperatures. The system chosen was dipalmitoylphosphatidylcholine/dimyristoyl phosphatidylcholine (DMPC/DPPC), the most common combination in biological membranes. This work may be considered as a starting point for further research into more complex lipid mixtures with functional biological importance.

1. Introduction

Liposomes are bilayer microstructures composed of natural or synthetic lipids [1] whose polar head group and long hydrophobic tail form an amphipathic environment. Lipid membranes, which are the structural basis of biological membranes, can serve as an appropriate model for many biophysical studies [2]. During the last decades, the approach to overcome the complexity of biological membranes relies in the use simplified biomimetic models consisting primarily binary lipid mixtures or ternary [3,4].

It is still disputed that the partially exotic phases occurring in synthetic lipid mixtures, such as tilted gel phases have any relevance for native membranes consisting of tenth, hundreds components. About the interest in lipid phase studies, lateral phase separation in biological membranes could be functionally important [5]. Lipid rafts or ordered lipid domains may play a role in the localization, transport, and function of different proteins. In spite of the relevance of lipid-protein interactions, the phase behavior of the lipid components of the cell membrane is believed to be of great importance in the efforts to find out some the principles that underlie the membrane function [6]. The characterization of phase boundaries has been conducted for mixed lipid dispersions using various techniques such as differential scanning calorimetry (DSC) [7], nuclear magnetic resonance (NMR), [8] X-ray scattering [9] Fluorescence Microscopy [10] Laser Ultrasound [11] and ultrasonic velocimetry [12]. Pre-transition temperatures of lipid mixtures are usually determined with relatively large uncertainty especially due to the typical broad peaks, for instance, in DSC. Aqueous dispersions of polar lipids are known to form a large variety of phases depending on the chemical structure, temperature, and dispersing media. Their phase behavior is dominated by the main (order-disorder) phase transition associated with the melting of the lipid hydrocarbon chains. At temperatures above the main transition, lipids arrange in liquid crystalline structures. Below the main transition there is a multitude of different possible phases, a basic equilibrium structure is the subgel (crystalline) Lc phase. In addition, a large

* Corresponding author.
E-mail address: mamorini@criba.edu.ar (M.A. Morini).

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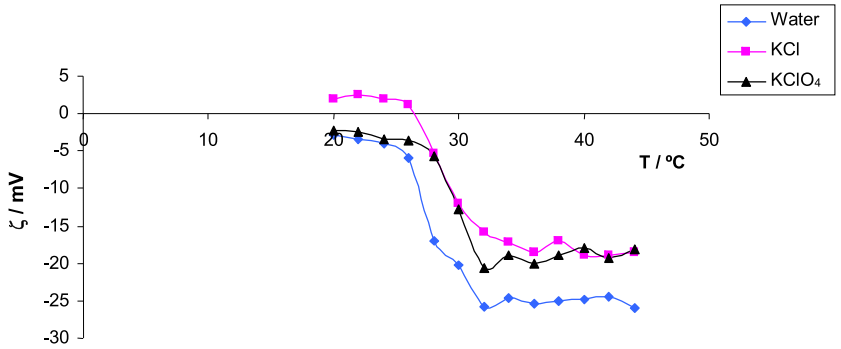

Fig. 1. Zeta potential as a function of T for $X_{DPPC} = 0.2$ mixture prepared and dispersed in water, KCl and $KClO_4$. In all cases, the standard deviation was lower than 10%, not shown for better viewing. Reported data were averaged over four different batches of liposomes.

number of intermediate stable, metastable, and transient lamellar gel structures are adopted by different lipids—with perpendicular or tilted chains with respect to the bilayer plane, with interdigitated, partially interdigitated, or non-interdigitated chains, rippled bilayers with various ripple periods, etc. Even so, the number of reported phases continues to grow. The formation of a subgel phase usually requires a prolonged low-temperature equilibration. The lipid polymorphism at low temperatures still appears to be far from clear [13].

Furthermore, liposomes pack concentrically hydrophilic and lipophilic portions thus rendering an internal vacuole which can serve as a storage compartment for an active agent. The rate limiting step in the use of this versatile system, for example for drug delivery, is the physical and chemical stability of liposomes [14]. Regarding stability, the surface charge of the aggregate is very important. This is also a factor to consider that influences the interaction of the liposome with a substrate.

In a previous work from our research group [15] it was shown that the surface charge of the liposome mainly depends on the kind of lipid and conditions such as temperature, phase state of the liposome and the presence or absence of ions in the medium.

Membrane properties such as surface potential, the dipole potential [16], structure and mechanical strength [17–19] are closely associated with ions present within the cell and its environment. Therefore, the study of the interactions of ions with the lipid bilayer is of considerable interest [20].

Temperature dependent Zeta Potential Studies are proposed in this work to analyze the thermotropic behavior of mixtures of synthetic phospholipids in unilamellar aqueous suspensions in the presence or absence of ions. Since the lipid phase transition influences the surface potential of the liposome reflecting a sharp ZP change during the phase transition [21], we propose this technique as a screening method for transition temperatures in complex systems, given its high sensitivity and small amount of sample required, that is, 70% less than that required in the use of conventional calorimeters. With data intervals transition temperatures obtained from zeta potential it was possible to construct plots of phase boundaries of the DPPC-DMPC mixture, similar to that obtained by calorimetric studies in literature [22–24]. The proposed use of ZP studies relies on the importance of continuous and gradual stabilization of the sample temperature, which ensures thermal equilibrium and thermodynamic study lipid mixture. The immediate consequence is to allow the system its conformational organization at a given temperature. This is particularly relevant in the transition zone where the system experiences continuous and large organizational changes in small temperature ranges. Based on the above mentioned, it is affirmed that the proposed technique allows to obtain accurate information about the phase transition of the system.

Despite the vast variety of lipid mixtures [25], this paper focuses on phospholipids, the most abundant structural elements present within cell membranes. In order to validate this approach, a kind of mixture of phospholipids was chosen, for which the phase behavior has already been studied experimentally [22,23,26–29] and computer simulations have been performed [23,27–29]. The chosen system is a binary mixture of saturated phospholipids with the same polar head and different length of the alkyl chain, such as 1,2-dimyristoyl-sn-glycero- 3-phosphocholine (DMPC, chain length n = 14, temperature Tm melting: 24° C) mixed with 1,2-dipalmitoyl-snglycero-3-phosphocholine (DPPC, n = 16, Tm: 41 °C). The mixture is an isomorphic system, both of its lipids components are miscible in both crystalline and liquid crystalline solid [23,30].

This work maybe considered as a starting point for further research into more complex lipid mixtures with functional biological importance.

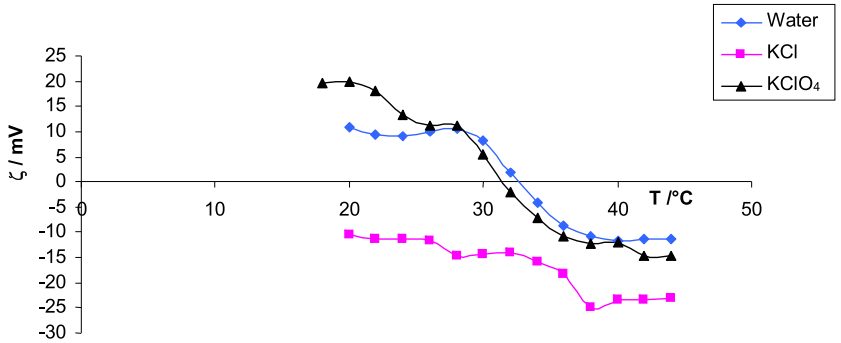

Fig. 2. Zeta potential as a function of T for $X_{DPPC} = 0.5$ mixture prepared and dispersed in water, KCl and $KClO_4$. In all cases, the standard deviation was lower than 10%, not shown for better viewing. Reported data were averaged over four different batches of liposomes.

2. Materials and methods

2.1. Materials

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and used as received. Chloroform, KCl and $KClO_4$ were of analytical grade. Water used for preparing solutions is ultrapure water (Super Q Millipore system, conductivity lower than 18 $\mu S\,cm^{-1}$).

2.2. Liposome preparation

Liposomes were prepared from binary mixtures of DMPC and DPPC lipids, weighing appropriate amounts of each one in order to obtain proportions X_{DPPC} of 0.2, 0.5, 0.6 and 0.8, where X_{DPPC} is the mole fraction of DPPC in the mixture of liposomes without considering the solvent.

Mixtures of lipids were dissolved in chloroform that was removed by evaporation with N_2 stream to obtain a dry lipid film. Remaining solvent was removed keeping 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 medium: 1 mM KCl, 1 mM $KClO_4$ and water, in all cases, pH 6.5, and homogenized with cycles of vigorous vortexing at around 10 °C above the transition temperature of the lipids. This heating-vortexing combination yields a polydispersed population of MLVs.

Unilamellar vesicles (LUVs) were obtained by sequential extrusion of the MLVs dispersions in an Avanti Mini-Extruder, through a polycarbonate membrane of 100 nm pore size at around ten degrees centigrade above the transition temperature of the lipid mixture in the appropriate proportion taking into account the literature transition temperatures [23,31–34].

The final conductivity and pH of the resulting suspension were the same as that found for pure water.

2.3. Methods

2.3.1. Zeta potential

The size distribution of liposomes before and after extrusion and the zeta potentials (ζ) of DMPC-DPPC liposomes were determined in a Zetasizer Nano ZS90 equipment (Malvern Instruments Ltd., UK). Measurements with DMPC-DPPC vesicles were performed in the range between 45 °C and 20 °C with successively lowered temperatures, allowing the sample to reach equilibrium between measurements, recording a point every 2 °C with a stabilization period of 10 min at constant temperature before measuring. Reported data are the average of four different batches of liposomes.

Sizes were measured before and after temperature stabilization with good reproducibility. Conductivity and pH values did not change during the determination of zeta potential.

2.3.2. Calorimetry

Calorimetry was performed using a DSC TA Instruments Q20 differential scanning calorimeter. Samples of each mole fraction were obtained by mixing the four replicates used in zeta potential, then centrifuged for 1 h to separate the lipid solvent. The solvent was removed with a Hamilton syringe and the remaining lipids mixture was centrifuged with a Thermo Scientific SPD11 V Speed Vac to evaporate the solvent. This procedure allowed the obtention of mixed lipids amount suitable for conducting DSC, of about 4 mg, which was placed in an airtight capsule for DSC. Calorimetry measurements were performed for each mixture between 18 and 60 °C, at a rate of 0.5 °C per minute. A similar procedure was carried out for every mixture at each solvent.

2.4. Mathematical treatment

DSC and ZP curves were fitted with mathematical models to obtain transition temperatures and transition temperature ranges in order to validate ZP method with DSC studies. Codes were written in MatLab version 7.11.0584, 2010.

2.4.1. Mathematical models to fit experimental data of zeta potential

Considering the graph of experimental data (T_i, z_i), $i = 1, \cdots n$different adjustment strategies are proposed. The first one is to interpolate only four nodes corresponding to the pre-transition zone or the transition zone (liquid crystalline-solid crystalline phases) using the three-degree Newton interpolating polynomial

$$f(T,A) = A_1 + A_2(T-T_1) + A_3(T-T_1)(T-T_2) + A_4(T-T_1)(T-T_2)(T-T_3). \quad (1)$$

Let A_* be the vector of parameters to interpolate the data, to get the abscissa of the inflection point the equation $f'(T, A_*) = 0$is solved; it is easy to show that the estimated transition temperature is

$$T_t = \frac{1}{3}\left(T_1 + T_2 + T_3 - \frac{A_3}{A_4}\right). \quad (2)$$

The second strategy is to interpolate entire dataset with a natural cubic spline and calculate the transition temperature in the range concerned.

The third strategy is to approximate the data in the sense of least squares with the linear model of harmonic functions.

$$f(T,A) = A_1 - \sum_{j=1}^{p} A_{2j} sen\left(\omega_j T\right) + A_{2j+1} cos\left(\omega_j T\right), \omega_j = \frac{2\pi j}{T_n}. \quad (3)$$

Let A_* be the vector fits data set, the transition temperature T_t is estimated by solving the nonlinear equation $f'(T, A_*) = 0$ with Newton's Method.

2.4.2. Mathematical models to fit experimental data of DSC

The experimental data $\left(T_i, C_{pi}\right)$, $i = 1, \cdots, n$ are approximate in the sense of least squares with three different models (3), (4) and (5):

$$f(T,A) = A_1 e^{-A_2\left(\frac{T-A_3}{A_4}\right)^2} + A_5. \quad (4)$$

$$f(T,A) = \frac{A_1}{A_2 + (T-A_3)^2} + A_4 \quad (5)$$

Models (4) and (5) are non-linear in the coefficients while (3) is linear with harmonic base functions. For each model the vector A_* that fits the data in the sense of least squares is obtained. The abscissa of the maximum value $f(T, A)$ that is the transition temperature T_t is estimated by solving the nonlinear equation

$$f'(T, A_*) = 0. \quad (6)$$

Then $f'(T_t, A_*)$ is calculated to ensure that $f(T_t, A_*)$ is maximized. It is easy to verify that for the models (4) y (5) $T_t = A_3$ As the model of harmonic functions (3) we should use the Newton's Method to solve the Eq. (6).

2.4.3. Plots of phase boundaries

In this section strategy for estimating the delimiting temperatures of both main phase transition and pre-transition of the binary mixtures in different solvents is presented. Selecting the model (3) adjusting the ζ versus T data, the curvature of the model function

Table 1
Transition temperature, pre-transition temperature and transition range obtained for ZP and DSC measurements of the DPPC-DMPC mixtures at each solvent studied. These results were obtained by interpolating and/or approximating in the least square sense the experimental data for each case studied.

X_{DPPC} Mixture	Solvent	Transition range(from ZP)	$T_{pretransition}$(from ZP)	$T_{transition}$(from ZP)	$T_{transition}$(from DSC)
0.2	Water	[24.2, 31.4]	–	27.4	26.3
	KCl (1 mM)	[25.1, 31.1]	–	27.9	26.8
	$KClO_4$(1 mM)	[26.6, 32.8]	–	29.7	28.9
0.5	Water	[27.9, 39.2]	–	31.8	32.5
	KCl (1 mM)	[33.2, 38.8]	26.3	36.1	34.6
	$KClO_4$(1 mM)	[27.2, 37.8]	22.7	30.7	31.7
0.6	Water	[27.5, 37.5]	–	34.4	34.7
	KCl (1 mM)	[31.3, 41.1]	27.4	35.0	35.5
	$KClO_4$(1 mM)	[29.7, 38.9]	23.4	34.1	34.8
0.8	Water	[34.8, 41.8]	–	38.1	37.1
	KCl (1 mM)	[29.0, 41.5]	22.0	37.5	36.2
	$KClO_4$(1 mM)	[30.0, 42.5]	24.7	35.9	36.3

in the range of experimental work was calculated. Then the temperatures of the transition region are determined by analyzing the sharp change of sign of the curvature in the range containing the estimated transition and pretransition temperatures.

Approaching the endpoints of each phase transition interval by the quadratic model

$$T = c_1 + c_2 x + c_3 x^2 \tag{7}$$

In the sense of least squares, subject to $T(0) = 24$ and $T(1) = 41$, being $T(0)$ and $T(1)$ the transition temperatures of DMPC and DPPC pure liposomes, respectively, is possible to construct the plot of phase boundaries. Similarly the Plot of phase boundaries for the pre-transition is obtained with $T(0) = 14$ and $T(1) = 34$.

In this work it was found that polynomial interpolation is not so appropriate for that rich set of data subject to experimental errors. Because of the shape of the ζ vs. T curves is was preferred to use the model of harmonic functions, calculating the parameter vector in the sense of minimizing the squared norm of the residual vector at the nodes. DSC data for all the models show similar values for the transition temperature.

Table 1 shows results obtained by interpolating and/or approximating, in the least square sense, the experimental data for each case studied.

3. Results

DMPC and DPPC (pure liposomes) transition temperatures have already being extensively studied by different methods. We have corroborated those values by ZP with continuous cooling (data not shown) obtaining similar curves as that obtained for mixtures, with similar sharp changes in the ZP during the phase transitions.

The results of zeta potential as a function of T for DPPC-DMPC mixtures for proportions $X_{DPPC} = 0.2$, 0.5, 0.6 and 0.8 in different solvents are shown in Figs. 1–4, respectively.

For the $X_{DPPC} = 0.2$ mixture it is shown in the graph of zeta potential that the phase transition takes place in the range between 24.2 and 31.4 °C, while for the $X_{DPPC} = 0.5$ mixture, transition phase is observed between 27.9 and 39.1 °C. The $X_{DPPC} = 0.6$ mixture shows the phase transition between 27.5 and 37.6 °C, while for the $X_{DPPC} = 0.8$ mixture, the phase change occurs between 34.8 and 41.8 °C.

It is noticeable that, except for the $X_{DPPC} = 0.2$ mixture, all the mixtures exhibit pre-transition temperature in ZP studies when the respective liposomes are in the presence of ions. The pre-transition is most noticeable in the presence of $KClO_4$. The pre-transition temperature appears close to 25.2 °C and 23.6 °C when the medium is 1 mM KCl and 1 mM $KClO_4$, respectively.

In all the mixtures studied with each technique, both the range and the corresponding phase transition temperature, are almost independent of the kind of solvent. Furthermore, Figs. 1–4 show that values and signs of zeta potential are dependent of the medium and the phase state of each mixture, similar to the behavior observed in liposomes from pure lipids. It is noticeable that in the absence of ions, liposomes of a mixture of zwitterionic lipids, exhibit surface charge, similar to that obtained for pure lipids in a previous work of this group [15]. For all the mixtures in fluid phase, negative zeta potential values were observed, the most negative corresponding to the $X_{DPPC} = 0.2$ mixture.

Regarding the crystalline solid phase, less negative values are observed, compared to the liquid crystalline phase, and are generally positive. The behavior of zeta potential as a function of T in the presence or absence of ions is similar in the $X_{DPPC} = 0.2$ and 0.6 mixtures, while the $X_{DPPC} = 0.8$ mixture shows a different behavior with respect to the mixtures above mentioned.

Fig. 5 shows the thermograms of the lipid mixtures in water, superimposed for comparative purposes. At the main phase

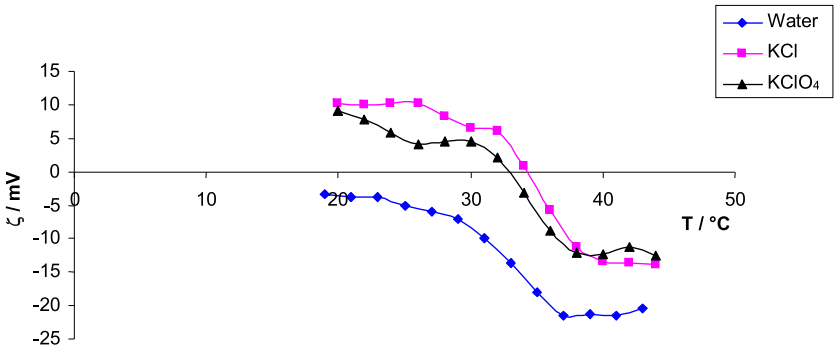

Fig. 3. Zeta potential as a function of T for $X_{DPPC} = 0.6$ mixture prepared and dispersed in water, KCl and $KClO_4$. In all cases, the standard deviation was lower than 10%, not shown for better viewing. Reported data were averaged over four different batches of liposomes.

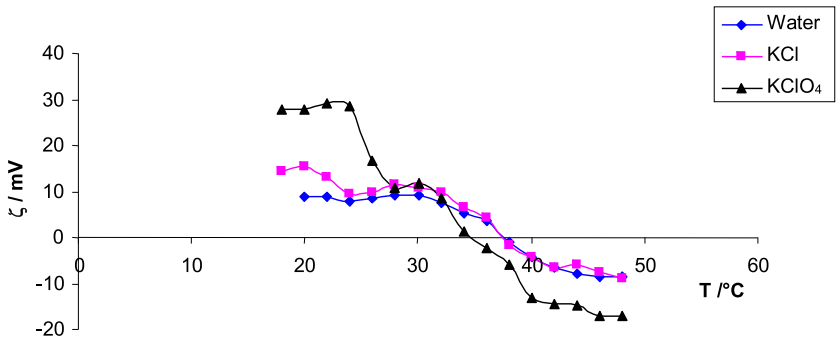

Fig. 4. Zeta potential as a function of T for X_{DPPC} = 0.8 mixture prepared and dispersed in water, KCl and $KClO_4$. In all cases, the standard deviation was lower than 10%, not shown for better viewing. Reported data were averaged over four different batches of liposomes.

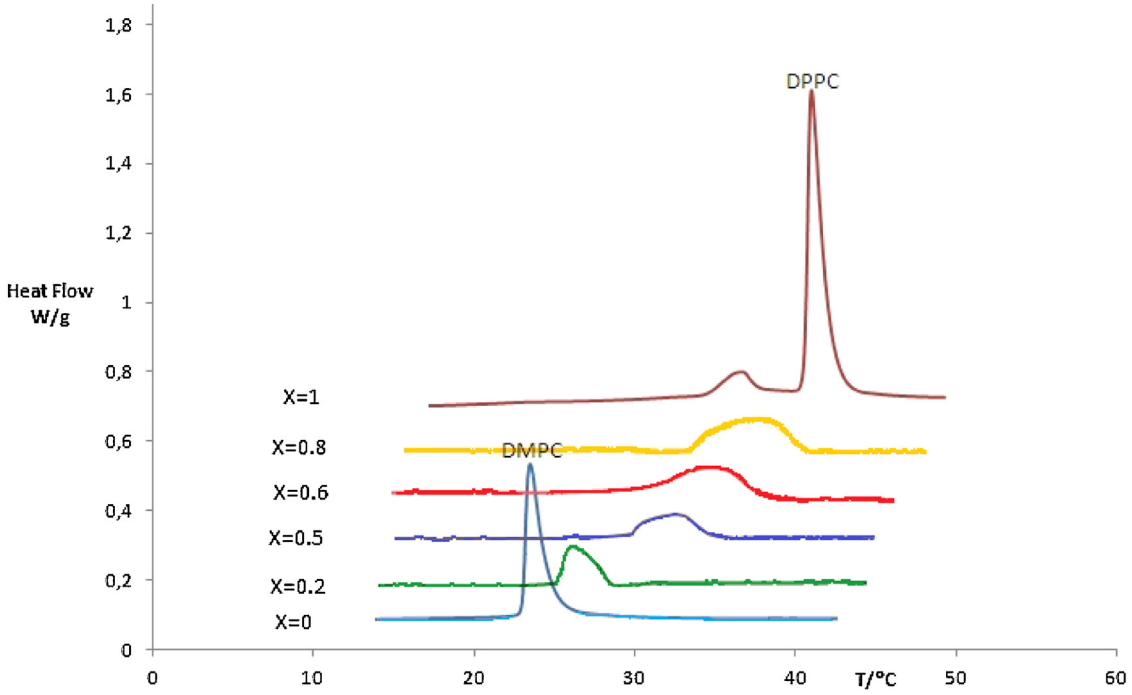

Fig. 5. Waterfall plot of thermograms of the DPPC-DMPC mixtures studied.

transition or melting, DMPC + DPPC mixtures exhibit a clear phase coexistence region, and the single peak characteristic of the main phase transition is shifted towards higher temperatures and broadens upon addition of DPPC. This contrast with the sharp transition in the main pure lipids, as shown in the fact that the mixtures in the beginning and the end of the transition region deviate from the melting temperatures of the pure compounds, suggests that the melting of the chains of each type of lipid is strongly affected by the presence of the other type of lipid in the membrane. As the peaks of the mixtures widen, the determination of the transition temperature is less certain than with pure lipids.

Table 1 shows transition temperatures, pre-transition temperatures and transition ranges obtained for ZP and DSC measurements of the DPPC-DMPC mixtures at each solvent studies. These results were obtained by interpolating and/or approximating in the least square sense the experimental data for each case studied.

4. Discussion

4.1. Zeta potential: continuous cooling experiment

Temperature dependence of the zeta potential as a method for studying phase transitions has the remarkable advantage of its high sensitivity considering not only the small amount of sample required but the possibility to detect both the main phase transition and the pre-transition of the lipid mixtures. This method allows the assessment of phase changes in the studied lipid system as well as their surface charge in different solvents.

The zeta potential measurements carried out in water and ionic medium show 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.

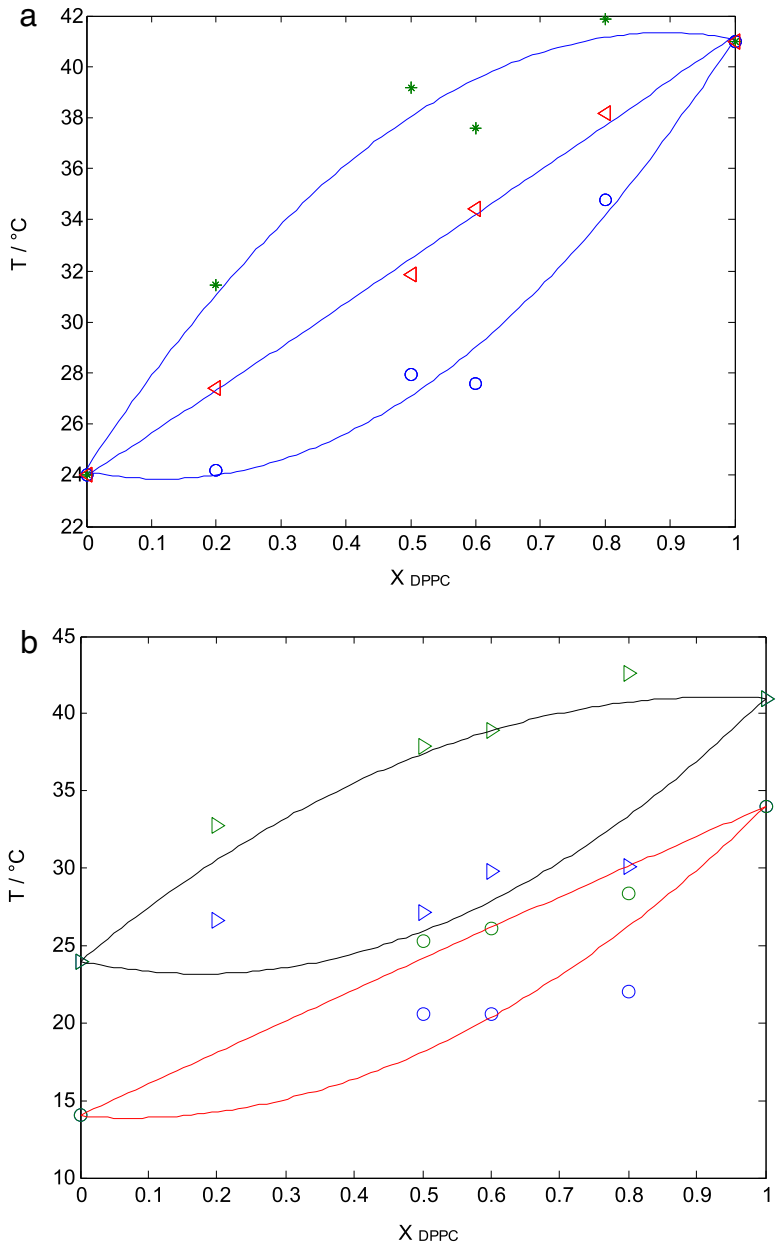

Fig. 6. (a) Plots of phase boundaries for DPPC-DMPC mixtures in water. These diagrams were constructed by plotting delimiting temperatures of phase transitions studied by ZP vs. the lipid composition of the liposome (∗,o). Also transition temperatures of the mixtures are shown (Δ). (b) Plots of phase boundaries for the binary system DPPC-DMPC in 1 mM $KClO_4$ with both two-phase regions. These diagrams were constructed by plotting delimiting temperatures of both phase transitions studied by ZP vs. the lipid composition of the liposome. Transition (Δ,Δ) and pre-transition (o,o) temperatures.
Delimiting temperatures were obtained for rigorous mathematical regression of ZP curves for the transition temperatures and transition temperature ranges.

When zeta potential measurements are analyzed, it should be considered that these measurements reflect the charge state of the surface and, in the case of mixtures, surface heterogeneity is also present. In $X_{DPPC} = 0.2$ and 0.6 mixtures, the more negative zeta potential values correspond to the liposomes in water, whereas in ionic medium, systems seem to be insensitive to the kind of ion. This reflects that lipid mixtures not fulfill the ion adsorption theory proposed for pure lipids. Equimolar mixture presents different behavior with negative zeta potential in the presence of ions, although with the expected trend (Cl^-–ClO_4^-) for more polarizable ions. This trend is evident in the $X_{DPPC} = 0.8$ mixture from but only in the solid crystalline phase. These results and analysis suggest that the sign in the surface of lipid mixture membranes could not be predicted directly from the lipid head group since it varies with ionic state and medium. From Figs. 1–4 it can be concluded that the main phase transition of each mixtures is a region of abrupt zeta potential changes reflecting the transition from the liquid crystalline to the solid crystalline state. This supports our results obtained for pure [15] lipids in the sense that the structural rearrangement of the head groups is the responsible of the surface potential that is reflected in the zeta potential.

4.2. Combined study by zeta potential and differential scanning calorimetry

Thermograms at each medium were performed with the samples obtained from the same solutions prepared for ZP studies. Those thermograms are similar despite of the medium only with slight displacements among the heat flow while the transition temperatures were practically unaltered. One could conclude that at the

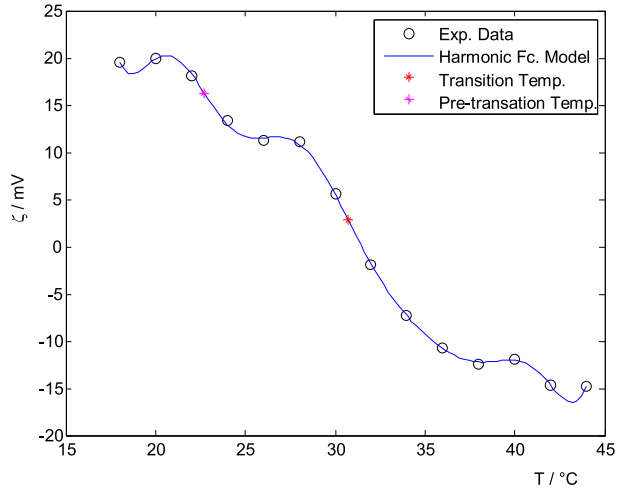

Fig. 7. Zeta potential as a function of T for the equimolar DPPC-DMPC mixture in $KClO_4$ 1 mM. Curve estimated by least squares of zeta potential. Highlighting points correspond to transition and pre-transition temperatures.

working ionic strength, adsorption of ions to the liposome surface, if any, is weak. It was shown [21,35,36] that, even at higher ionic concentrations and using pure lipids, mainly chloride ions remain in the aqueous phase as potassium ions. The latter supports a previous proposal of these authors [15], further complemented with the zeta potential values in ionic medium.

Slow scanning rates during ZP measurements keep the sample close to thermal and thermodynamic equilibrium, allowing the precise determination of the Plot of phase boundaries. The cooling rates during ZP measurements (0,2 K/min) are even lower than the corresponding rates in DSC measurements (0,5 K/min). ZP also allowed studying low-temperature transitions, in the regions corresponding to those of the gel to ripple transition, being better detectable upon addition of DPPC in the mixture and with the presence of ions in the medium.

As expected, this temperature did not match the pre-transition temperature of the pure phosphatidylcholines and did not considerably changed with neither the increasing amount of DPPC nor the presence of ions. Losada-Pérez et al. [6] studied phase transitions of DPPC–DMPC mixtures in NaCl 100 mM by calorimetry and could observe low-temperature transitions. It is noticeable that a pre-transition was detected in this work by temperature-dependent ZP measurements even with ionic concentrations 100 times lower. The low-temperature phases of pure phosphatidylcholines are quite well studied [37], and, in addition to the prototypical Lc → Lβ → Pβ → Lα phase sequence lipids, extra complexity is present in the form of intermediate phases and metastable states [37,38]. On the other hand, literature data for the lipid mixture studied here seem scarce.

In this work, in the studied temperature range, it could be detected in ZP curves, only a single phase transition below the coexistence region. These data cannot give any definite assignment of the phases.

In literature low-temperature transitions of phosphatidylcholine mixtures are reported only in ionic medium. In this work, the fact of having studied liposomes in the absence of ions revealed that the presence of ions favors the occurrence of a transition at low temperatures for this lipid mixture. In order to find possible explanations, our group currently studies this phenomenon.

Temperature dependence of the ZP made it possible to get enough information for constructing the DPPC-DMPC plots of phase boundaries. The delimiting temperatures of both phase transitions studied by ZP were determined. Plots of phase boundaries shown in Fig. 6a for these mixed-lipid vesicles in water were constructed by plotting these delimiting temperatures vs. the lipid composition of the vesicle. It is also shown the quasi-lineal correlation made by mathematical calculations of phase transition temperatures. Data from temperature dependence of the ZP are in good agreement with those from DSC [39–41]. Fig. 6b presents the plots of phase boundaries for the binary system DPPC/DMPC in 1 mM $KClO_4$ with both two-phase regions. The combined data suggest a plot of phase boundaries in which the main two-phase region is either symmetric, or nearly so, about a line connecting the two pure-component transitions, while at low temperature the two-phase region, is asymmetric and with smaller area. This could be an evidence of the phase transition of a small fraction of lipids at low-temperature [42]. This would also explain the apparent absence of a low-temperature transition. Fig. 7 shows temperature-dependence curve estimated by least squares of zeta potential of the equimolar mixture in $KClO_4$ 1 mM. Highlighting points correspond to transition and pre-transition temperatures.

Based on the analysis and correlation of the data presented, the technique of zeta potential with successively reduced temperature, allow to asses transition and pre-transition temperatures of lipid mixtures with good accuracy even with small amount of sample. This work can be taken as a departure point for further studies on more complex lipid mixtures displaying biologically relevant phases.

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